HAEMATOLOGICAL AND IMMUNOLOGICAL STUDIES OF HEALTHY AND EPIZOOTIC ULCERATIVE SYNDROME AFFECTED FRESHWATER TELEOST, *Cirrhinus mrigala.*

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This is to certify that Mr. Sumanta Bagchi has worked under my supervision in the Department of Zoology, University of North Bengal for Ph.D. the thesis entitled, "Haematological and immunological studies of epizootic ulcerative syndrome affected freshwater teleost, *Cirrhinus mrigala*". I am forwarding his thesis for the Ph. D. degree (Science) of the University of North Bengal.

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Sumanta Bagchi

(Sumanta Bagchi)

Dedicated
To

Those who become elated at my success and depressed at my failure.

Contents

Introduction

Fishes are the most primitive and numerous of vertebrates. More than 24000 species of fishes are found on this earth. Out of these more or less 10500 species inhabit the freshwater.

Since time immemorial, fishes are indispensable food item of human. Fishes are major source of protein diet of human all over the world, especially in south-east Asia. To meet the increasing demand of fish-proteins, fish culture has received a great attention throughout the world, especially in developed countries.

India is a country which is gifted with an abundance of all types of fishery resources. There are huge rivers and their tributaries, creeks and canals. Besides big rivers and their tributaries, creeks and canals, there are a number of ideal places for fish production such as oxbow-lakes locally known as beels and boars. Along with the natural water bodies, impounded water bodies such as ponds and canals are also found abundantly. The state of West Bengal is also a no exception. The state is highly enriched with different fishery resources resulting a substantial fish production. The state has the largest brackish water fishery resources in India. At least twenty two species of economically important fishes have been identified in the rivers of West Bengal. Beels and boars of West Bengal harbour at least twelve marketable species (Jain, 1990).

Offering wide variety of tastes as well as nutritive qualities, aqua-culture has already become a global commercial activity. This has led the intense rearing of fishes through high stocking densities, artificial feed and fertilizer use. This intensive rearing of fishes using high stocking densities, artificial feed and fertilizers has not only increased the production of fishes by many folds but also has created conditions that favour outbreak and spread of infectious diseases. As the fishes are vulnerable to various types of infectious agents, fish disease has already become a global problem causing large scale death of freshwater and marine fish, wild, cultured, sport fish and finally ornamental fishes (Trust 1986).

Fish diseases which have already been diagnosed across the world are bacterial diseases such as furunculous (Ghittino, 1972; Ferguson and McCarthy, 1978; Dalsgaard,

1994; Ford et al., 1994), vibriosis (Levin et al., 1972; Lewis, 1985; Egidius et al., 1986; Liu et al., 1994), bacterial kidney disease (Bruno and Munro, 1982; Bruno, 1986; Magnusson et al., 1994), enteric septicemia (Plumb and Sanchez, 1983; Plumb and Hilge, 1987; Kasornchandra et al., 1987; Chen and Kumlin, 1989; Morrison and Plumb, 1994), streptococcicosis (Ferguson et al., 1994; Al-Harbi, 1994), bacterial gill disease (Lumsden et al., 1994), viral diseases e.g. infectious carp dropsy or viral haemorrhagic septicemia (Schaperclaus, 1965; Ghittino et al., 1984; Trust, 1986; Meyers et al., 1992), fungal diseases (Noga et al., 1991; Kumar and Dey, 1991), metazoan and protozoan diseases (Paperna, 1980; Mishra et al., 1982; Kabata, 1985; Landsberg and Paperna, 1987) etc.

But prior to 1988 fish diseases in India had not been a menance. The diseases which were common in freshwater fishes were haemorrhagic septicemia, dropsy, ulcerative disease, columnaris disease, microsporidiasis, dactlyogyrosis, gyrodactylosis, ligulosis, argulosis and saprolegniosis (Kumar and Dey, 1992; Das and Das, 1995).

In May, 1988 Epizootic ulcerative syndrome (EUS) broke out as one of the most destructive fish diseases, India have ever experienced. The disease first appeared in some north-eastern states of India such as Tripura, Meghalaya and Assam (Das, 1988; Pal and Pradhan, 1990). In October 1988, severe out breaks of the EUS occurred in northern districts of West Bengal such as Coochbehar, Jalpaiguri, West Dinajpur, plains of Darjeeling district and Maldah (Pradhan and Pal, 1990).

Subsequently, the disease spread to some southern districts of West Bengal such as Murshidabad, Nadia and 24 Parganas and Midnapur. In 1989 the disease affected almost all the districts of West Bengal. In the same year the disease spread to some areas of other states of India such as Orissa, Bihar and Uttar Pradesh. Further it spread to almost all the states of India except Jammu and Kashmir, Punjab, Himachal Pradesh and Gujarat (Das and Das, 1993). Over 100 species of fish have been confirmed by histological diagnosis to be affected by EUS (Lilley et al., 1992). But some important culture species including tilapia, milkfish and Chinese carp have been shown to be resistant.

