Iranian Journal of Fisheries Sciences 22(4) 771-789 2023
DOI: 10.22092/ijfs.2023.129755

Research Article

Histology of the inflammatory response of carp (*Cyprinus carpio* L.) to fungus, *Aphanomyces invadans*, infection

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Received: November 2022 Accepted: March 2023

Abstract

The present investigation was carried out to study the inflammatory response of carp (*Cyprinus carpio* L.) to the fungus *Aphanomyces invadans* infection which is known as a causative agent of Epizootic Ulcerative Syndrome (EUS). Thirty two carps with an average length of 13cm (±2cm) were injected intramuscularly with 0.1 ml of the *Aphanomyces invadans* spore suspension. Two injected fish were sacrificed by an overdose of 10% benzocaine, at 6 hours, 1, 2, 3, 4, 5, 6, 7, 10, 14, 18, 22, 28, 35 and 42 days after inoculation. Blocks of tissue were fixed in 10% buffered formalin then processed and stained with haematoxylin and eosin, Grocott and periodic Acid Schiff (PAS) for histologic examinations. A chronic inflammatory response, consisted of cellular infiltration, vascularization, fibrosis and granulomata formation, occurred after inoculation of spores of *Aphanomyces invadans* at water temperature of 27°C (± 1.5°C). Macrophages which infiltrated the lesion area in early stages took on an epithelioid configuration at 3 days post injection. In addition to classical Langhans and foreign body type of giant cells which were observed 3 days after infection, also intermediate type was seen in the lesion area. With continuing healing, giant cells reduced in number by 14 days and disappeared at 18 days after injection. Muscle regeneration started at 3 days and the defect area was filled by new muscle bundles by 14 days. Fibroplasia, along with vascularization, started at 3 days and well developed granulomata formed by 10 days and then fully matured granulomata which filled the whole defect area and were surrounded by the normal muscle bundles were observed by 18 days post injection. It is apparent from the results of the present study that healthy carp resist *Aphanomyces invadans* infection at high temperatures (27°C) by an active defence mechanism and employing a strong inflammatory response.

Keywords: Infelamtory response, Fungus, *Aphanomyces invadans*, Histological examination, EUS, Carp

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Introduction

Mycotic infections of fish by fresh water Oomycetes can develop at all stages of the fish's life cycle and as such are of considerable economic significance to the intensive fish cultivation industry (Pickering and Willoughby, 1982). Fungi are distinguished principally by their complete lack of chlorophyll. They are responsible for a range of serious and economically important diseases of teleosts. Absence of chloroplasts means that they cannot make use of photosynthetic pathways for energy production and therefore are bound to live a saprophytic or parasitic existence (Roberts, 1989). There are at least 100,000 species of fungi, which show great diversity in their morphology. In relation to fish diseases, they are conveniently divided into two large grouping, those with cross cell walls, the septate fungi, and those which are aseptate or without cross walls. Fungi with aseptate mycelium are placed in grouping *Phycomycetes*; these include *Saprolegnia* spp., probably the most important fish pathogenic genus (Willoughby, 1994).

Fungal infections in fish may arise as primary infections or as secondary invasion of tissues which are already damaged by viral, bacterial or other mechanical agencies (Khulbe and Sati, 1981). Mycotic diseases of fish have been reported in both fresh and salt water environment (Wolke, 1975). Most living organisms under certain circumstances become subject to the attack of fungi, and fishes are no exception. It is well known that whenever freshwater fishes are roughly handled or even slightly bruised, fungus infection and a high rate of mortality are likely to follow. In addition, under both natural and hatchery conditions, almost all fish eggs are susceptible to fungus attacks (Scott, 1964).

The family Saprolegniaceae contains the majority of fungi that have been associated with diseases in fish and shellfish (Willoughby, 1994). Members of family Saprolegniaceae which have been recorded as parasitic in fish are *Saprolegnia*, *Achlya*, *Aphanomyces*, *Dictyuchus*, *Pythium*, *Leptolegnia*, and *Leptomites* (Alderman, 1982). The most important genera of the family Saprolegniaceae in terms of pathogenicity, are *Saprolegnia, Achlya* and *Aphanomyces* (Willoughby, 1994).

Slow-growing *Aphanomyces* strains have been isolated from fish suffering from red spot disease (RSD) in Australia (Fraser *et al*., 1992), mycotic granulomatosis (MG) in Japan (Hatai *et al*., 1977), and epizootic ulcerative syndrome (EUS) in South East Asia (Roberts *et al*., 1993). Lilley and Roberts (1997) showed that a single species of *Aphanomyces* is involved in RCD, MG and EUS. Willoughby *et al*. (1995) have proposed the name *Aphanomyces invadans* for this species.

EUS caused by fungus *Aphanomyces* has been reported in over 100 species of fish (Frerichs *et al*., 1988; Lilley *et al*., 1992) and to date, more than 160 fish species belonging to 54 families under 16 orders are reported to be susceptible to the disease (Afzali *et al*., 2015).

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Aphanomyces invadans, an oomycyte fungus most frequently recognized as a causative agent of epizootic ulcerative syndrome (EUS) is a seasonal epidemic pathogen of great importance in wild and farmed fish in both freshwater and estuarine environments. EUS is a complex infectious etiology which leads to necrosis ulcerative lesions and granulomatous response in fishes. It is a cause of death of approximately 92 species that has been recorded in wild as well as in commercial culture systems worldwide. Several environmental and biological factors are responsible for the growth and establishment of *A. invadans*, which further attracts secondary pathogens to enter the lesions thus, increasing the severity of the infection (Kumar *et al.*, 2020).

