Research Article

Histology of the inflammatory response of carp (*Cyprinus carpio* L.) to *Aeromonas hydrophila* infection

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Abstract

The inflammatory response is the basic mechanism protecting tissue from damage from whatever cause. It can be considered as a fundamental aspect of pathology because of its frequent occurrence in many diseases and also its protective role. The present investigation was carried out to study the inflammatory response of carp (*Cyprinus carpio* L.) to a biological stimulus, *Aeromonas hydrophila* infection at 27.5± 1°C. The treated fish were sacrificed over a while, and sequential tissue samples were processed for histopathological examinations. Fish challenged with *A. hydrophila* exhibited an acute inflammatory response which was lethal for some fish within 48 hours post-injection.

Polymorphonuclear cells (PMNs) were infiltrated in the lesions 1 h post-challenge, and revealed a remarkable response to the infection. Polymorphonuclear cells remained up to 7 days post-injection, and participated in myophagia and contributed in micro-abscess formation. The acute inflammation progressed into a chronic inflammation characterized by fibroplasia which was active 5 days post-infection. The wound healing progress initiated and developed in 5-10 days and was completed by scar formation on 28 days post-challenge. In addition, the regeneration of muscle bundles increased and filled the defect area.

Keywords: Inflammatory response, Bacterial diseases, *Aeromonas hydrophila*, Histological examination, Carp (*Cyprinus carpio*)

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Introduction

Bacterial diseases are responsible for high mortality in wild and cultured fish (Frerichs and Roberts, 1989). The normal bacterial flora of fish is a direct reflection of the bacterial population of the water in which they swim (Horsley, 1973; Sakata *et al*.,, 1980). These normal micro-organisms are essentially opportunist pathogens which infect susceptible fish to infection by stress factors or other disease processes. Several aeromonads have been cited as causing major problems for carp aquaculture. Although motile aeromonads are fish pathogens, it is important to note that these bacteria also form a part of the normal gut microflora of healthy fish, and thus stress is often considered a factor in the outbreak of diseases caused by these bacteria. The most important micro-organism in this respect is *Aeromonas hydrophila*, which is generally considered as a facultative pathogen, invading the tissues of a fish severely stressed or physically traumatised by another agent. The genus *Aeromonas* belongs to the aeromonadaceae family, which consists of 14 different species. *Aeromonas hydrophila* is known to infect fish and occur ubiquitously and spontaneously in aquatic environments. A. *hydrophila* is an opportunistic invasive bacterium which has a variety of hosts (Pachanawan *et al*.,, 2008). *Aeromonas* is a facultative bacterium that has pathogenic potential for fish and found everywhere. The bacterium is found in fish hatcheries and farms, and can easily develop colonies on the skin, gills, fins and gut of fish. Most common diseases in fresh water are abdominal dropsy, columnaris, furunculosis, fin-rot and tail-rot (Fern´andez-Bravo and Figueras, 2020). *A. hydrophila* is a Gram-negative bacterium which is widely distributed in fresh water systems. It has been reported from a wide range of fish species in temperate and tropical water systems (Snieszko and Axelrod, 1971; Plumb *et al.,* 1976; Allen *et al.,* 1983). *Aeromonas hydrophila* is mostly appeared in waters with high organic load than in relatively polluted waters (Jeney *et al.,* 1995).

Recently, fresh-water fish species including common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), blunt snout bream (*Megalobrama amblycephala*) and mandarin fish (*Siniperca chuatsi*) has been contaminated by A. *hydrophila* (Xia *et al.,* 2017) and the results revealed that the non-specific immunity of fish played an important role in self-protection after pathogens infection. The organism has been identified in all countries where pond and

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ornamental fresh-water fishes are cultured (Frerichs and Roberts, 1989). *A. hydrophila* is a motile species of aeromonads which are often ubiquitous microbial flora of aquatic animals and maybe pathogens of poikilotherms, homoiotherms and even man (Salton and Schnick, 1973; Fraire, 1978). In warm-water aquaculture, *A. hydrophila* is considered to be a major problem, but it is difficult to distinguish direct losses from those caused by secondary infections (Amin *et al.,* 1985; Ruangapan *et al.,* 1986). Environmental factors such as crowding, low concentrations of dissolved oxygen and high organic content in the water, industrial pollution, abrupt temperature changes, physical injuries, and spawning may also contribute to infections by *A. hydrophila* (Pippy and Hare, 1969; Shotts *et al.,* 1972). The pathogenicity of *A. hydrophila* has been investigated in many species of fish, mainly due to heterogenic strain and differences in enterotoxic and adhesive mechanism, the prevalence of infection in fish aquaculture (Podeti and Benarjee, 2017). One of the most common fish bacterial disease syndromes is motile aeromonas septicaemia (MAS) caused by *A. hydrophila*. An outbreak of MAS among wild or pond-raised fish is difficult to control despite corrective action by fish biologists. It appears that once the infection is established, rapid growth of the bacterium and elaboration of its toxic products may cause irreparable systemic damage which leads to death, and can occasionally cause devastating losses in both wild and farmed fish population (Brenden and Huizinga, 1986; Roberts, 1993). This infectious disease is also known to reduce catfish production by nearly 10% every year and is considered to be one of the common diseases accounting for the decrease of fish production (Alagappan *et al.,* 2009).

Common carp is one of the reasonable candidates for the application of intensive aquaculture systems; however, the intensive rearing strategies are among the main reasons for impairing the immune system and the susceptibility to diseases with a resultant of high economic loss (Abdel-latif *et al*., 2020)**.**

This study was conducted to investigate the progress of inflammatory response and the pathogenesis of the inflammatory lesion induced in healthy carp (*Cyprinus carpio*) by a strain of *Aeromonas hydrophila* during 42 days.

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Materials and methods

Fish

One hundred mirror carps with average length of 13±2 cm was stocked in a stock tank with a recirculating water supply and aquarium water heaters. Sixty-five fish were transferred from the stock tank to the glass aquaria in an isolated challenge room and kept for 7 weeks to acclimatise before the experiment began. They were fed with pelleted food once per day.

Aquaria and water system

Recirculating glass aquaria systems were used for stocking fish, and during the course of the experiment. The average temperature of water was 27.5°C (± 1°C). Faecal materials were removed from aquaria every day and between 20-30% of aquaria water was changed as necessary. As live bacteria were used in this experiment, a drainage system with two tanks was used to disinfect the water before draining to waste. Effluent water from the aquaria was treated with sodium hypochlorite (1400 ppm) to kill pathogens present.

Preparation of inoculum

A highly virulent strain of *A. hydrophila* (T4), isolated from EUS affected Indian major carp (*Labeo rohita*) in Bangladesh (Millar, 1994), was used as the inoculum. The bacterium was cultured in Tryptone Soya Broth (TSB) medium for 24 hours at 22°C. Broth culture was plated out onto Trypton Soya Agar (TSA) medium for 24 hours at 22°C to check culture purity. Individual colonies from the TSA culture were suspended in sterile 0.85% saline. This bacterial suspension was adjusted to contain 10^6 organisms/mL using the results of pilot experiment. Viable bacterial numbers for this inoculum were determined by making ten-fold dilutions from 10^{-1} to 10^{-8} of the saline suspension and spreading 100 mL of each dilution on separate TSA plates. Following incubation at 22°C for 24 hours, resultant colonies were counted on each plate. The number of viable bacteria for the inocula was determined as 5.3×10^6 organisms/ml and the optical density (OD) was measured as 0.010 at 680 nm on a WPA S105 spectrophotometer (WPA ltd. England).

Injection procedure

Sixty fish were inoculated into the left side of dorsal myotomal muscle with 0.1 mL of the 5.3×10^6 organisms/mL suspension, using a 1 mL syringe and 25-gauge needle. The fish were anaesthetised with 10% benzocaine prior to inoculation. The injection was made in line with the first ray

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of the dorsal fin, below the scale row, as a marker for subsequent sampling. Control fish were injected intramuscularly on the left flank with the same volume of sterile saline. All infected fish and control fish were returned to the aquaria following injection for observation of clinical signs of infection and subsequent sampling through to the end of the experiment. Fish were observed during the early hours following injection and thereafter daily. Dead fish were removed from the aquaria and a detailed record of the gross pathology obtained.

Histological sampling

The infected fish were sacrificed by an overdose of 10% benzocaine at 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48 hours, thereafter at 3, 4, 5, 6, 7, 10, 14, 18, 22, 28, 35 and 42 days after inoculation. Due to mortalities following inoculation, two fish were sacrificed in the early stages from 1 hour to 48 hours, thereafter one fish was sampled between 3 and 42 days post-injection. A block of tissue, including the lesion area, was immediately cut out from the injected site of each sacrificed fish and fixed in cold 10% neutral buffered formalin for at least 24 hours before cassetting. During the fixation time, buffered formalin was changed at least once.