The name epizootic ulcerative syndrome (EUS) was adopted by the FAO consultation of experts meeting in Bangkok in 1986 (FAO, 1986). It was accepted that

the condition was primarily an infectious disease and it was a complex condition involving certainly fungal, bacterial elements and probably one or more viruses. Epizootic ulcerative syndrome was redefined at a DFID Regional Seminar in Bangkok in 1994 as a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious etiology characterized by the presence of invasive *Aphanomyces* and necrotizing ulcerative lesions typically leading to granulomatous response (DFID 1994). The disease has been given various names, but is most commonly known as mycotic granulomatous (MG) in Japan, red spot disease (RSD) in Australia and epizootic ulcerative syndrome (EUS) in South-Asia. At present MG, RSD and EUS are considered synonymous.

Immune system of animals is the defence machinery to fight against the diseases and the immune system is a remarkably efficient defence system that has evolved into a well organized system in vertebrates to protect them from invading pathogenic micro-organisms. Fishes which lie on the base line of vertebrate evolution have developed a primitive but efficient immune system which during course of evolution reaches to its highest in mammals notably in humans. The immune system of fishes must be an effective system, because a bewildering array of infectious agents with multitudes of chemical moieties.

So along with the development of different effective treatment of different diseases, it is imperative to develop a better understanding of the immune system of fishes.

Both the innate and acquired immune system of teleost fishes appear increasingly similar to mammalian immune systems although the different evolutionary pressures teleosts have faced, may be expected to generate unique aspects of immunity. The greatest difference between the two taxa is the absence of lymph nodes and the ontogeny of leucocytes. In fish spleen, kidney (Pronephros) and thymus combinedly compensate the function of bone marrow in mammals. The spleen and kidney are the main haemopoietic organs in teleosts. In some fishes both organs function equally whereas in others any of two remains more active than the other (Catton 1951). However, in some fishes only the spleen (e.g. in Parch) or the head kidney shows activity. There are a few

reports on ontogeny of hoemopoiesis in fishes (Mahajan and Dheer, 1980; Weining, 1990).

There is evidence that the thymus plays a vital role in the ontogeny of immunologic competence (Miller, 1961; Good et al., 1962; Cooper, 1973). Studies by Beard (1994) on elasmobranch suggested that the Thymus is the source of lymphoid cells. However, there is a need for better understanding of organization of the lymphoid tissue resulting the better understanding of the functions of the lymphoid tissue. The main objectives of the present work are:

I. Histological studies of lympho-haemopoietic organs, head kidney, spleen and thymus of both healthy and EUS affected *Cirrhinus mrigala.* Electron microscopic studies of lympho-hoemopoietic organs of healthy and EUS affected *C. mrigala.*

II. Studies on blood cell profile in healthy *C. mrigala.*

III. Cytochemical studies on erythropoiesis in healthy *C. mrigala.*

IV. Studies on erythropoietic efficiency of head kidney and thymus of healthy and EUS affected *C. mrigala.*

V. Comparison of percentage of mature and immature erythrocytes in peripheral blood of healthy and EUS affected *C. mrigala.*

VI. Total counts of erythrocytes and leucocytes in peripheral blood of healthy *C. mrigala* and EUS affected *C. mrigala.*

VII. Measurement of Hb content, total erythrocyte count (TEC), total leucocyte count (TLC), differential count of three subpopulations of leucocyte in peripheral blood of healthy *C. mrigala* throughout the year.

VIII. Studies on the humoral immunity of the fish, *C. mrigala.*

IX. Count of antibody secreting "B" cells in healthy fish, *C. mrigala.*

Review of Literature

Disease is a function of more than one factor which includes host, parasite and environment. To produce disease a pathogen must overcome host defenses. The host-parasitic interaction with fish, unlike that with homeothermic animals, is strongly influenced by the environment. As fish is an ectothermic aquatic animal, it is very much susceptible to environmental stresses compared to the homeothermic animals. Sublethal changes in water environment make fish more susceptible to pathogens and this is true in case of Epizootic ulcerative syndrome also. To understand any disease it is imperative to understand the different aspects of host biology which include histopathological changes, heamatological changes and immunological status.

The main purpose of this review is to assemble in brief the observations of previous workers in view of understanding the present state of epizootic ulcerative syndrome. The main features of this review are:-

- History of the disease.
- Fish species affected.
- Socio-economic impact.
- Etiological investigations.
- Environmental factors.
- Signs of the disease.
- Management of the disease.
- Lympho haemopoietic organs.
- Cytological studies.
- Haematological studies.
- Immunological studies.