It has been shown that *Aphanomyces invadans* can produce haemorrhagic lesions and high mortalities during the outbreaks of EUS (Chinabut *et al*., 1995), but there is no report to indicate that *Aphanomyces invadans* can breach the fish skin integrity, primarily.

Epizootic ulcerative syndrome (EUS), caused by the oomycete *Aphanomyces invadans*, is one of the most devastating diseases of fresh and brackish water fishes. The disease is of international concern and reportable to the World Organization for Animal Health (Verma *et al.*, 2021). Although the major group of species including murrels, cyprinids, barbs, gouramis, clarids are reported to be susceptible, but some species like common carp (*Cyprinus carpio)*, milkfish *(Chanos chanos)* and tilapia (*Oreochromis niloticus*) are resistant to this disease (Pradhan *et al.*, 2014; Herbert *et al.*, 2019).

The present investigation was conducted to study histology of the inflammatory response of healthy carp (*Cyprinus carpio*) to fungus *Aphanomyces invadans* which is known as a causative agent of Epizootic Ulcerative Syndrome (EUS).

Materials and methods

Fish

Sixty-eight mirror carp were kept in glass aquaria for acclimation for 3 weeks before the experiment began. The average length of fish was 13 cm (±2 cm) and they were fed with pelleted food once per day.

Aquaria and water

The fish were held in a recirculating glass aquaria system, 122×32×42 cm. The water system consisted of dechlorinated, aerated fresh water, at an average temperature of 27°C (± 1.5°C). Aeration, circulation and filtration of water was supplied via a standard Fluval 303 external power aquarium filter system (ASKOLL, Italy) and air pump. Although filtration allowing mechanical, biological and chemical water quality control, further monitoring was carried out using the Dry-Tablet Master Test Kit (Aquarium Pharmaceuticals, Inc.). This allowed measurement of ammonia (NH_3 / NH_{4+}), nitrite (NO^{2-}), nitrate (NO^{3-}) and pH. Faecal material was also

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removed from aquaria every day. Depending on the results of the water quality measurement, between 10-50 percent of the water was changed, as necessary.

Preparation of inoculum

An *Aphanomyces invadans* isolate (PA7) was obtained from the collection maintained at the Institute of Aquaculture, University of Stirling, UK. The culture was grown in petri dishes containing glucose-pepton-yeast (GPY) broth ($3gl^{-1}$ glucose, $1gl^{-1}$ peptone, $0.5gl^{-1}$ yeast, $0.128gl^{-1}$ $MgSo_4.7H_2o$, $0.014gl^{-1}$ KH_2Po_4, $29.3mgL^{-1}$ $CaCl_2.2H_2o$, $2.4mgL^{-1}$ $FeCl_3.6H_2o$, $1.8mgL^{-1}$ $MnCl_2.4H_2o$, $3.9mgL^{-1}$ $CuSo_4.5H_2o$, $0.4mgL^{-1}$ $ZnSo_4.7H_2o$). After 3 days at 20°C, mycelial mats were washed in five changes of autoclaved pond water (APW: 1 part filtered pond water, 2 parts distilled water). Thereafter mycelial mats were incubated overnight at 20°C to allow for production of secondary zoospores. After the incubation period, mycelia were examined under phase contrast microscope to confirm the presence of motile secondary zoospores. A suspension of these zoospores was used as inoculum for injection.

Injection procedure

Thirty two mirror carp with an average length of 13cm (±2cm) were injected intramuscularly on the left flank with 0.1 mL of the spore suspension. The fish were anaesthetised with 10% benzocaine (0.5 mL/L) before injection. Inoculation was made using a 1 ml syringe and 25-gauge needle. The injection was made in line with the first ray of the dorsal fin, below the scale row, as a marker for subsequent sampling. Control fish were injected intramuscularly on the left myotome with the same volume of sterile normal saline. After injection, the fish were kept in the aquaria until they were sacrificed.

Histological sampling procedures

Two injected fish were sacrificed by an overdose of 10% benzocaine, at 6 hours, 1, 2, 3, 4, 5, 6, 7, 10, 14, 18, 22, 28, 35 and 42 days after inoculation. Blocks of tissue encompassing the injected site were immediately cut out and fixed in cold 10% buffered formalin for at least 24 hours before processing. Formalin was changed and refreshed at least once during the fixation period. 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.

Staining procedures

The standard H& E staining method was used for the routine examination of sections. Special stains were used for

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observing the fungus in tissue sections namely Grocott, and periodic acid Schiff (PAS). The stained sections were mounted in Pertex mounting medium (CellPath plc, Herts, England) with a cover glass for light microscopic examinations.

Results

Gross pathology

There were no gross signs visible of any change indicating inflammatory responses in the surface of the body of the injected fish with *Aphanomyces invadans*, during the course of the study.

Histopathology

6 hours

At 6 hours after inoculation of the spore suspension of *Aphanomyces invadans*, there was sarcoplasmic degeneration throughout the area of injection. Also initiation of myophagia was evident. There were haemorrhages in the damaged area and some hyperaemic blood vessels with melanin granules around at the edges of the lesion. Infiltration of inflammatory cells such as PMNs, lymphocytes, macrophages, and thrombocytes with fibrin strands in the lesion area and between the necrotic myofibres was obvious (Fig. 1). Swelling of some fibroblasts in the intermyotomal fascia adjoining the effected myotome at this stage indicated the initiation of fibroblast activity.