The fixed and cassetted tissues were processed in an automatic tissue processor using standard procedure. After processing, tissues were cut down the middle into two pieces to expose the site of injection for sectioning. These trimmed blocks were then embedded into wax, decalcified by RDC (Rapid Decalcifier from CellPath plc, Herts, England), and 5 μm sections prepared using a Leica Rotary Microtome. The control fish were sampled, processed and sectioned using the same procedures as the experimental fish.

Bacterial isolation

A piece of lesion muscle from freshly sacrificed fish was sampled aseptically for histology, and from this, a very small area was cut out and chopped into smaller pieces using sterile blade and disinfected forceps for bacterial isolation. The samples for bacterial isolation were prepared from all the sacrificed fishes for histological examination. The chopped tissues were cultured in TSB for 24 hours at 22°C. Following this, the TSB was plated out onto TSA plates and incubated at 22°C for 24 hours to produce individual colonies. Identification of the isolated colonies was confirmed by a range of morphological and biochemical tests. First basic identification tests (primary tests) namely Gram stain,

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microscopic morphology, motility, oxidase, oxidation-fermentation (O/F) and 0/129 vibriostat sensitivity were carried out as well as determining growth at 37°C. After the basic identification of bacteria, the API 20 E microbial identification kit (bioMeriéux, UK ltd), was used to determine biochemical characteristics. These tests were also carried out on the stock bacterium used as inoculum. Results for inoculum and isolates were compared to determine if both were identical. The same procedures were also used for control fish.

Staining procedures

In addition of the standard H and E staining for the routine microscopic examination of the sections, Gram staining, was used specifically to identify Gram negative bacteria in the sections. The stained sections were mounted in Pertex mounting medium (CellPath plc, Herts, England) for examination.

Results

Gross pathology

During the first few hours after inoculation, behaviour of the infected fish was normal. Thereafter the general findings from the study were as follows;

By 10 hours after injection, most of the infected fish were lethargic and remained at the bottom of the aquaria. They showed slight swelling, small white patches and pin-point haemorrhages (in some fish) at the site of injection.

At 12-24 hours after injection, the white patch and swelling became quite extensive and fluctuant. Severe generalised erythematous reaction was obvious all over the body surface. The scales above the site of injection showed severe protrusion and readily sloughed off. One quarter of the injected fishes died at this period.

After 36-48 hours, initial ulceration occurred. An oval or irregular white or pale ulcerative lesion was clearly seen and necrotic muscle at the centre of the lesion sloughed off, caused an open concave lesion about 1cm diameter. The fish with ulcerative lesions lost their scales close to the site of injection. Five more fish died at this stage. From 48 hours onwards, no further mortalities occurred.

After 3-4 days, the ulcerative lesion became so extensive and deep that a part of spinal column was obvious. The diameter of the lesion was approximately 1.5 cm. The edge of the ulcerative lesion was irregular and white.

By 5-10 days after inoculation, a healing process was observed. The edge of the lesion was irregular and distinct due to its

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dark pigmented border, and the size of the lesion decreased to about 0.5 cm.

At 14-22 days post-injection, most of the infected fish showed a black coloured area and a dark depressed scar at the site of injection on the skin.

After 28-42 days, the remaining fish showed a dark smooth depressed scar with a black coloration and an irregular attached border at the injection area on the skin. A small number of new small scales grown on the scar were evident.

None of these changes were observed in the control fish.

Histopathology

1 hour

At 1 hour after inoculation of bacterial suspension, the principal feature was myofibrillar degeneration which started with central migration of sarcoplasmic nuclei, flocculation of sarcoplasms and loss of sarcolemmal integrity (Fig. 1). Large or small foci of haemorrhage along with hyperaemic blood vessels associated with melanin pigments were seen in the lesion area. Thrombocytes, lymphocytes and a few polymorphonuclear cells (PMNs) were the inflammatory cells within the defect area. No bacteria were evident in the sections stained by H and E or Gram staining methods, but Gram-negative bacterial bacilli, *A. hydrophila*, were re-isolated from the muscle of the injection area, and identified using bacteriological tests.

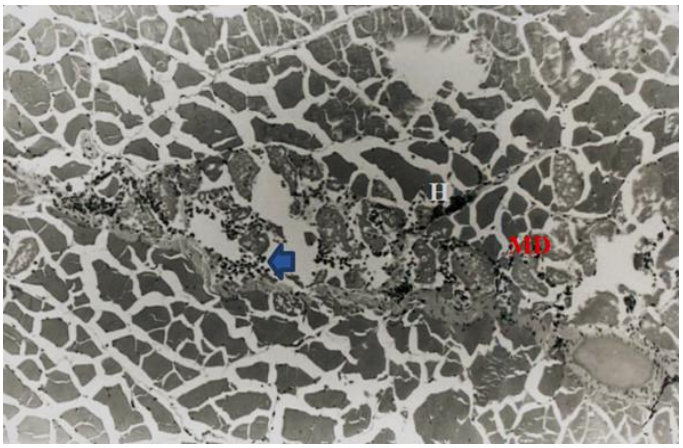

Figure 1. At 1 hour post-injection of *A. hydrophila*, muscle degeneration (MD), foci of haemorrhage (H), and cellular infiltration (arrow head) were observed in the lesion area. (H & E, X 110).

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2 hours

By 2 hours after the bacterial injection, the degenerated sarcoplasms were more fragmented and also muscle degeneration area was actively more extended to the adjacent tissue. Generalised and localised haemorrhages were found extensively in the lesion area, and also hyperaemic blood vessels with melanin granules around, either isolated or in dense aggregates, were obvious in the tissue. Lymphocytes, PMNs, and thrombocytes were infiltrated into the area. No bacteria were found in the tissue with H and E and Gram stain, but the gram-negative *A. hydrophila* was re-isolated and identified from the muscle of the infected fish by bacteriological tests.

3 hours

At this stage, more muscle degeneration and flocculation of sarcoplasms with loss of membrane integrity and nuclear pyknosis were seen (Fig. 2). Localised haemorrhages and also engorged blood vessels with red and white blood cells inside and melanin granules around were obvious. There were more lymphocytes and PMNs comparing with the last stage. A few Gram-negative bacilli were seen within the sarcoplasms in Gram stain. *A. hydrophila* was re-isolated from the site of injection and identified using bacteriological tests.

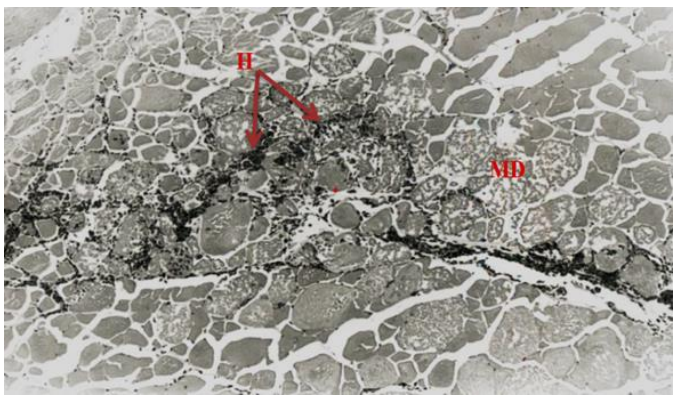

Figure 2. By 3 hours post-injection more degeneration and flocculation of muscles (MD), and haemorrhages (H) were obvious in the area of the lesion. (H & E, X 110).

4 hours

The picture of this stage of the lesion area was more cellular especially with further increase in PMNs numbers. Also, for the first time some macrophages were evident in hypodermis and adjacent to the damaged muscles and also some of them, along with PMNs, were located inside the damaged muscles which could have represented the initiation of myophagia (Fig. 3). Flocculation of the degenerated muscles was obvious in the lesion area.

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6 hours

At this stage the main feature was evidence of myophagia in the lesion area (Fig. 4). The dominant PMNs and some macrophages were seen within the degenerated muscles engulfing the remains of the damaged sarcoplasms. Further degeneration and flocculation of sarcoplasms was seen. Some haemorrhages and fibrin strands with thrombocytes were seen in some parts of the damaged area. Unlike macrophages, the number of lymphocytes were increased.

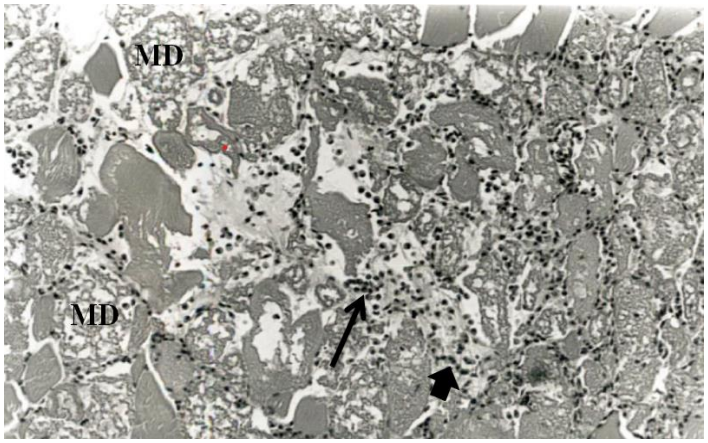

Figure 3. The picture of the lesion area at 4 hours post-injection was more cellular especially with further increase in PMNs number (arrow). PMNs were located inside the damaged muscles along with macrophages (arrow head). Also, more degenerated muscles (MD) were observed in the defect area. (H & E, X 220).