History of the disease

Prior to 1971, there was no report of the out break of Epizootic Ulcerative Syndrome, in different parts of Asia and Asia-Pacific region. In 1971 from Japan an ulcerative condition of fish was first reported in farmed ayu (*Plecoglossus altivelis)* in

Oita prefecture and it was named mycotic granulomatosis (MG) (Egusa and Masuda, 1971). In 1972 a similar ulcerative disease in fish was reported from Central Queensland, Australia with recurrence in subsequent years and the disease was known as red spot disease (RSD) (Rodgers and Burke, 1981).

FAO consultation of experts meeting was organized in Bangkok where the name Epizootic ulcerative syndrome was adopted and it was also accepted that the condition was primarily an infectious disease of mixed etiology (FAO, 1986).

During 1975-76 the ulcerative disease spread to Papua New Guinea (Haines, 1983). Anon (1981) reported the outbreak of EUS from Java in 1980. Subsequently, the disease spread to Indonesian states like Sumatra, Swaweri and Kalimantan (Wadagdo, 1990) as well as in northern Malaysia (Jothy, 1981). Thailand first witnessed the outbreak of EUS in 1981 with subsequent recurrences (Ulcerative Fish Disease Committee, 1983; Chulalongkorn University, 1983, 1985, 1987; Tonguthai, 1985). During 1983-84 the disease entered Myanmar, Laos PDR and Cambodia from Thailand and Malaysia (Roberts et al, 1986; Lilley et al., 1992). A severe outbreak of EUS in December 1985 was reported from Laguna de Bay in the Philippines affecting snakeheads, gobies, gouramies, catfish, crucian carp etc. (Llobrera and Gacutan, 1987).

China (Lian, 1990; Guizhen, 1990), Vitenam (Xuan, 1990) and Hong Kong (Wilson and Lo, 1992) also witnessed the outbreak of EUS. Outbreak of EUS first took place in Sri Lanka in December 1987 (Costa and Wijeyaratne, 1989).The disease entered into Chandpur district of Bangladesh crossing the Myanmar in February 1988 (Kar and Dey, 1990; Roberts et al, 1992; Hossain et al, 1992; Ahmed and Rab, 1995; Ahmed and Hoque, 1998). According to Rahim et al. (1985), five species of brackish water fish of Bangladesh were affected with ulcerative disease prior to the outbreak of EUS in February 1988.

India first experienced the outbreak of epizootic ulcerative syndrome in the month of May 1988 in some states of northeastern India. Navgao, Karimganj, Kamrup, Cachar and Silchar areas of Assam were initially affected. Of the affected areas of Assam severe outbreak of EUS was reported from Borak valley which is situated in the districts of

Cachar and Karimganj. In the same year i; e in 1988 two adjoining states of Assam such as Tripura & Meghalaya were also affected.

After a few months, in October 1988 the disease spread to some northern districts of West-Bengal (Das, 1988; Pal and Pradhan, 1990). In the same year the disease extended to some southern districts of West-Bengal such as Nadia, Murshidabad and 24 Parganas and Midnapore (Pradhan and Pal, 1990). Slowly the outbreak of EUS spread to almost all the districts of West-Bengal except Purulia (Jain, 1990). From West-Bengal the disease first spread to the adjacent districts of Bihar like Katihar and Kishanganj but very quickly it spread to the other districts of Bihar (Prasad and Sinha, 1990). In Orissa also the disease spread the way it had spread in Bihar starting from adjacent districts of West-Bengal such as Balasore and Mayurbhanj to all the districts of Orissa (Prusty and Nayak, 1990).

From North-east India the disease gradually spread to Uttar Pradesh, Madhya Pradesh, Maharastra, Tamil Nadu, Andhra Pradesh, Kerala, Hariyana, Rajasthan and Karnataka. By 1993, the disease spread to almost all the states of India except Gujarat, Punjab, and Jammu and Kashmir (Das and Das, 1993). Though the disease first appeared in wild water of rivers and canals but in the long run it affected all types of water bodies like reservoirs and culture ponds.

In spite of declining trend of EUS in recent years the occurrence of the disease is taking place every year till today especially during the winter months in various areas of North-Bengal. Occurrence of the disease in every winter suggests that lowering of temperature during winter months may bring on some degree of changes in the immune status of the fish. So it is a crying need to explore the immune status of fish for providing better management of the disease.