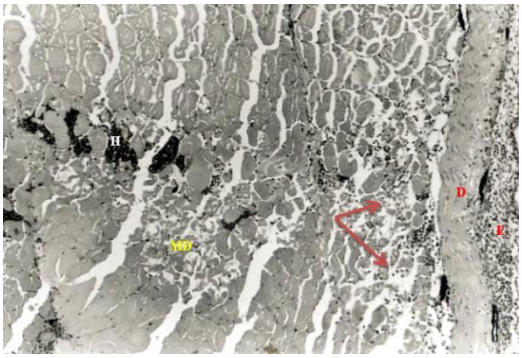

Figure 1: At 6 hours post-inoculation of *Aphanomyces invadans*, muscle degeneration (MD), haemorrhage (H) and myophagia (arrows) were observed in the damaged area. (H&E, 110X) E=epidermis, D=dermis

1 day

By this time, muscle degeneration was increased and spread throughout the injected area. Myophagia was active. Macrophages, lymphocytes, PMNs and thrombocytes with fibrin strands had increased in the inflammatory area. Some new swollen fibroblasts, with pale staining nuclei, were evident in the intermyotomal fascia and around the blood vessels (Fig. 2).

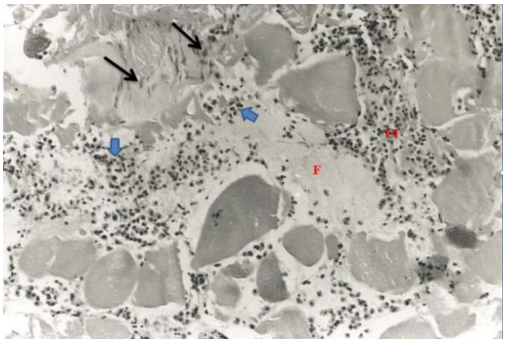

Figure 2: By 24 hours p.i., myophagia was active (arrow heads) and cellular infiltration (CI) with fibrin (F) was increased. Also some swollen fibroblasts with pale staining nuclei (arrows) were observed. (H&E, 220X)

2 days

The dominant feature at this stage was myophagia and presence of active macrophages. The necrotic myofibrils

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were spread throughout the lesion area. Also small number of fibroblasts had migrated in the defect area.

3 days

By the 3rd day, macrophages were present in a whorling epithelioid cells pattern in the lesion area and around the fungus. Also myophagic macrophages were active and myophagia was still in progress in some areas. Degenerated muscles had been removed by myophagia and were replaced by epithelioid cells and some fibrous tissues. Fibroplasia was active along with vascularization. Blood vessels and new capillaries were hyperaemic and some local haemorrhages were seen in the lesion area. Initiation of muscle regeneration with redeveloping peripheral nucleation in myofibrils which had been slightly damaged and also with presence of some new basophilic small buds of muscle was evident. First presence of giant cell types (Langhans and foreign body types) in the defect area was clearly evident. Also giant cells of intermediate type were seen. There were some fungus hyphae in the infected area which were surrounded by epithelioid cells (Figs. 3, 4 & 5).

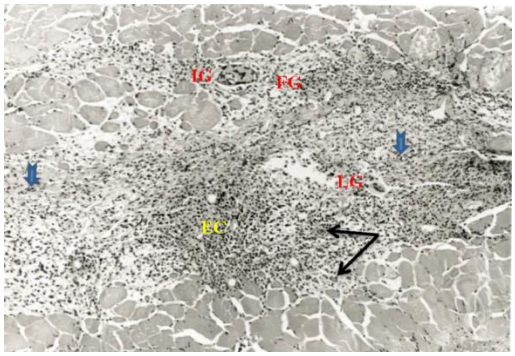

Figure 3: This picture shows a general view of the lesion area at 3 days p.i. Macrophages are seen in a whorling epithelioid cells pattern (EC). Three types of giant cell, Langhans (LG), foreign body (FG) and intermediate (IG), and also new capillaries (arrow heads) and new muscle buds (arrows) are observed in the lesion area. (H&E, 110X)

4 days

At this stage the most defect area was filled with epithelioid cells which was dominant feature of the lesion. Myophagia was still in progress in the small area of necrotic muscle fibres. Myofibrils regeneration and also some new muscle buds were evident near the damaged muscle areas and in the fibrous tissue. Fibroplasia and fibrosis were obvious in the area, along with formation of new capillaries. Hyperaemic small blood vessels and some haemorrhages were present in the area. Lymphocytes, macrophages and epithelioid cells were inflammatory cells present in the lesion. More giant cells which mostly engulfed the fungal hyphae, and also were surrounded by epithelioid cells was evident. Fungus was obvious in the infected area surrounded by epithelioid and giant cells (Fig. 6).

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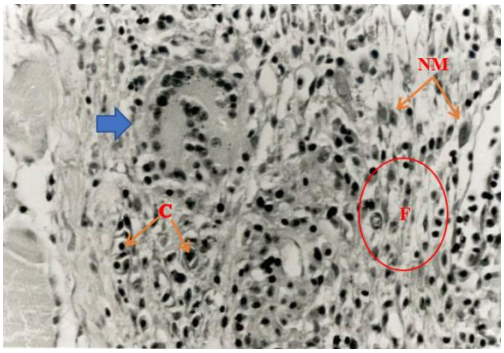

Figure 4: In addition of Langhans and foreign body type giant cells, intermediate giant cells (arrow head) were observed at 3 days p.i. Fibroplasia (F), new capillaries (C) and new muscle buds (NM) were also seen. (H&E, 440X)

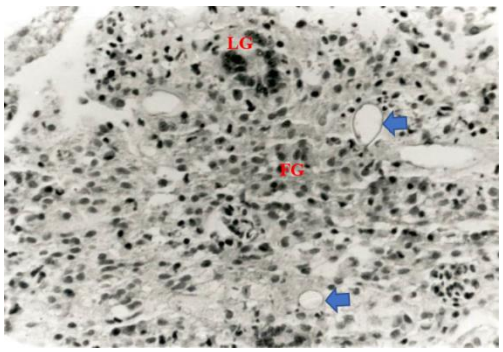

Figure 5: This picture demonstrates some fungus hyphae (arrow heads) surrounded by epithelioid cells. Langhans (LG) and foreign body (FG) giant cells are observed in the lesion area. (PAS, 440X)