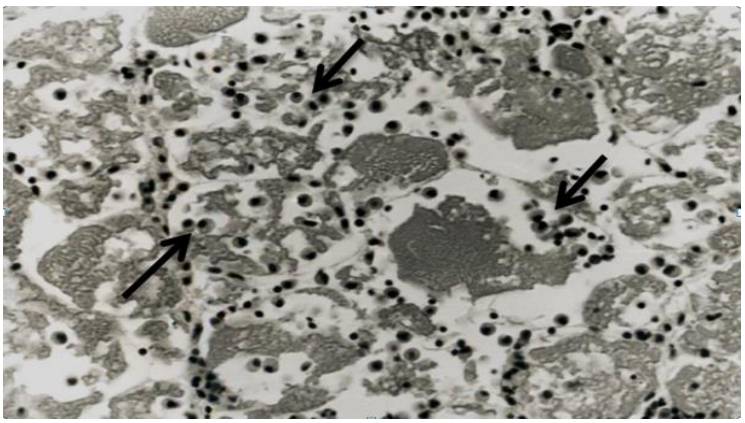

Figure 4. Evidence of the active myophagia (arrows) was the main feature at 6 hours after inoculation. (H & E, X 440).

8 hours

By 8 hours, myophagia was active. The number of macrophages were increased but PMNs were still dominant cells in the area.

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Further degeneration of sarcoplasms which were formless was seen (Fig. 5). Dermis was, to some extent, oedematous and blood vessels were hyperaemic with red and white blood cells and also there were melanin pigments around them. Local and scattered haemorrhages were seen throughout the damaged area.

10 hours

At 10 hours after injection, the lesion was still dominated by PMNs activity which in some areas were very dense, aggregated and, to some extent, degenerated along with macrophages, some amorphous suppuration, degenerated muscles and nucleic debris formed micro-abscesses (Fig. 6). Myophagia was in progress in the defect area. Inflammatory cells infiltration was in high level and globular sarcoplasms were obvious in the damaged area. Although the bacteria were not seen in the sections of the lesion area, *A. hydrophila* was re-isolated from the infected fish using bacteriological tests.

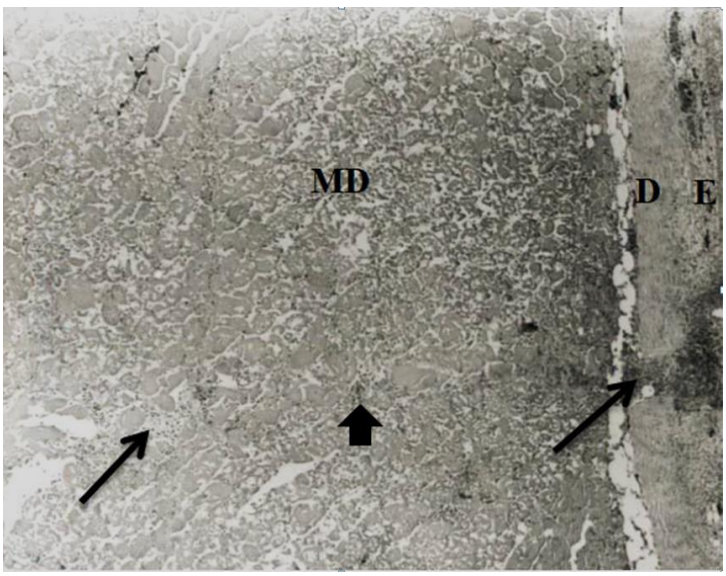

Figure 5. This picture shows a general view of the site of injection in the dermal area at 8 hours p.i. which was infiltrated with inflammatory cells (arrows), and also muscle area with extensive muscle degeneration (MD), and active myophagia (arrow head). (H & E, X 44). E=epidermis, D=dermis

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Figure 6. This picture demonstrates a micro-abscess (arrow head) at 10 hours p.i., comprising a very dense aggregation of inflammatory cells with PMNs dominated, some amorphous suppuration, degenerated muscles and nucleic debris. (H & E, X 220).

12 hours

The picture of this stage was mostly similar to that of 10 hours post injection The lesion was still dominated by PMN activity. The number of macrophages was increased and myophagia was active. Large area of degenerated muscles with cellular debris was obvious. Also hyperaemia of blood vessels associated with melanin pigments was seen. Gram negative bacteria were seen within the degenerated muscles and among them (Fig. 7). They were also re-isolated from the freshly sampled tissue and identified as *A. hydrophila*, usingbacteriological tests.

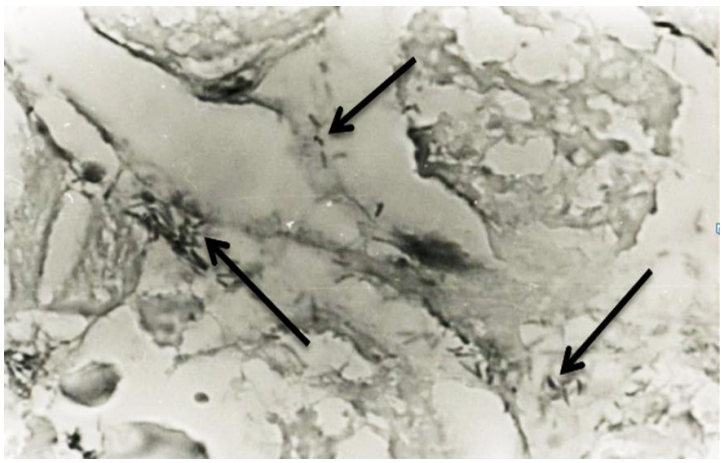

Figure 7. Gram negative *A. hydrophila* were seen within the degenerated muscle and among them (arrows) at 12 hours p.i. (Gram, X 1100).

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18-30 hours

At these stages largely extended degenerated muscles were obvious in the lesion area and myophagia was active so that from the some degenerated sarcoplasms only an outline was remained (Fig. 8 and 9). The blood vessels were hyperaemic and containing inflammatory cells, and some of them were filled with fibrinogen and thrombocytes. They were also associated with melanin pigments. Inflammatory cells such as PMNs, macrophages and lymphocytes were seen in the damaged area. Masses of Gram-negative bacterial colonies were dispersed within the damaged muscles and among them (Fig. 8). They were also observed in cytoplasm of some macrophages (Fig. 10). Presence of these bacteria in the injected site of the freshly sacrificed fish was also proved by re-isolating and identification them as *A. hydrophila*, using the bacteriological tests.

36-48 hours

By this time sever myonecrosis and sarcolysis was observed extending throughout the lesion area. Floccules of the degenerated muscles, degenerated inflammatory cells, debris and pyknotic nuclei showed an ulcerated area extended towards the dermis. Meanwhile the dermis was swollen due to a sever inflammatory oedema (Fig. 11). The presence of some pale swollen fibroblasts in the myotomal fascia and around the blood vessels showed the beginning of fibroblast activity. The blood vessels in the lesion area were seen with different appearance, some of them filled with fibrinogen and thrombocytes, some with inflammatory cells and were also hyperaemic but all were associated withmelanin pigments.

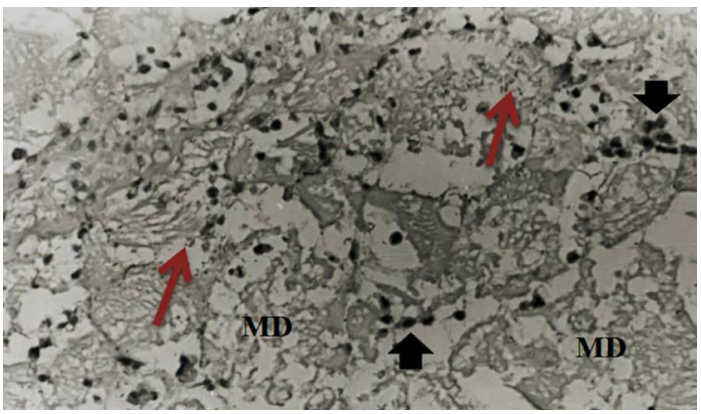

Figure 8. By 18 hours p.i. largely extended degenerated muscle (MD), active myophagia (arrow heads) and masses of Gram-negative bacteria (arrows) were observed in the infected area. (Gram, X 440).