Table 1. Fish species affected by EUS in India

Freshwater

Cultured	Wild
Cirrhinus mrigala	*Anabas testudineus*
Catla catla	*Heteropneustes fossilis*
Labeo rohita	*Clarias batrachus*
L. calbasu	*Channa punctatus, C. striatus, C. gachua*
Cyprinus carpio	*Chanda chanda*
Puntius javanicus.	*Mastocembelus* sp.
Ctenopharyngodon idella	*Callichrous pabda*
Hypophthalmichthys molitrix	*Mystus* sp.
	Nandus nandus
	Colisa fasciata
	Amblypharyngodon mola
	Ambassis ranga
	Glossogobius giuris
	Gadusia chapra
	Macrognathus aculeatus
	Notopterus sp.
	Mastocembelus armatus
	Rhinomugil corsula
	Trichogaster sp.
	Acrossocheilus hexagonolepsis
	Wallago sp.
	Salmostomo bacaila
	Monopterus cuchia
	Amphipnous cuchia
	Ailia coila
	Ompak sp.
	Rasbora sp.

Brackishwater

Mugil parsia	*Scatophagus* sp.
Mugil cephalus	*Epinephelus* sp.
Mugil subviridis	*Platycephalus* sp.
Sillago sp.	*Etroplus* sp.

Fish species affected

A large number fish species, freshwater as well as brackish water has so far been confirmed by histological diagnosis to be affected by EUS. Surprisingly, it not only produces high mortality in fishes of all ages within a very short period, but also it attacks a huge number of species of fish of both wild and cultured water.

More than 100 fish species have so far been found to be affected by EUS (Lilley et al., 1992). Das and Das (1993) reported that in India, more than 30 species were affected by EUS. But among those 30 species, 26 are indigenous fish species and remaining 4 species are exotic. Along with very susceptible fish species under the genera *Channa, Puntius* and *Osphronemus,* the other susceptible genera are *Mystus, Mastocembelus, Glossogobius, Anabas, Clarius* and *Heteropneustis.* Indian major carps are also affected frequently by EUS. Among the Indian major carps, *Cirrhinus mrigala, Catla catla* and *Labeo rohita* are the most common victims of EUS (Jhingran and Das, 1990; Pal and Pradhan, 1990; Kumar et al., 1991; Abdul Hameed, 1996; Mukherjee, 1996; Das, 1997). However, Saha (1998) reported that at least 46 fish species were found to be affected by EUS in India.

In Philippines the fish species which were affected badly in a large scale, were snakehead (*Ophiocephalus streatus),* catfish (*Clarias barouches)* gorily (*Trichogoster pectoralis),* goby (*Glossogobius giurus),* crucian carp (*Carassius carassius),* Manila sea catfish (*Arius manilensis)* and silvery theraponid (*Therapon plumbius)* (Llobrera, 1987). Brackish water fish species or estuarine fish species affected during the outbreak of December 1990, in Brgueey lagoon, Cagayan Province, northern Philippines, were mullet (*Mugil* sp.), flatfishes (*Platyceptralus* sp. and *Psethodes* sp.), goat fish (*Upeneus bensan),* Croaker (*Johnius* sp.) and spad fish (*Scaptophogus* sp.).

In Australia yellow fin bream (*Acanthopagrus austtralis)* and striped mullet (*Mugil cepholus*) were the most susceptible fish species (Calinan et al., 1995a). The affected fish species in EUS outbreak in Thailand included snakehead fish (*Ophiocephalus strialies*), serpaint fish (*Channa micropeltis),* sand goby (*Oxydeotus marmoratus),* three spotted gourami (*Trichogaster trichopterus),* striped croaking gourami (*Trichopsis vittatus),*

Siamese fighting fish (*Betta splendens)* and wrestling half beak (*Dermogenns pustillus)* (Sailendri et al., 1986).

In Sri Lanka, Subhasinghe et al. (1990) found 19 affected fish species which included *Ophiocephalus striatus, Ophiocephalus punctatus, Heteropneustes fossillis* and *Mastacembelus armatus* etc. Ahmed and Rab (1995) from Bangladesh reported that Thai Silver barb, *Puntius gonionolus* was the most susceptible fish species.

Most surprisingly, three culture species of fish such as Nile tilapia, milkfish and Chinese carp have been shown to be resistant to EUS.