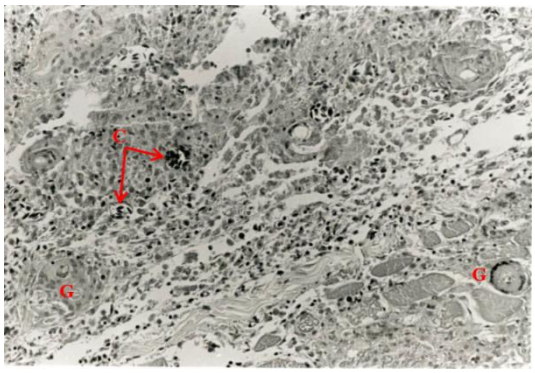

Figure 6: At 4 days p.i. most of the defect area was filled with epithelioid cells. Giant cells (G) which mostly engulfed the fungus hyphae, and also new capillaries (C) were observed in the area of the lesion. (H&E, 220X)

5-7 days

At the beginning of this period myophagia was completed and no necrotic muscle fibres were seen in the lesion, while the regeneration of muscle fibres was active. Presence of the epithelioid cells and granulation tissue was dominant picture in this period which filled all of the defect. Some layers of tightly bound epithelioid cells, surrounded by fibrous tissue, in some areas were evident. More giant cells comprising Langhans, foreign body and intermediate types, were evident in the area of the lesion. Fibrosis with active collagen formation were seen accompanied by forming new capillaries, while the previous formed new capillaries were active and nurishing the fibrosis area. Fungus was seen engulfed by giant cells and whorling epithelioid cells (Figs. 7-11).

10 days

After 10 days of inoculation, the main feature in the lesion area was granulation process. This process was well in progress and epithelioid cells accompanied by fibroblasts filled the whole defect area where muscle tissue had been largely removed by myophagia, this also extended up between the intact muscle fibres (Fig. 12).

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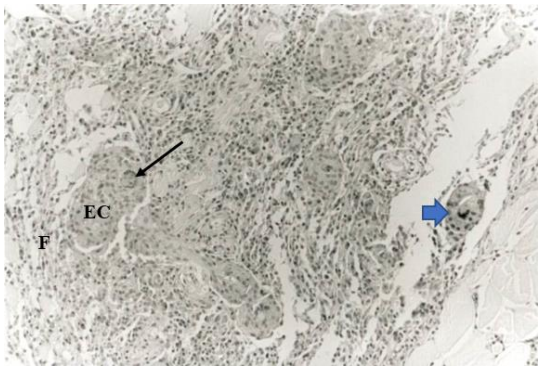

Figure 7: By 5 days p.i. myophagia was completed and presence of tightly bound epithelioid cells (EC) with surrounding fibrous tissue (F) were observed. An intermediate giant cell engulfing a fungus hypha (arrow head), and another one within a granuloma of epithelioid cell (arrow) are seen in this picture. (PAS, 220X).

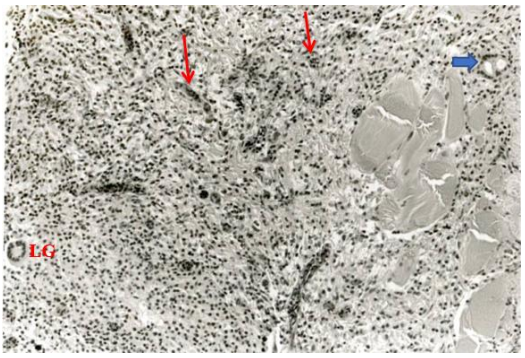

Figure 8: At 5 days after injection, vascularization was very active and new capillaries (arrows) were hyperaemic. A giant cell is observed in this picture engulfing fungus hyphae (arrow head), and another Langhan giant cell (LG) with no engulfed fungus. (H&E, 110X)

Some granulomata, consisting of dense layers of epithelioid cells which surrounded fungus, pink materials, nuclei debris, and also in some area degenerated giant cells were obvious in the lesion, surrounded by fibrous layer. Fibrosis with collagen formation was active along with marked new capillaries. More active giant cells which had engulfed fungus were observed between epithelioid cells. Repairing of certain damaged muscles was still in progress in some areas. New formed capillaries and small blood vessels were very active in the area of fibrosis; also large blood vessels were associated with melanin pigments. Fungi were seen distributed in the whole area of the defect but were surrounded by giant cells and epithelioid cells.

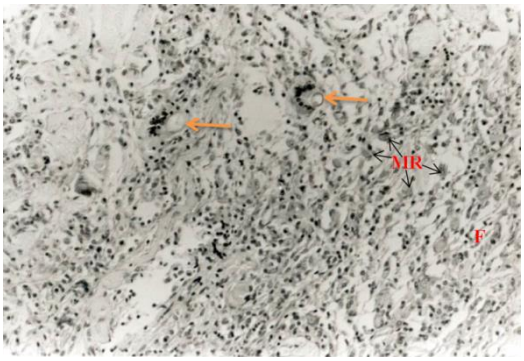

Figure 9: Fibroplasia (F) and muscle regeneration (MR) were active at 6 days p.i. Fungus hyphae are seen in this picture engulfed by giant cells (arrows). (PAS, X 220)