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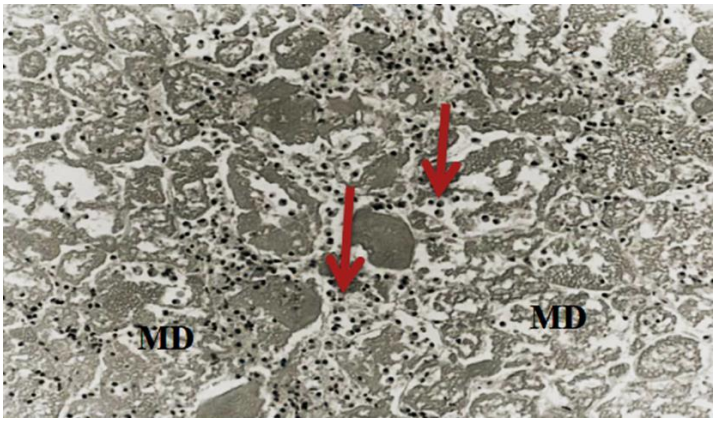

Figure 9. An active cellular infiltration and myophagia (arrows) with extensive muscle degeneration (MD) were the main features at 24 hours p.i. (H & E, X 220).

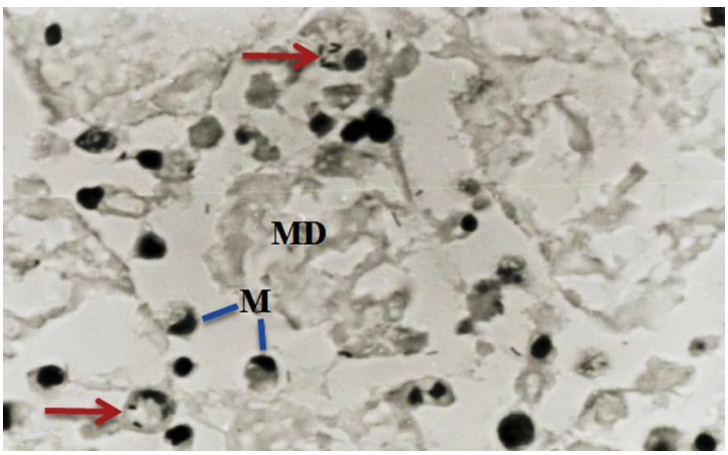

Figure 10. This picture shows the presence of Gram-negative *A. hydrophila* within the cytoplasm of macrophages (arrows) at 24 hours p.i. (Gram, X 1100). DM=degenerated muscle, M=macrophage

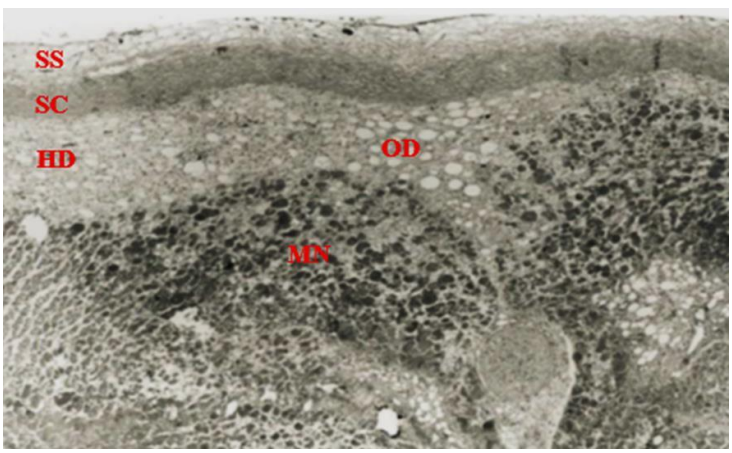

Figure 11. Severe extended myonecrosis (MN) and oedema (OD) of stratum spongiosum and hypodermis were observed at 48 hours after inoculation. (H & E, X 44). SS=stratum spongiosum, SC=stratum compactum, HD=hypodermis

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3 days

The picture of this stage was mostly similar to those of second day, except that the number of fibroblasts and their activity especially around the destroyed area was increased.

4 days

The main feature of this time was fibroplasia in the damaged area, especially in the edge of the lesion. Fibroblasts activity was increased and these cells along with macrophages which were in an epithelioid form, appeared encapsulating the necrosis area (Fig. 12).

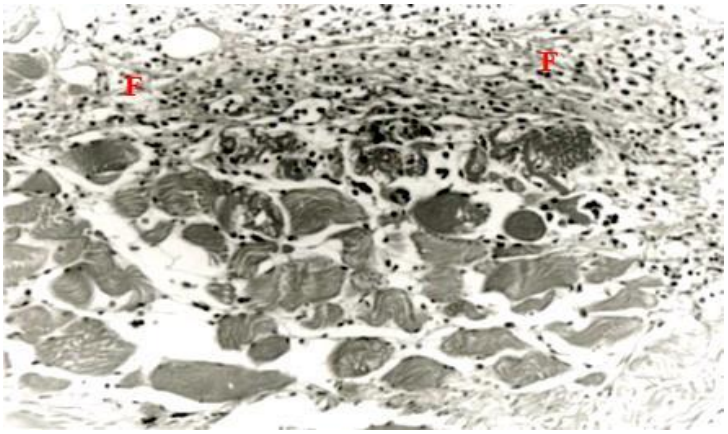

Figure 12. The main feature of the lesion at 4 days p.i. was fibroplasia (F) specially in the edge of the lesion. (H & E, X 220).

Also, myophagia was, to some extent, in progress and removed necrotic muscles being replaced by fibroplasia. The first presence of some new small basophilic muscle buds was evident in the damaged area. Inflammatory oedema caused dermal swelling showing a large space between dermal layers.

5 days

At this stage fibroplasia and fibrosis was active and boosted by new capillaries which appeared in the fibrous tissue. Small number of macrophages was observed. New muscle buds were increased in the damaged area (Fig. 13). The bacteria were neither seen in the lesion area nor re-isolated from the fish by bacteriological tests.

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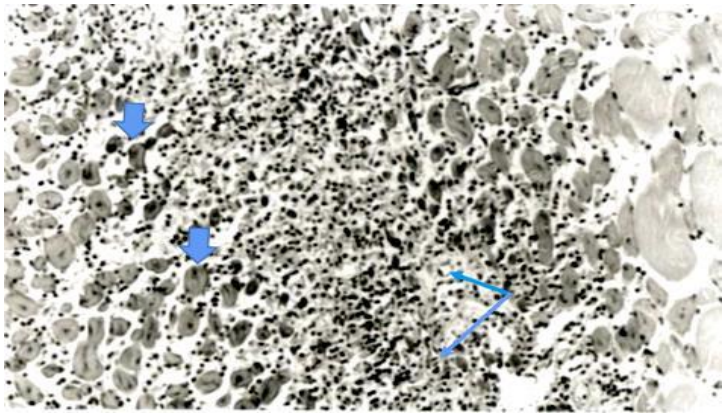

Figure 13. At 5 days p.i. new muscle buds (arrow heads) and new capillaries (arrows) were increased in the damaged area. (H & E, X 220).

6 days

The dominant features in this time were active fibroplasia, fibrosis and vascularization. Also new myofibrillar buds were seen extending in the damaged area (Fig. 14). In the centre of the lesion an area consisted of degenerated muscles and some amorphous pink staining substance with some macrophages and PMNs within, was seen surrounded by active fibrosis and muscle regeneration which was also occurring within this area. Myophagia was in progress in this part of the lesion and also in the rest of the area. New capillaries were active and nourishing the fibrous replacement area. Also, some haemorrhages were scattered within the lesion area. Inflammatory cells such as macrophages, PMNs, lymphocytes, eosinophilic granular cells (EGCs), and fibroblasts were obvious in the whole lesion area. No bacteria were detected either in the sections or in bacteriological tests.

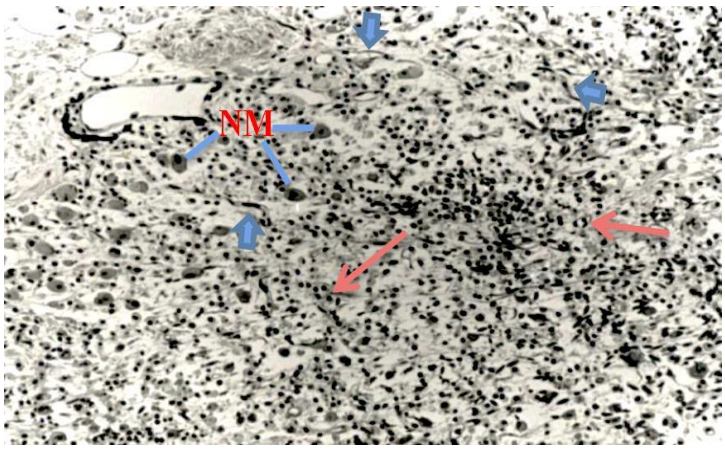

Figure 14. The dominant features at 6 days p.i. were active fibroplasia and fibrosis (arrow heads), vascularization (arrows) and new myofibrillar buds (NM). (H & E, X 220).