Socio-economic impact

There is hardly any confusion about the huge social damage and economic loss caused due to outbreak of Epizootic ulcerative syndrome or EUS. Estimations of situations in five districts of West-Bengai gave an indication of the actual depth of damage, both social and economic, done by Epizootic ulcerative syndrome or EUS. It showed that 73% of aquaculture units were badly affected by EUS and decline in fish consumption rate by 28.7%, 23.3% and 20.5% in urban, suburban and rural sectors respectively resulting a sharp decline in fish trade. More or less 70% aquaculturists suffered a huge economic loss, 50% of them incurred a loss amounting a range of rupees 1001 to 5000 and remaining 20% culturists suffered a greater loss amounting a range of Rs. 5001 to Rs 10,000. A section of fish farmers searched for alternate job because from fish farming they could no longer manage their livelihood. Almost 90% fish traders were affected and suffered economic loss to some extent during affected period (Bhowmik et al., 1991). Among the southern states of India, Kerala was the worst affected where outbreak of EUS almost paralyzed the inland fish markets. Consequently, a huge number of fishermen became jobless. The women fish vendors in particular were the worst sufferers (Sanjeevaghosh, 1992). A report from Abdul Hameed (1996) stated that during February, 1994 EUS caused mortality of 25 tones of fish valued at approximately Rs 5.00 lakhs in about 20 tanks in the Dhanwad district in the state of Karnataka. The report also

informed the large scale mortality of murrels in the rivers, pond etc. In Goa farmers incurred a loss of 20 metric tones fish which valued Rs 8.00 lakhs in 1993. Fish losses in the same state i.e. in Goa were 30 metric tones amounting to as 12.0 lakh and 15 metric tones amounting Rs 6.0 lakh in 1994 and 1995 respectively (Sardesai, 1996). Study in Assam, one of the north-eastern states of India, showed that a total loss of fish owing to EUS had been estimated at 10,625 metric tones affecting 81,400 numbers of fish farmers (Das, 1996). Fish trade in Bihar experienced the loss of fish which valued about Rs 48.00 lakhs during 1989-90 when initial outbreak took place (Prasad and Sinha, 1990). Orissa also suffered a loss of Rs 30.0 lakhs during 1989-91 (Das, 1994). Epizootic ulcerative syndrome or EUS has not only caused a huge damage in fish farming in India, but it has also affected fish farming in other South-eastern countries.

Llobrera (1987) estimated the huge economic and social impact owing to outbreak of EUS in Philippines. Lake shore peoples were the worst sufferers. Near about 15,000 lakes shore families in Laguna Lake were badly affected resulting a 30% decline in average daily income of fishermen. The situation was worse around 5000 lakes in Margabol Swamp in Pangasian province of Philippines where at least 75,000 people were affected incurring a loss over 50% and 40% during 1989 and 1990 outbreaks of EUS respectively. Not only the huge economic loss faced by fishermen and lake shore people, even a panic spread far and wide among the consumers and farmers in Philippines.

Outbreak of EUS in Sri Lanka also compelled the fishermen and fish traders to incurr a heavy economic loss which amounted to Rs 1 million during 1988-89 (ADB/NACA, 1991) and increased upto Rs 20-40 million in Sri Lankan currency (Balasuriya, 1994).

Spread of EUS had more than one fold negative influence in societies of different south-east Asian countries. It created a huge panic and unprecedented fear of disease transmission from fish to human among common people resulting a sharp decline in the demand of fish. It had heavily affected the socio-economic status of fish farmers and fish traders in Bangladesh (Rahaman et al., 1988). Consequently, fall of price of fish upto

75% led to economic loss of about 118 million and 88.2 million taka in Bangladesh in the year 1988 and 1989 respectively (Barua, 1990).

During 1982-83 in Thailand the economic loss due to EUS was upto 200 million in Thailand currency (Tonguthai, 1985) and over ten years i.e. from 1983-93 the loss was about 3600 million in Thailand currency (Chinabut, 1994).

Our neighbouring countries like Nepal and Pakistan were also no exceptions. During 1989-90 the total economic loss in eastern Nepal only was about Rs 30 million (ADB/NACA, 1991). In Pakistan the fishermen and fish traders were also badly affected. In 1996 the total economic loss incurred was about Rs 15 million (AAHRI, ACIAR, 10A and NACA 1997).

Etiological Investigations

Opinions differ regarding the exact etiology of epizootic ulcerative syndrome and it has been accepted that epizootic ulcerative syndrome is a complex condition involving certainly fungal and bacterial elements in its later stages and probably one or more viruses (Chinabut, 1995). Some environmental factors also play the role of predisposing factors of EUS. Naturally, etiological studies have been the subject of major interest for ichthyologists in the countries where the outbreak of EUS has occurred. A lot of works have been carried out by different researchers on the role of various etiological agents, such as viruses, bacteria, fungi and animal parasites on the outbreaks of the EUS.