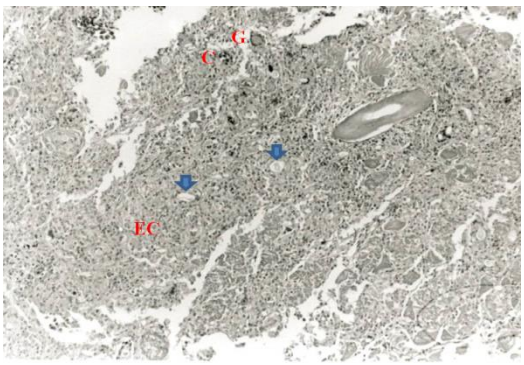

Figure 10: This picture shows a general view of the lesion area at 7 days p.i. comprising epithelioid cells (EC), giant cells (G), capillaries (C) and fungus hyphae (arrow heads) sequestrated by epithelioid cells. (H&E, 110X)

14 days

By 14th day post-inoculation, well developed small and large granulomata were dominant, composed of layers of bound epithelioid cells surrounded by variable thicknesses of fibrous

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encapsulations and often with a central area of necrotic materials. Also fusion of some granulomata had begun in the area (Fig. 13). Foci of lymphocytes aggregation, accompanied, to some extent, by macrophages and fibroblasts were seen in the fibrous tissue and around the granulomata. The number of lymphocytes and the level of their activity was considerable.

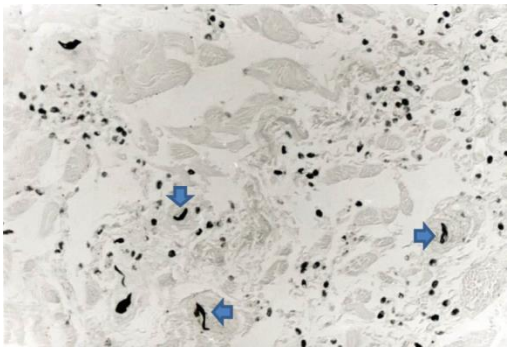

Figure 11: Fungus hyphae (arrow heads) sequestrated in the lesion area at 7 days p.i. are observed in this picture. (Grocott, X 220)

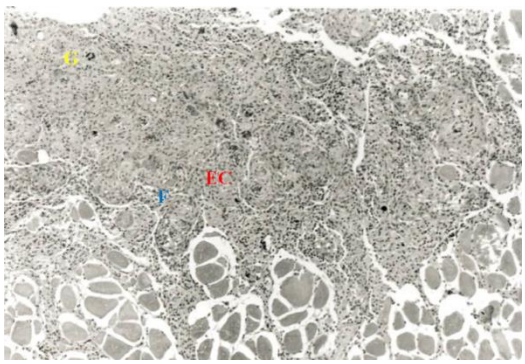

Figure 12: At 10 days p.i. granulation process was well in progress and epithelioid cells (EC) accompanied by fibroblasts (F) filled the whole defect area. Giant cells (G) also were observed. (H&E, 110X)

Also macrophages with lipofuscin in the cytoplasm were seen throughout the defect area. New capillaries were obvious, and small blood vessels were active in the area. An extreme reduction in the number of giant cells was evident as the lesion healing progressed, and a very few, if any, giant cells present in the area were surrounded by granulomata. Fungus was seen in the centre of granulomata.

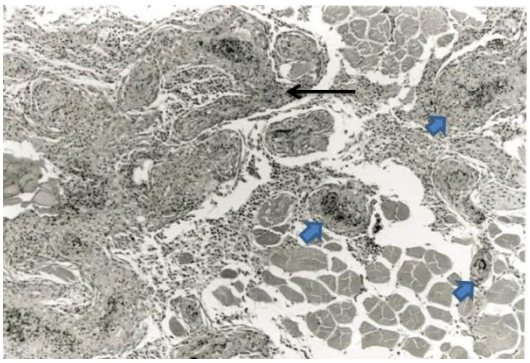

Figure 13: By 14 days p.i. well-developed small and large granulomata (arrow heads) were dominant in the lesion area. Also fusion of some granulomata was began (arrow). (H&E, 110X)

18-22 days

During this period the dominant feature was developing granulation process with more solidification. Fully mature granulomata of different sizes from small to large, separated or fused were evident and filled the whole defect area (Fig. 14).

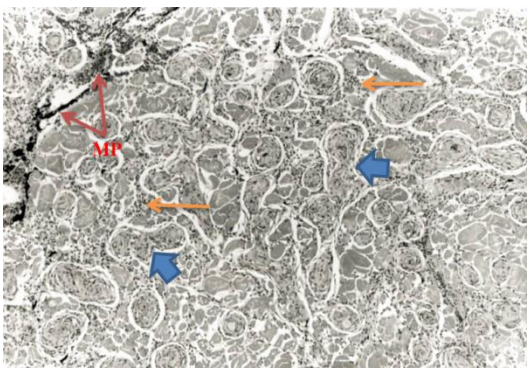

Figure 14: Mature granulomata of different size separated or fused (arrow heads) filled the whole defect area at 18 days p.i. Increasing in number of lymphocytes was obvious (arrows). Blood vessels were associated with melanin pigments (MP). (H&E, 110X)

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The epithelioid layers in granulomata were reduced, while fibrous layers around them increased. The centre of these granulomata was filled by effete macrophages, nucleic acid debris, pink deposit materials, and fungus which was degenerate in some granulomata (Fig. 15).

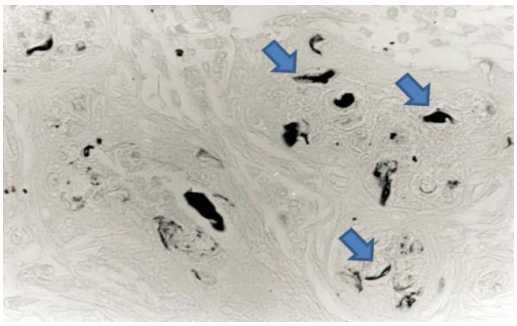

Figure 15: In addition to necrotic cells, nucleic acid debris and pink deposit materials, sequestrated fungus hyphae (arrow heads) were also observed at 22 days p.i. (Grocott, 440X).