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7 days

By 7 days, fibroblasts and capillaries activity, and also growth of myofibrillar elements into the granulation tissue were dominant features. Myophagia was completed. Lymphocyte activity was in a high level and macrophages in the form of epithelioid cells were seen in the lesion area (Fig. 15). The presence of some PMNs and EGCs was evident. No bacteria were seen in the damaged area but Gram-negative *A. hydrophila* was re-isolated from freshly sampled muscle tissue by bacteriological tests.

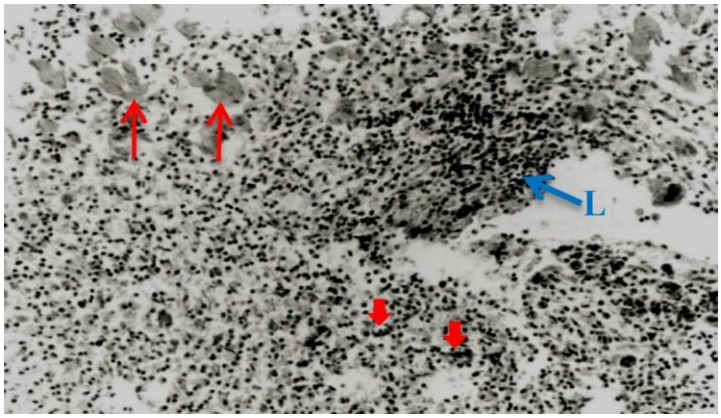

Figure 15. By 7 days p.i., myophagia was completed and lymphocyte infiltration (L) was increased. New muscle fibres (arrows) and new capillaries (arrow heads) were also observed. (H & E, X 220).

10 days

At this stage the process of wound healing was well developed. The regenerated muscle fibres actively developed and infiltrated into the fibrous area. There was more dense fibrous tissue replacing the damaged area and collagen forming was in a high level of activity. New epidermis was oedematous and some inflammatory cells migrated through it, and the cut edges of dermis were linked by fibrous tissue (Fig. 16).

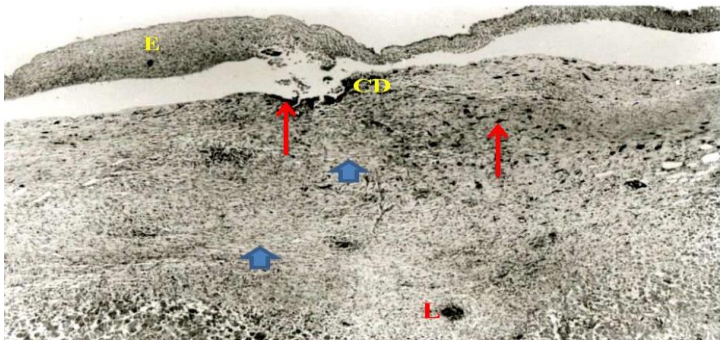

Figure 16. This picture shows a general view of the injected area 10 days p.i. More dense fibres tissues (arrow heads) are replacing the damaged area. New epidermis (E) is oedematous and the cut edges of dermis (CD) are linked by fibrous tissue, which is associated with melanin pigments (arrows). (H & E, X 44). L=lymphocytes aggregate

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Capillaries were very active in nourishing the fibrosis area and also more new capillaries were seen. A large granuloma was seen comprising an amorphous pink staining substance, similar to a newly grown small scale, in the centre area, macrophages, epithelioid cells and some nucleic aciddebris, encapsulated by fibrous tissue and also lymphocytes (Fig. 17).

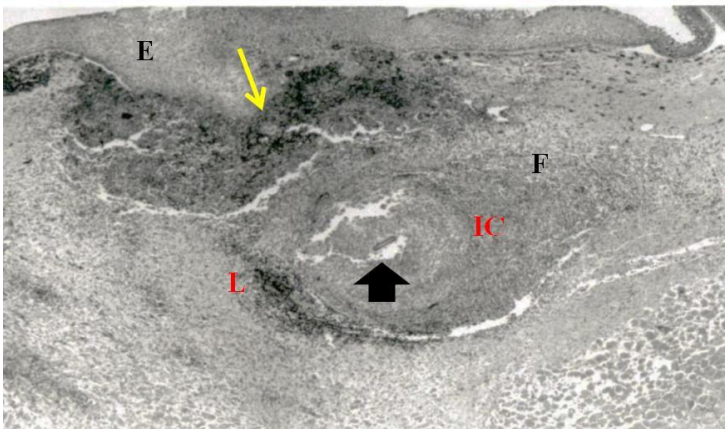

Figure 17. A large granuloma comprising an amorphous pink substance in the centre (arrow head), and inflammatory cells (IC), nucleic debris, fibrous tissue (F) and lymphocytes accumulation (L) was observed at 14 days p.i. Also, Langhans giant cell (arrow) was seen between the granuloma and new oedematous epidermis (E). (H & E, X 44).

A large Langhans type giant cell in an area between this granuloma and oedematous epidermis was observed (Fig. 18). Lymphocytes accumulation, some active macrophages and some EGCs were observed in the area. In some sites a ring of fibrous tissue and inflammatory cells separated the normal muscle and damaged area. Dense melanin pigments and isolated melanin granules were scattered in the damaged dermis and replaced fibrous tissue in the muscle area and also around the blood vessels. Some haemorrhage was observed in the defect area. No bacteria were seen either in the infected area or re-isolated from the fish in bacteriological tests.

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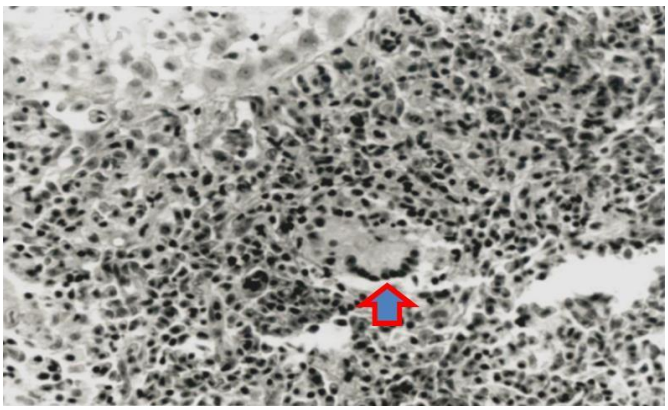

Figure 18. The enlargement of Figure 18 shows a large Langhans giant cell (arrow head) located between granuloma and epidermis (E). (H & E, X 440).

14 days

By the day 14, the progress of the dermal regeneration led to integrity in the area of the defect being almost completed. Epidermis was normal and two separated edges of dermis were completely closed and linked together. Also, muscular regeneration was, to some extent, in progress but in most of the defect area regenerated muscle fibres actively developed into the fibrous area (Fig. 19). Fibrosis was active and well developed. New capillaries were active in the area and some scattered haemorrhages were seen. Stratum spongiosum was developing normal melanocyte structures, so that this component was scattered throughout the whole lesion area and around the blood vessels. The lesion area was still more cellular than the normal tissue. Melanin and lipofuscin containing macrophages, lymphocytes in a high level of activity, some EGCs, and macrophages in the form of epithelioid cells between the normal and regenerated muscles and also in the fibrous area, were evident. No bacteria were seen in the lesion area, and neither was *A. hydrophila* re-isolated from the infected fish using bacterial culture medium.

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Figure 19. By 14 days p.i., regenerated muscle fibres actively developed into the fibrous area in most of the lesion area (Circles). (H & E, X 110).

18-22 days

The dominant features in this period were mostly similar to those of 14th day. Muscle regeneration was still in progress and all the defect area underside the dermis was filled by new muscle bundles. Fibrous replacement with scarring of the lesion area was obvious. More cellular components, especially lymphocytes in a high level of activity, and macrophages laden with lipofuscin, melanin granules and ceroids were dominant throughout the healing lesion. Fibrosis and new capillaries were still active and melanin pigments were scattered throughout the joined dermis and fibrous replacement areas (Fig. 20 and 21). New scales were seen well-formed above the newly re-joined dermis (Fig. 20).