Virus

Virus like particles was detected in different tissues of affected fish during 1982-83 outbreaks in Thailand (Rattanaphani et al., 1983; Wattanavijarn et al., 1983 a, b, 1984). Rhabdovirus was isolated from diseased fish in some south-east and south Asian countries by Frerichs et al. (1986, 1989). They suggested that it could be the initiating factor in the outbreak of EUS. But Frerichs et al. (1986, 1989) could isolate the virus from not more than 5% fish examined and the virus could not induce the disease in

healthy fish experimentally. Saitanu et al. (1986) isolated a new virus, snakehead fish virus (SHV) from infected *O. striatus, C. micropeltes, Oxyeleotris marmoratics, T. trichopterus, T. vittatus* etc. This virus was not affected by ether or chloroform and was found resistant at 60°C for 30 min. SHV also produced cytopathic effects, rounded cells and complete destruction of cell sheet on BB, BF_2 and FHM cells. Hedrick et al. (1986) from cultured sand goby (*Oxyeleotris marmoratus)* and Subramaniam et al. (1993) in Singapore from infected fish isolated birna virus. Ahne et al. (1988) also isolated a rhabdovirus from snakehead (*O. striatus)* in Thailand. He also showed that this rhabdovirus was serologically different from VHSV, IHNV, RVC, PERV or EVX. Rhabdoviruses were also isolated from the diseased fish collected from Thailand, Myanmar, Australia (Roberts et al., 1989; Roberts et al., 1994; Lilby and Frerichs, 1994) and the viruses were named as ulcerative disease rhabdovirus (UDRV).

Siddhi (1989) carried out virological studies on EUS affected fish species in Assam, West-Bengal and Tripura and found no relationship of any virus with EUS. Electron microscopic studies by Kar et al. (1990) showed the presence of viruses in muscles and gills of ulcerative disease affected fish in Assam.

Bacteria

The constant and relentless search for pathogens responsible for outbreak of EUS by different scientists from different affected species of fish has led the isolation of a number of bacteria from the ulcers as well as the internal organs such as kidney, liver, spleen etc. Though the bacteria isolated from infected fishes by different workers differ, yet it has been established that *Aeromonas sp.* is the most predominant pathogenic bacterium.

Llobrera and Gacutan (1987) showed the consistent association of *Aeromonas hydrophila* with necrotic ulcers and lesions in snakehead (*Ophiocephalus striatus)*, Thai catfish (*Clarias batrachus)*, crucian carp (*Carassius carasius)* and goby (*Glossogobius giurus)* in Laguna de Bay, Philippines, from December, 1985 to February, 1986. They also isolated *Aeromonas hydrophila* from body lesions as well as from internal organs from affected fish. Later Boonyaratpalin (1989) found the association of primarily

Aeromonas hydrophila and occasionally *Pseudomonas sp.* with the outbreak of EUS in Burma, Indonesia, Lao Peoples Democratic Republic, Malaysia, Singapore and Thailand. Association of specially *Aeromonas hydrophila* was also established with EUS affected fishes in Sri Lanka (Costa and Wijeyaratne, 1989). During the extensive examinations of 19 species of EUS affected fishes such as *Ophiocephalus striatus, Ophiocephalus punctatus, Heteropneustes fossillis* and *Mastacembelus armatus* etc. in Sri Lanka. Subashinghe et al. (1990) showed the consistent association of *Aeromonas hydrophila* and occasional association of both *Aeromonas sp.* and *Pseudomonas sp.* with haemorrhagic lesions and open necrotic ulcers on the body.

Jhingran and Das (1990) had been able to induce the haemorrhagic ulcers inoculating pure bacterial isolates in healthy murrels within 72 hours after inoculation. Kar et al. (1990) also isolated *Pseudomonas aeruginosa* from the surface muscle lesions.

Four types of bacteria, two fluorescent Pseudomonads (R_1 and R_2), one Aeromonad (R_3) and one *Microceus sp.* (C) were isolated from skin lesions of air breathing fishes by Pal and Pradhan (1990) where R1, resembled *Pseudomonas fluorescens,* R2 resembled *Pseudomonas aeruginosa* and R3 showed strong resemblance with *Aeromonas caviae* (Pradhan, 1992). When a mixed culture of bacteria was inoculated, severe ulcers were produced but pure cultures of the fiuorescent Pseudomonads and Aeromonad induced only superficial ulcers while pure culture of *Micrococcus sp.* did not produce any ulcers. Pradhan et al. (1991) isolated two Pseudomonads (R_4 and R_5) which resembled *Pseudomonas fluorescens,* one Aeromonad (R_6) and another coccus (C_1) from the Indian freshwater major carp, *Cirrhinus mrigala. Aeromonas hydrophila* was only isolated from EUS affected fishes of more than 70 species by Chattopadhya et al. (1990). Several researchers in India and abroad reported associations of bacterial pathogens with EUS (Mc Garey et al., 1991; Ali and Timuli, 1991; Mukherjee et al., 1991; Lio-Po et al., 1992). Chakraborty and Dastidar (1991) repeatedly isolated chemoautotrophic nocardiofarm (CAN) bacteria from different types of skin lesions of EUS affected fishes.