Fusion of small granulomata was obvious and these fused granulomata were encapsulated by layers of fibrous tissue. High levels of lymphocytes activity, with increasing in the number and foci of lymphocytes aggregation especially around the granulomata and in the fibrous tissue, was obvious. Macrophages with lipofuscin in cytoplasm were seen in the area. Melanin pigments were distributed throughout the healing lesion area (at 18 days p.i.) and around the blood vessels. Some new capillaries were formed in the fibrous tissue and all the blood vessels were congested along with local haemorrhages (at 18 days p.i.) in the lesion area. No giant cell was evident in the lesion area at this period.

28-35 days

During this period, presence of the typical granulomata was the most obvious feature in the defect area (Fig. 16). The granulomata consisted of a masses of necrotic materials in the centre and a thick fibrous layer around. Almost no epithelioid cell remained intact in granulomata, and those that were observed were degenerate. Granulomata of different sizes, mostly small sizes, were fused together in different areas, forming larger granulomata surrounded by layers of fibrous tissue (Fig. 17). Macrophages with lipofuscin, melanin granules or ceroid in cytoplasm, and also foci of lymphocytes were obvious in the lesion area especially close to the granulomata. Some capillaries were seen in the fibrous tissue, and blood vessels in the area were congested, with surrounding melanin pigments. Degenerate fungus was observed in some granulomata.

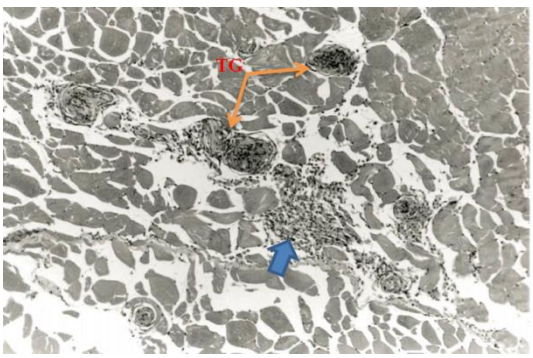

Figure 16: Presence of the typical granulomata (TG) among the normal muscles was the most obvious feature at 28 days p.i. A focus of cellular aggregation with lymphocytes domination is obvious in the picture (arrow head). (H&E, 110X)

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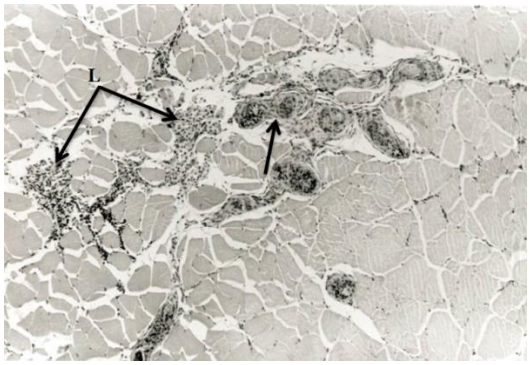

Figure 17: At 35 days p.i. granulomata of different sizes were fused (arrow), forming larger granulomata. Foci of lymphocytes (L) were seen in the lesion area. (H&E, 110X)

42 days

This was the last sampling time in this experiment. At this time more consolidation of the process of encapsulation was evident. The lesion was healed and regardless of the presence of granulomata in the area, the structure of the tissue was almost similar to a normal tissue. The granulomata were only composed of a massive amount of necrotic materials such as; epithelioid debris, pink materials, nucleic acid debris and degenerate fungus in the centre, and dense fibrous layers around. Most of granulomata in different areas fused together, formed large granulomata with surrounding dense fibrous layers (Fig. 18). Although the cellular components were reduced in the area, macrophages with lipofuscin, melanin and ceroid in cytoplasm, and also lymphocytes were present especially around the granulomata. Some capillaries were found in the fibrosis areas. All these capillaries and larger blood vessels were hyperaemic and some larger blood vessels were associated with melanin pigments. Degenerated fungi were seen in some granulomata.

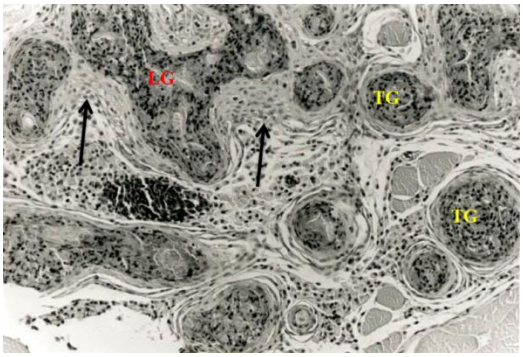

Figure 18: This picture shows the lesion area at the final sampling time, 42 days p.i. Typical granulomata (TG) are mostly composed of a massive amount of necrotic materials and surrounding dense fibrous layers. Fusion of granulomata (arrows) resulting formation of large granulomata (LG) is obvious. (H&E, 220X).

Control fish

No significant changes were detected in the fish injected with normal saline.

Discussion

Observations of the inflammatory changes in carp, in the present study, after intramuscular inoculation of *Aphanomyces invadans*, indicated the occurrence of the characteristic features of a chronic inflammation. The main features of this chronic inflammation were cellular infiltration, fibrosis and vascularization in the lesion area. Floccular and cloudy degeneration of the muscle fibres, and also moderate haemorrhage associated with the line of injection produced at the early stages of the infection, maybe traumatic damage caused by the passage of the needle inserting the inoculum, and the zoospore activity.