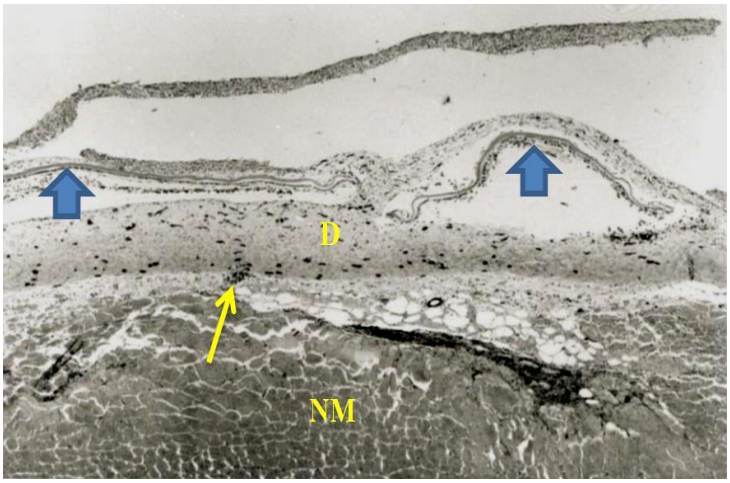

Figure 20. This picture shows the lesion area at 18 days p.i. All the defect area underside the dermis was filled by new muscle bundles (NM). Cellular components (arrow) are seen throughout the healing area, and melanin pigments are scattered throughout the joined dermis (D). New scales (arrow heads) are seen above the newly re-joined dermis. (H & E, X 44).

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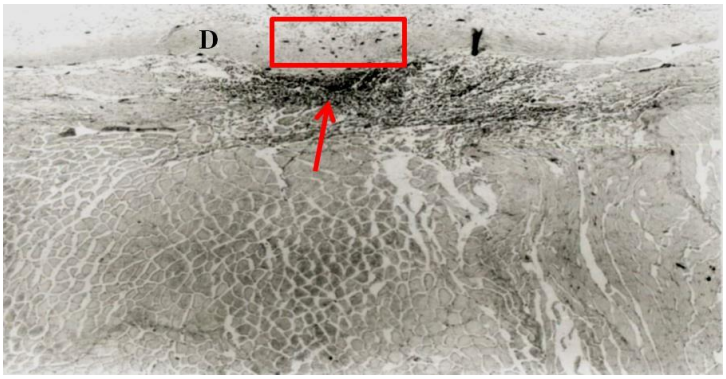

Figure 21. The healing area under the newly re-joined dermis (D) was still more cellular (arrow) at 22 days p.i. Melanin pigments (Rectangle) were observed throughout the joined dermis and healing area. (Gram, X 44).

28 days

At this stage a scar replaced the destroyed area by infection, but the scar tissue was reduced, and re-grown muscle bundles were increased and filled most of the defect area. The cellular components were dramatically reduced and only a small accumulation of active lymphocytes and a small number of active macrophages were present in the healing lesion. Small blood vessels were active and nourishing the healing lesion and also some new capillaries were formed. Melanin pigments were scattered throughout the healed dermis and replaced fibrous tissue in the muscle area and around the blood vessels. Newly formed scales were growing on the damaged area within the stratum spongiosum exactly above the scar tissue (Fig. 22).

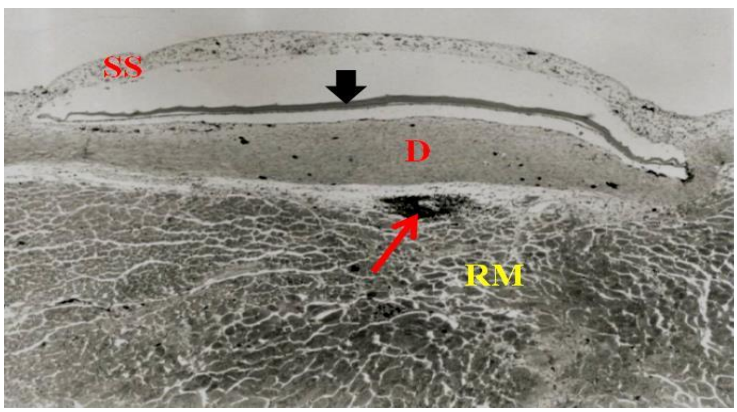

Figure 22. This picture demonstrates a general view of the lesion at 28 days p.i. The scar tissue is reduced and re-grown muscles (RM) filled almost the whole defect area. Only a small accumulation of lymphocytes and macrophages (arrow) are present under the dermis (D) in the lesion area. A new scale (arrow head) is well grown over the scar area within the stratum spongiosum (SS). (H & E, X 44).

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35-42 days

At this period the lesion area almost recovered. The scar had joined up with the dermis especially at 42 days. The re-grown muscles were indistinguishable from the normal muscles, and filled the entire defect with a highly reduction of the connective tissue and cellular components. The epidermis and dermis had recovered their normal form but dense melanin pigments and isolated granules were dispersed throughout the healed area and around the blood vessels. A small number of macrophages with ceroid and melanin within their cytoplasm were still present in the scar area and adjacent to the congested blood vessels. Some new scales were seen above the healed lesion area well formed (Fig. 23 and 24).

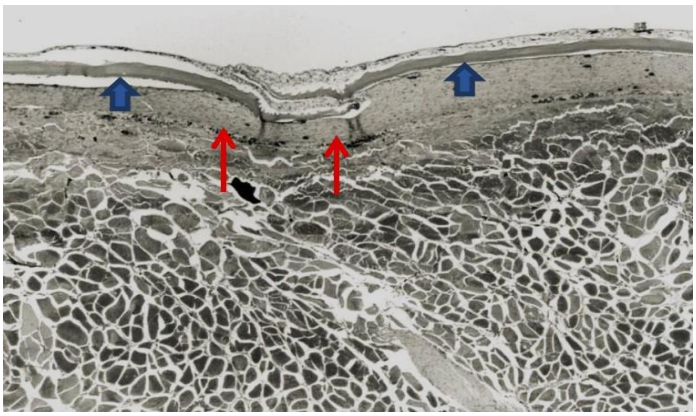

Figure 23. At 35 days p.i. the lesion area almost recovered. The scar had joined up with the dermis (arrows). Re-grown muscles were well developed and filled the defect with highly reduction of the connective tissue and cellular components. Two well developed new scales (arrow heads) are seen above the healed area of the lesion. (H & E, X 44).

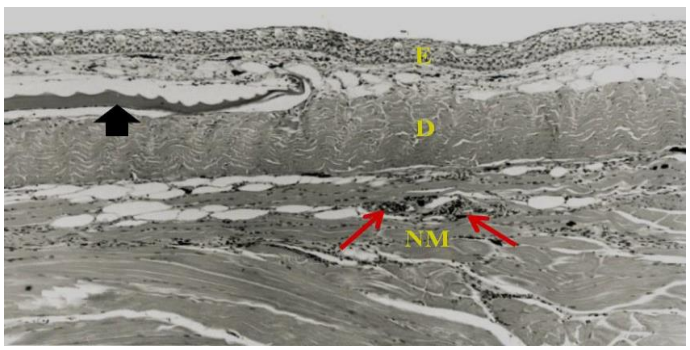

Figure 24. By 42 days p.i. lesion area was similar to a normal tissue. The scar completely filled with new muscles (NM) and joined with dermis (D). A few cellular components remained in the area (arrows). The epidermis (E) and dermis had recovered their normal form. A new scale (arrow head) above the healed area was obvious. (H & E, X 110).

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Control fish

No histological changes were observed in tissues of control fish injected with sterile saline. Also *A. hydrophila* was seen neither in the histological sections of tissues nor isolated from the sampled tissues of control fish by bacterial tests.

Discussion

Fish diseases are the outstanding reason of fish mortality in aquaculture, which is mainly caused by *Aeromonas* (Hamouda *et al.*,, 2019). Universally, *A. hydrophila* is the most common freshwater bacterium are integrated with disease exposure and cause of economic detriment for aquaculture industry. This bacterium has earned resistance to a wide domain of antibiotics, so strong preventive measures are necessary to control its spread (Marinho-Neto *et al.*, 2019).

Infectious diseases are becoming destructive factor especially in developing countries, where aquaculture is operational with limited financial resources. One of the constraints faced in major carp culture in these countries is the high temperature and humidity during Monsoon season, which provides a desirable environment for *A. hydrophila* to grow in ponds resulting in high risk of infectious diseases in fish (Kousar *et al.*, 2020). Keeping in view the increasing importance of vaccination as immunoprophylaxis measure for disease prohibition, Sughra *et al.* (2021) were developed inactivated vaccines against pathogenic *A. hydrophila* for cultured fish species.

Bacterial diseases caused inflammations in organs of many cultured fish. The histopathological studies on different target organs such as; gill, kidney and liver, are useful to assess the intensity of damage to fish due to infection. Adanir and Turutoglu (2007) studied the pathological findings of *A. hydrophila* in carp (*Cyprinus carpio*). They isolated pure cultures of *A. hydrophila* from the skin, liver, heart and kidney of carp.