Torres et al. (1993) performed virulence screening of 54 species of *Aeromonas* and found the *Aeromonas hydrophila* was the most pathogenic. Qureshi et al. (1995) also

performed the virulence test of eight bacterial isolates from EUS affected fishes and found *Aeromonad* and *Pseudomonad* were highly pathogenic while micrococcus and cytophogans were less pathogenic. Lio-Po et al. (1998) isolated four species of bacteria from EUS affected fishes from Philippines and Thailand and *A. hydrophila* was proved to be most pathogenic. Saha and Pal(2000) isolated sixteen (16) strains of bacteria belonging to the genus *Pseudomonas*, *Aeromonas*, *Micrococcus*, *Bacillus*, *Moraxella* and *Vibrio*. Out of these 16 strains belonged to Pseudomonad and Aeromonad were found to be pathogenic.

Fungus

Fungal species were consistently isolated from lesions of EUS affected fishes.

Different fungal species were isolated from the lesions of affected fishes, of which *Achlya sp.* and *Saprolegnia sp.* were the most common (Pichyangkura and Bodhalamik, 1983; Limusuwan and Chinabut, 1983).

Roberts et al. (1993) first isolated fungus, Aphanomyces from EUS affected fish of Thailand. An inflammatory response and severe myonecrosis were observed after the inoculation of a mycelium from this fungal strain below the dermis of healthy fishes. Chinabut et al. (1995) reported that at 19°C the fungal species induced more pathogenicity than 26°C and 31°C temperature.

Willoughby et al. (1995) first named the fungus *Aphanomyces invaderis*. Miyazaki and Egusa (1972, 1973a, 1973b and 1973c) were the first to isolate the fungus from affected fish in Japan. Their efforts did not draw the attention of wider community of scientists as their publications were entirely in Japanese (Chinabut, 1995). Hatai et al. (1977) isolated a fungus from fish *Plecoglossus altiveliss* from Shiga Prefecture, Japan. It was named *Aphanomyces piscicida* (Hatai, 1980).

Later involvement of *Aphanomyces sp.* was also reported from Australia, Philippines, Indonesia and Bangladesh (Fraser et al., 1992; Paclibare et al., 1994; Callinan et al., 1995a, b; Lilley and Roberts, 1997).

Analysis of protein banding profiles (Callinan et al., 1995b; Lilley et al., 1997b), growth characteristics (Lilley and Roberts, 1997) and chemical susceptibility (Lilley and Inglis, 1997) of *Aphanomyces sp.* isolated from EUS affected fishes in different countries showed that the same *Aphanomyces sp.* was involved in each case and finally the species was named as *Aphanomyces invadans* (David and Kirk, 1997).

In India also workers most frequently isolated *Saprolegnia sp.* and *Aspergillus sp.* from EUS affected fishes (Das et al., 1990; Kumar et al., 1991; Patra, 1992).

Karunasagar et al. (1994) traced out the existence of fungi deep into the musculature below the EUS affected ulcers in both freshwater and estuarine fish, but no existence of fungi was detected in early stages of lesions prior to development of ulcers.

Histopathological studies of EUS affected freshwater and estuarine fishes showed the presence of numerous non-septate, highly invasive fungal hyphae (Mohan and Shankar, 1995).

Qureshi et al. (1995) isolated seven species of fungi belonging to the genera *Saprolegnia, Aphanomyces, Achyla* from lesions of EUS affected fishes from Bhopal.

Pal (1996, 1997) also isolated three species of fungi but he also stated that no fungi were found in the primary stage of ulcer formation. Viswanath et al. (1998) assumed that EUS specific fungus can only enter into the fish after the primary damage to the skin Mohan et al. (1999) suggested that an invasive fungus *A. invadans* is the primary pathogen of EUS. Roy (2003) reported isolation of an aseptate fungus, *Aphanomyces* sp. from infected *C. mrigala* and experimentally fungal zoospores induced ulcer in healthy *C. punctatus*. Routh (2006) reported isolation of *Apanomyces* sp. from infected *C. striata*, *C. punctatus*, *L. rohita* and *L. bata*. Pathogenicity studies with the zoospores of fungus, *Aphanomyces* sp. (F_{CS1}) isolated from ulcer of *C. striata* induced ulcer at the site of injection and caused 44% mortality in experimental *C. punctatus*.