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The main components of the inflammatory response were the inflammatory cells comprising macrophages, lymphocytes, epithelioid cells, giant cells and fibroblasts. Corbel (1975) stated that the cellular response to mycotic infection in fish is quite varied, ranging from granulomata formation to complete absence of response. Along with myofibrilar degeneration and presence of the pathogen in the tissue, inflammatory cells began to infiltrate into the lesion area resulting the initiation of myophagia at 6 hours after inoculation in this study. Myophagia was completed at 5 days post-injection. Initiation of myophagia in carp in this study was faster than 12 hours for early myophagia in experimentally infected snakehead with *A. invadans*, at all temperatures used (i.e. 19, 26 and 31°C) (Chinabut *et al*., 1995).

Perimysium and inter-myotomal fascial were observed limiting the spread of necrosis in muscle in the present study which supports the findings of Finn and Nielson (1971a) in rainbow trout.

Some PMNs infiltrated into the lesion area at early stages and remained less than 2 days while the number of macrophages which had migrated to the necrotic area by 6 hours, was increasing. These times for infiltration of PMNs and macrophages were faster than that reported by Chinabut (1989) in snakehead infected with *Achlya debaryana* at 28°C. Pradhan *et al*. (2014) reported that higher number of inflammatory cells and more efficient epithelioid cell layer formation are believed to play an important role in the defence mechanism of yearlings of Indian major carps and *Cyprinus carpio* against *A. invadans* infection.

Lymphocytes were observed in the lesion area from the early stages. Their number and activity increased along with developing healing process and remained in the area until the final sampling time. This long-term presence and gradually increasing in the number and activity of lymphocytes could be an immunological reaction related to responsibility of lymphocytes for the development of cell-mediated immunity (Ellis, 1977, 1989). However, as a result of such effective cellular defence mechanisms against the invading fungus, limited growth of the fungus inside tissues occurred which was then halted rapidly by fish defence mechanisms.

First presence of giant cells in the lesion area was observed 3 days after injection. Thereafter their number and activity well increased by 10 days post-injection. With improvment in lesion healing, the number of giant cells was extremely reduced at 14th day, so that no giant cells were evident from the 18th day after injection onwards. In addition to two very well-known types of giant cells, Langhans and foreign body types, intermediate giant cell type was also observed. Intermediate giant cell was described by Timur (1975) in plaice, injected with talcum powder. These cells have their nuclei at periphery of the cytoplasm and also

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scattered in the middle of the cytoplasm.

Production of giant cells was reported neither in snakehead, experimentally, infected with *A. invadans* (Chinabut *et al*., 1995), nor in snakehead, infected experimentally with *Achlya debaryana* (Chinabut, 1989). Neither was it observed in dwarf gourami infected by *Aphanomyces* sp. (Wada *et al*., 1994). Presence of giant cells in UM in Atlantic menhaden was reported by Noga *et al*. (1988). Also it has been found in MG in freshwater fishes, goldfish and Ayu (Miyazaki and Egusa, 1972, 1973). Miyazaki and Egusa (1972) defined two types of giant cells, one type with engulfed hyphae were always within the granulomas of muscle tissue, and another type without hyphae usually appeared in the loose connective tissue of the dermis or lamina propria. In this study, giant cells containing fungus in their cytoplasm were seen located either within the stroma of epithelioid cells or within the granulomata. Also giant cells with no engulfed fungus were either seen lying free in the lesion area, or in the granulomata. Richards *et al*. (1978) suggested that giant cells appear to be common in fungal granulomas of fish, and Secombes (1985) demonstrated that they are capable of phagocytosis. The findings of the present study support these views.

It is obvious from literature that only Langhans type giant cells are produced in piscine tuberculosis and foreign body types do not normally occur. Timur (1975) found both types in plaice in response to Freund's complete adjuvant and concluded that foreign body types were in response to the mineral oil of adjuvant rather than the *Mycobacterium* sp. Both Langhans and foreign body types and also intermediate type were produced in the present study. It could be suggested that in addition of Langhans type, foreign body type giant cells could also be produced either in response to inert materials such as mineral oil (Timur, 1975), and talcum powder (Sharifpour 1997), or even biological materials such as fungus as used in this study.

Fibrosis and vascularization are two important components of a chronic inflammation which allow the lesion to heal. In the present study, new fibroblasts began to appear in the fascia and around the blood vessels by the first day after injection. Actual fibroplasia, which is a major element for encapsulation the irritant and repairing the damaged areas, along with vascularization was active by 3 days post-injection which is similar to that of snakehead infected by *Achlya debaryana* at 28°C (Chinabut,1989), but much faster than 8 days in *A. invadans* infected snakehead at 26 and 31°C, reported by Chinabut *et al*. (1995). Connective tissue proliferation in this study resulted in joining and repairing the damaged and separated parts of the lesion area, and also encapsulation of the spores. Formation of new capillaries in the lesion area is an important source to nourish the newly developing granulation tissue. Their number and activity in the lesion

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area was gradually increased by the day 18th, as healing progressed. Thereafter they began to decrease but never totally disappeared by the end of the sampling time. New capillary buds were observed within the supporting stroma around the granulomata in snakehead, 2 days p.i. of *A.invadans* at 31°C (Chinabut *et al*., 1995).

Mycotic granulomata formation as a defence mechanism of fish to wall off and kill the pathogen began by surrounding the fungus by epithelioid cells at 3 days post-inoculation. Then, along with an increasing layer of tightly bound epithelioid cells and their activity, granulation tissue began to surround dense layers of epithelioid cells, resulting in the formation of the well-developed granulomata at 10 days after injection. Thereafter the number and the rate of consolidation of the typical granulomata increased. As the healing process developed (i.e. 18-22 days p.i.) the epithelioid layers in granulomata decreased, while fibrous layers increased so that in the late stages of the lesion, granulomata were cystic and consisted of only a necrotic core of basophilic and eosinophilic debris, and remnants of fungus, with thick fibrous layers around. Chinabut *et al*. (1995) reported that mycotic granulomata were well developed in snakehead infected with *A. invadans* and kept at 26 and 31°C, by 4 days p.i., and extensive mycotic granulomatosis was observed by 8 days. From the findings of this study it could be suggested that fibrous proliferation and intense granulomata formation in response to *A. invadans*, might be an important factor inhibiting fungal growth.