Also the pathomorphological changes in A. *hydrophila* infection in carp provoked by different field strains of this bacterium were investigated in another research by Stratev *et al.* (2015) .The strongest histopathological damages were seen in the functional epithelium of many organs such as liver and kidneys, intestine, followed by heart damages in addition to various hemorrhages in interstitial tissues of visceral organs and some skin hemorrhages on the ventral surface of the body and the anal region. Generally, pathological damages in internal organs and

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hemorrhages were vigorously in experimental fishes exposed to higher levels of the respective pathogens and less pronounced damages were seen in fishes infected with of A. *hydrophila* isolated from a silver carp with marked indications of septicaemia.

In another study, the intestinal swelling in grass carp (*Ctenopharyngodon idella*) due to *A. hydrophila* strain was investigated and histopathological examinations exhibited that severe intestinal lesions and villus blending and peeling were caused by *A. hydrophila.* Also, the pathological variations in the intestines were observed after 21 days (Song *et al.*,2014).

The researchers had proved that when A. *hydrophyla* were introduced, the phagocytic activity of fish blood cells, neutrophil activity, adhesion indices and the bactericidal activity of blood serum increased. Among the markers of homeostasis constancy, immunological indicators mirror the mechanisms of initiation, development, and output of the infectious phases facilitated by the interaction of adhesive molecules of multicellular eukaryotes and adhesives of infectious disease pathogens (Lenchenko *et al.*, 2022).

The gross pathological changes of the infected carp in this study were generally similar to those described by Brenden and Huizinga (1986) in gold fish and Chinabut (1989) in snakehead in relation to intramuscular injection of *A. hydrophila*. These changes also were similar to those described by Roberts (1993) as clinical signs of motile aeromonad septicaemia.

The first 48 hours post-inoculation of *A. hydrophila* was the most critical and fatal period in this study. During this period more than one quarter of the infected fish died with a severe generalised erythematous reaction over the whole-body surface, which indicated, probably, a toxaemic condition. Haemorrhage is a conspicuous feature of *A. hydrophila* infections in poikilothermic animals (Snieszko, 1974; Hazen *et al.,* 1978). Haemolysin is suggested as the major active toxin and virulent factor (Rigney *et al.*, 1978; Thune *et al.*, 1986) which is a major lethal factor rather than protease (Allan and Stevenson, 1981). Aoki and Hirono (1991) cloned and characterised haemolysin as the causal agent of haemorrhages in *A. hydrophila* infections, and Olivier *et al.* (1981) reported that haemolysin can haemolyse erythrocytes resulting in anaemia in fish. However, haemorrhage in *A. hydrophila* infections maybe an outcome of involving some of the extracellular products of this bacterium

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together such as; haemolysin, elastase and protease.

Extensive haemorrhage and mortalities in carp in the present study, therefore could be a result of the ability of *A. hydrophila* to damage the elastic and collagenous fibres of blood vessels, and also lysis of red blood cells and breakdown haemoglobin by lytic toxins properties. Death of the infected fish may also be resulted from a systemic disturbance involving the haematopoietic system and liver. The recovery of those fish which survived following inoculation of *A. hydrophila* showed that they might have developed immunity after inoculation, since from 7 days after infection onward, presence and activity of lymphocytes increased in the lesion area. Lymphocyte cells are immunocompetent cells which are responsible for initiating and mediating the aspects of specific immunity (Ellis, 1989). Baba *et al.* (1988*a*) showed that the defence mechanism in crude LPSDP (lipopolysaccharide)-immunised carp is composed of a thymus-derived sensitised lymphocyte-macrophage system. Baba *et al.* (1988*b*) also showed that the protection against *A. hydrophila* infection in carp is not dependent on humoral immunity. This indicates the probability of differences in individual fish defence mechanism, since the bacterial strain, dose of inoculum, route of infection, the techniques used, water system and water temperature were the same for all infected fish in the present study. Also, all lesions in the surviving fish healed, and prolonged infections limited to skin and muscle did not occur. This is similar to that of channel catfish experimentally infected by *A. hydrophila* (Ventura and Grizzle, 1988).

This study showed that the bacteria were present in the tissues of lesion of the fish and also re-isolated from the infected tissues after injection, but not after 5 days post-injection, which is similar to that of walking catfish reported by Angka *et al.* (1995). The disappearance of the injected micro-organisms could be due to the onset of development of effective immunity (Angka *et al.,* 1995). Also, clearance of bacteria from tissue has been shown to be temperature-dependent, and the time taken for clearance is much slower at low temperature (Finn and Nielson, 1971*a*).

Fibroblasts activity for healing the lesion began by 2 days after injection with presence of some pale swollen fibroblasts in the myotomal fascia and around the blood vessels. Their number and activity increased by 4 days and fibroplasia and fibrosis was active at 5 days after injection. The process of wound healing began and developed clinically and histologically by

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5-10 days post-injection. Ventura and Grizzle (1988) reported that in channel catfish infected with *A. hydrophila* at 18°C, fibroblasts had proliferated in necrotic areas by day 8. Chinabut (1989) reported that in snakehead, intramuscularly infected by *A. hydrophila* at 28.5°C, wound healing began by day 7-10. She reported that healing area was distinguished grossly as being darking colour, which is the same as that seen in the healing area in the present study. The rate of wound healing in this study was faster than that of above-mentioned channel catfish and snakehead, respectively.

The PMNs infiltrated into the lesion area at the first hour after infection in only a few numbers and increased gradually while the lesion progressed. They were one of the dominant inflammatory cells in the lesion area especially throughout the time of major activity, and remained until 7 days post-injection. These observations correlate with the findings of Brenden and Huizinga (1986) in gold fish (temperature not available) and Chinabut (1989) in snakehead at 28.5°C, who found PMNs at the early hours after injection and the dominant role of them throughout the sampling period. Anderson and Roberts (1975) reported the appearance of PMNs in small numbers around blood vessels at 55 hours after wounding in *Salmo salar* at 23°C, and remained for 6 days, then disappeared. They suggested that different time scale in appearance of PMNs in fishes may have been the result of either a species difference becoming evident at higher temperatures, or the presence of bacterial factors providing a powerful attraction at high temperature. They concluded that attraction of PMNs to bacterial factors appeared only to function at high temperature. Roberts and Bullock (1976) also noted that the exact period of the presence of PMNs in an inflammatory response depending on the type of insult and the temperature. They also stated that the role of the PMNs in inflammatory response in fish is relatively minor comparing to that in mammals.

One of the roles of PMNs documented in this study was participating in myophagia along with macrophages. At 4 hours after infection some PMNs and macrophages were observed located within the degenerated muscles which could be considered as the initiation of myophagia. Thereafter they were observed within the damaged muscles engulfing the remnants of the damaged sarcoplasms. Myophagia was completed by 7 days. Ventura and Grizzle (1988) reported the presence of large numbers of lymphocytes and macrophages as early as 24 hours after injection of *A.*

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hydrophila in channel catfish at 18°C. Chinabut (1989) reported the presence of small numbers of macrophages in snakehead at 28.5°C in the damaged muscle area by 4 hours which increased by 6 hours. She stated that they were not principally attracted to the bacteria, but she did not mention the presence of PMNs along with macrophages in the damaged muscles.

PMNs in mammals are major phagocytic cells which appear rapidly at the inflammatory area (Ellis, 1977), while there are different comments about their function in fish. Roberts and Bullock (1976) believed a relatively minor role of the PMN leukocytes in fish response as a significant difference from the mammalian response. Klontz (1972) and Ellis (1976) reported that plaice and rainbow trout PMNs appeared not to be phagocytic towards carbon particles or bacteria. While Finn and Nielson (1971a, b) reported PMNs in rainbow trout as a phagocytic cell in a bacterial infection. Also, it has been reported a phagocytic role for gold fish PMNs towards thorotrast (Weinreb and Weinreb, 1969) and bacteria (Watson *et al.*, 1963) and for three-spined stickleback towards carbon (Phromsuthirak, 1977).

The other role of PMNs seen in this study, was contributing in micro-abscess formation. A dense aggregation of PMNs in some areas of the lesion along with some amorphous substance, macrophages, degenerated muscles and nucleic debris formed a micro-abscess. Although the bacteria were not seen in the sections of some cases, *A. hydrophila* was re-isolated from the infected fish. As described by Anderson and Roberts (1975), the presence of bacteria could be a powerful attraction for PMNs to infiltrate, dominate and aggregate in the lesion area producing micro-abscess, especially considering the average ambient temperature (27.5°C) in this study. It also could be concluded from this study that the nature of the lesion in *A. hydrophila* infection, especially in early stages is suppurative. Chinabut (1989) also reported micro-abscess formation in snakehead in response to the *A. hydrophila* infection. She described micro-abscess as the most unexpected finding in her study which was exactly similar to that in higher animals, and mentioned that it is most uncommon in fish lesions. Roberts and Bullock (1976) stated that abscessation does not occur spontaneously in fish, although aggregates of PMNs which could be described as micro-abscesses, are very occasionally seen. Roberts (1989) described that liquefactive necrosis is a result of rapid enzymatic digestion of cells in tissue. He stated that liquefaction is

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produced either by enzymes released from host cells such as PMNs or by lytic toxins released by infecting bacteria.