Willoughby and Roberts (1994b) showed that motility of *Aphanomyces* zoospores could be inhibited temporarily by physical or chemical shock, but resumed after 4.5 hours without an intervening encystment phase. Therefore, they might be transported by water currents, immobile and encyst only on a favourable surface. It is likely that most of the injected zoospores in the lesion of carp tissue (present study) gradually ceased their motility when were sequestrated by epithelioid cells and giant cells, but thereafter they could not regain their motility and consequently lost their viability when granulomata formed and developed. Meanwhile a limited growth of some of the zoospores during the early stages after inoculation and before epithelioid cells formation, was also completely ceased by defence system of fish.

From the results of the present study it could also be suggested that in addition to physical encounter of the fish tissue, and the fungus as a foreign body that could not be phagocytised, (surrounding the fungus and granuloma formation), carp might inhibit and suppress biological activities of the fungus by some physiological, biological and/or immunological factors, which requires further study.

Muscle regeneration in carp (present study) initiated at 3 days p.i. with redeveloping peripheral nucleation in those myofibrils with slight damage

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along with appearance of new basophilic muscle buds mostly at the edges of the lesion. Then these muscle buds developed into sarcolemmal tubes and thereafter into muscle bundles. As the healing process developed, the new muscle bundles and regenerating muscle fibres filled the whole damaged area and replaced the fibrous tissue by about 14 days after injection. Chinabut *et al*. (1995) did not report the time at which muscle regeneration commenced in snakehead infected experimentally with *A. invadans*, but did state that in the late stages of infection, regenerating muscle fibres were observed replacing the fibrous tissue, and from 14-28 days p.i., the healing process became well established in fish kept at higher temperatures (i.e. 26 and at 31°C) which is similar to the observations in the late stages of this study. Muscle regeneration was recognised by day 4 in snakehead infected with *Achlya debaryana* at 28°C, and the rate of repair of the lesion was very rapid so the infected site became normal by around day 6 p.i. (Chinabut, 1989). Yadav *et al*. (2016) explained that the muscle at the site of injection revealed well developed granulomas at 12 days post infection, with subsequent regeneration of muscle fibres. They resulted from their study that innate defence mechanisms of common carp are able to neutralize the virulence factors secreted by *A. invadans*, thereby, preventing its invasive spread and containing the infection.

The delicate *A. invadans* of EUS is slow-growing at all temperatures, but dies at 37°C (Willoughby *et al.*, 1995). The spores of the EUS-specific *Aphanomyces* can grow in muscle of snakehead fish at temperatures ranging from 19 to 31°C. Mortalities from EUS occur when water temperatures are low (Chinabut *et al*., 1995). Low temperatures reduce the resistance of the fish so much that even small numbers of zoospores can initiate infection (Neish, 1976). In addition to the activity of the spores, the temperature effects on wound healing (Anderson and Roberts, 1975), macrophage response, clearance of necrotic muscles, fibroblasts activity (Finn and Neilson, 1971a), metabolic activity and protein synthesis (Prosser, 1962), and immune response of fish (Ellis, 1989). All stages of the inflammatory process are slower to develop and reduced in extent at low temperatures (Chinabut *et al*., 1995), and also the immune response of fish is considerably reduced at low temperatures (Rijkers, 1982; Ellis, 1989). Therefore, opportunities exist for any of a wide variety of viral or other stressors, which might increase the susceptibility of fish to invasion (Chinabut *et al*., 1995). Regarding these explanations and as a result of effective defence mechanisms of carp, in the present study, against the fungus, the inoculated fungus showed no continuing viable growth in carp tissue. Therefore such resistance against fungus infection and the fast rate of chronic inflammatory response and wound healing at 27°C (average water

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temperature in this study) would be expected. The key mechanisms of common carp in resisting *A. invadans* infection, include efficient processing and presentation of *A. invadans* antigens, enhanced phagocytosis, activation of NLRP3 inflammasome and increased recruitment of leukocytes to the site of infection. Transcriptome analysis reveals immune pathways underlying resistance in the carp against the oomycete *Aphanomyces invadans* (Verma, 2021). As the warm water carp is immunologically suppressed at temperatures below 15°C (Ellis, 1989), further study is necessary to determine the effects of *A. invadans* on carp at low temperatures to find whether this fungus is able to infect carp severely and cause mortalities as in snakehead fish. Afzali *et al*. (2015) reported that Gourami, koi carp, and catfish are highly susceptible while goldfish and climbing perch were found to be moderately susceptible to the EUS infection. They also suggested that the cellular response of fish to mycotic infection and granulomatous reaction varied in different fish species, which could not be an indicator of susceptibility or resistant to the EUS itself, although it was shown that the granulation rate and the level of maturity or solidification (consolidation of granulomas) were higher in resistant fish. Corbel (1975) described that cellular response of fish to mycotic infection varied, ranging from granulomata formation to complete absence of response. The results of the present study agree and support the granulomata formation component of Corbel's findings.

It is apparent from the results of the present study that healthy carp resist *A. invadans* infection at high temperatures (27°C) by an active defence mechanism and employing a strong inflammatory response. The general pattern and development of the chronic inflammatory response was similar to those described by previous workers.

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