The pathological changes at the site of injection occurred mainly in the muscular layers and also in the dermis because of their first contact and encounter with the bacteria, therefore the lesion could be considered as a dermal and hypodermal lesion. As the lesion developed, there was an accumulation of necrotic tissue and tissue fluid exudate with some inflammatory cells under the dermal layer, extending to the deeper muscular layer causing a swollen lesion to the outside. This swollen and fluctuant lesion ultimately ruptured and released exudate, containing breakdown products of necrotic tissue and bacteria into the water. The epithelial layer at the point of injection and around, was also necrotic and destroyed. The early lesion showed massive muscular degeneration with less infiltration of inflammatory cells indicated a toxic condition.

The histopathological changes at the site of injection were similar to those reported by Thorpe and Roberts (1972) in rainbow trout, Brenden and Huizinga (1986) in gold fish, Ventura and Grizzle (1988) in channel catfish, Chinabut (1989) in snakehead, and Suthi (1991) in *Pontius* sp. and *Oreochromis niloticus*.

Kaper *et al.* (1981) reported that severe damage to the muscle at the area of injection may have been caused by the enterotoxin produced by *A. hydrophila*. Brenden and Huizinga (1986) suggested that the progressive skeletal muscle necrosis at the site of inoculation maybe due to elastase and protease which are produced *in vitro* by *A. hydrophila*, and elaboration of these enzymes *in vivo*. Scholz *et al.* (1974) suggested that the unusually low inflammatory cells in some necrotic tissues maybe caused by leukocidins produced by *A. hydrophila*.

Anderson and Roberts (1975) reported that scale regeneration in wounded salmon began at the wound edge at 10 days, and within the wound by 22 days after wounding at 23°C, while Chinabut (1989) reported the first appearance of the scale buds 5 days after wounding of snakehead at 28°C, and were well formed within 14 days. Protrusion of the scales close to the lesion area due to the dermal inflammatory oedema was begun at the early stages of inflammation in the present study. Some of these scales sloughed off as the lesion developed. From the 18th day after inoculation onwards, some small newly formed scales were seen growing above the newly re-joined dermis in the stratum

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spongiosum. They were later seen above the healed lesion on the edges of the scar, well-formed and arranged in an overlapping pattern in the same position as the original scales. These small new scales were formed and grew in the area of the healed lesion where originally no scales grow. It is necessary to mention that experimental fish in this study (mirror carp) has only one row of scale on the flank close to the dorsal fin along the lateral line. The relation between healing the lesion and these new scales was not investigated in this study. It could be a matter for further investigation, especially considering that in integumental wound healing no new scale grew around the scar of the healed skin of mirror carp (Sharifpour, 2004).

At 10 days after inoculation, in one of the sampled fish a typical foreign body granuloma was found in the lesion area. It was consisted of a limited amorphous pink staining substance, similar to a newly grown small scale, located in the centre of the granuloma and macrophages, epithelioid cells, nuclear debris which was encapsulated by fibrous tissue and lymphocytes. A distinct horse shoe shaped Langhans giant cell was also observed around this granuloma. Roberts (1989) suggested that if an acute inflammatory lesion does not resolve quickly, then chronic inflammation with proliferation of neighbouring support tissues develops. He also explained that chronic inflammation, with development of a proliferative lesion progressing to fibrosis is called granuloma which may be caused by different foreign bodies and may have different colour and consistency. Roberts and Bullock (1976) described those certain types of particularly resistant foreign bodies within the skin, produce a very distinctive chronic inflammatory response characterised by presence of epithelioid cells around the stimulus and also subsequent formation of giant cells by fusion of epithelioid cells. Also, Timur (1975) reported that the Langhans type giant cell is frequently seen in foreign body granulomata due to a variety of irritants. Considering these explanations, acute inflammation occurred during the early hours after inoculation of the fish in this study and then developed into chronic inflammation characterised by fibroplasia and fibrosis in the lesion area. The foreign body granuloma formation in the lesion area with presence of a giant cell is an unusual response to *A. hydrophila* infection which to this author's knowledge has never been reported before. It could be described that the limited amorphous, pink staining substance in the centre of the granuloma, maybe a newly grown scale

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which penetrated into the hypodermal area, considering that from the 18th day new scales were evident in the lesion area. İf it is a new scale, it is difficult to explain and remained unclear how the newly grown scale penetrated into the subdermal area. However, whatever it is, the defence mechanism of fish considered it as an irritant which is resistant and could not be phagocytised by phagocytic cells, so it was surrounded by granuloma and giant cell formation to prevent irritation of the tissue. The macrophages loaded with melanin granules, lipofuscin and ceroids were commonly found at late stages in the fibrous replacement area throughout the healing lesion. Melanin is often associated with lipid residues from the breakdown of cell membranes (Edelstein, 1971).

Lipofuscin is a visceral melanin (Cappell and Anderson, 1971), a peroxidised and polymerised breakdown product of lipid catabolism and tissue destruction (Miquel *et al.,* 1977; Ventura and Grizzle, 1988). It maybe the by-product from the oxidation of tissue lipids during an inflammatory response as a consequence of peroxidase, released by PMNs (Roberts, 1975*a*). One of the main sources of lipofuscin maybe degenerating mitochondria, with their high content of polyunsaturated fatty acids (Agius and Agbede, 1984). Lipofuscin in association with bacterial and viral diseases can indicate phagocytic clearance of necrotic lesions (Wood and Yasutake, 1956). Undigested lipid-rich substances are usually accumulated as lipofuscins (Goudie, 1988). Any metabolic disorder could make lipids difficult to digest and, in consequence, the lipids would accumulate in the fish tissue (Lamas *et al.,* 1996). Under normal conditions, lipofuscins are endogenous pigments which accumulate in several organs with age and are yellowish-brown (Woolf, 1986). However, depending on the original lipid and where the oxidation takes place, it can be a highly coloured product (Pearse, 1985). Oxidised lipids are toxic for fish (Roberts, 1989). Usually, these lipids are acid-fast, and in consequence are identified as ceroids. Ceroids are considered to be typical lipofuscins in an early stage of oxidation (Pearse, 1985). Ceroid usually accumulates in the liver, spleen and kidney of fish fed with rancid lipids or which is vitamin E deficient (Smith 1979; Roald *et al.,* 1981; Moccia *et al.,* 1984).

The acid-fast pigment ceroid is commonly associated with metabolic disorders in laboratory animals (Wood and Yasutake, 1956). It has been reported that fish with their high content of unsaturated fatty acids are particularly susceptible to formation of

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ceroid (Yasutake *et al.,* 1965).

Muscle regeneration began at the day 4 after infection with the presence of some new small basophilic muscle buds, along with fibroplasia in the lesion area, and their growth progressed as the lesion developed. At the last stage of sampling, the regenerated and regrown muscles were well developed and filled all the damaged area of the lesion so that they were indistinguishable from the normal muscles. Chinabut (1989) reported that the regeneration of myofibrillar buds in snakehead infected by *A. hydrophila* at 28.5°C was seen around day 6 in the area around the centre of the lesion and considered it as a distinctive feature which has not been described elsewhere in other species. However, the muscle regeneration in carp at 27.5°C in the present study was faster than in snakehead.

Chen *et al.* (2018) surveyed histopathological assays and the immune related gene expression profiles of mandarin fish (*Siniperca chuatsi*) infected with *A. hydrophila*. Histopathological examinations exhibited that inflammation, vacuolization and wide necrosis appeared in the gill, liver, spleen and head kidney of the diseased fish; so, these organs might be target of A. *hydrophila* infection. The results of the present study in skin and muscle of carp showed the same inflammatory response they reported from internal organs of mandarin fish.

It is described in literature that physiological and anatomical differences between fish species, different species defence mechanism, virulence of the bacterial strains, route of the introducing infection, and accuracy of the techniques used, are major factors in the degree of bacterial infection and subsequent effects on fish. In this study, it was determined that difference between individuals of a species was also another major factor.

Intramuscular route of infection was used in this study to inoculate the bacterial suspension. It could be said that the intramuscular injection is a suitable route for induction of the experimental *A. hydrophila* infection, considering that the normal route of infection in the wild is more likely to be via trauma or other damages to the skin, and not by the vascular, intraperitoneal or oral (via stomach) routes. The results of this study indicated that the rate and degree of infection by the T4 strain of *A. hydrophila*, used in this experiment, in healthy carp, depends on the individual defence mechanism of fish, and those fish which survive from the infection by 48 hours after infection, have a well-developed capacity for dealing with bacteria. Wound

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healing, and muscle regeneration in these fish were faster than those infected experimentally by *A. hydrophila* mentioned before.

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