Animal Parasite

Reungprach et al. (1983) examined 273 EUS affected fishes during 1982-83 outbreaks in Thailand and found a number of metazoans (*Dactylogyrus sp.* and *Gyradactylus sp.*) and protozoans (*Chilodnella sp., Trichodena sp., Costia sp., Henneguya sp.* and *Folthyopthirus sp.)* associated with the ulcers. A considerable number of protozoans belonging to genera *Epistylis sp.* were also reported from fishes with tiny red spots on the skin before the outbreak of the EUS in Thailand (Tonguthai, 1985). Callinan et al. (1989) and Pearce (1990) reported the presence of protozoan and metazoan parasites on some affected fish in Australia.

Jhingran (1990) detected the presence of some common animal parasites such as *Tripartiella sp., Phlebitis sp., Trianchortus sp.* and *Dactylogyrus sp.* in and around the ulcers of EUS affected fishes in a very low intensity. As the animal parasites were found in a very low intensity, Jhingran (1990) did not consider them as primary cause of ulceration of EUS affected fishes. Mondal et al. (1990) reported the presence of *Costia necatrix* in the ulcer of EUS affected fish in West-Bengal, India. Kumar et al. (1991) noticed the existence of myxozoan parasites in the skin and internal organs of *Clarias sp.* Ram (1992) reported that myxozoans, *Myxobolus sp.* and *Thelohanellus sp.* were associated with EUS outbreak in Haryana, India.

Subasinghe (1993) performed experiments with an aim to know the relationship, if there any, between some animal parasite infection like *Trichodina sp.* infection and induction of EUS in *Clarias striata* by feeding with infected fish and by direct contact of water from infected environments. The results showed that heavy infection of animal parasites accelerated the formation of ulcers in EUS affected fishes.

Environmental factors associated with EUS outbreak

Fishes, being poikilothermic aquatic animals, are much more influenced in their physiology by the variation and changes in the environment than the homeothermic vertebrates. The role of environment is regarded of prime importance in the outbreak of

the fish epizootic by many authors (Sneirzko, 1974, 1983; Wedemeyer et al., 1977; Walters and Plumb, 1980; Csaba et al., 1981; Ahne et al., 1982; Schaperclaus, 1986). Epizootic ulcerative syndrome or EUS is not an exception. Tonguthai (1985) reported that EUS outbreaks are frequently predisposed by adverse environmental parameters.

Temperature

Rodgers and Burke (1981) observed that maximum EUS prevalence in estuarine fish was due to fall of temperature. Roberts et al., (1986) recorded that the occurrence of EUS outbreaks in cyclic manner coincided with fall of water temperature after heavy rainfall. Jhingran (1990) also reported that EUS outbreaks took place when water temperature fell significantly. Ahmed and Hoque (1998) reported that outbreaks of EUS were highly related with fall of water temperature. Many other workers also found the some relationship of water temperature and EUS outbreaks (Phillips and Keddie, 1990; Das et al., 1990; Lilley et al., 1992; Das and Das, 1993; Mohan and Shankar, 1994; Lumanlan-Mayo et al., 1997; Sanaullah et al., 2001; Roy and Pal, 2005).

Water quality variables

Jhingran (1990), Jhingran and Das (1990) found that EUS outbreaks were also related with lowering of alkalinity of water.

Bondad-Reantaso et al. (1992), Palisoc and Aralar (1995) and Sanaullah (2001) showed that EUS outbreaks took place when alkalinity and chloride were reduced in water. Mohan and Shankar (1994) reported that during rainy season of 1993 when the salinity was very low. EUS outbreaks took place in estuaries of Dakshina and Uttara Kanoda districts of Karnataka, India. It was also suggested by Callinan et al. (1995) that exposure of fishes to acidified runoff water in Australia was a causal factor for EUS outbreak in estuarine water.

Sardesai (1996) suggested that decrease in salinity of estuarine water due to heavy rainfall was on of the causal factors for EUS outbreaks in 1993 in Goa.

Pathiratne and Jayasinghe (2001) showed that low amount of dissolved oxygen helped in outbreaks of EUS. Roy and Pal (2003) mentioned that outbreak of EUS occurred in the ponds of three areas of North Bengal when dissolved oxygen content, hardness and total alkalinity of water remained low.

Heavy metals

An attempt to find out the relationship between the concentrations of heavy metals e.g. Fe, Zn, Cu, Cr, Cd, Pb and Hg in water and the outbreaks of EUS was made by Jhingran and Das (1990). But till today no significant relationship between concentrations of heavy metals in water and EUS outbreaks has been established.

Pesticide and other agrochemical

Kurup (1992) performed an extensive study in the EUS affected regions of north-eastern Kuttanand in Kerala, India and found that indiscriminate application of pesticides have aggravated water pollution problem exposing the fishes to more stressful conditions which played a predisposing factor for EUS outbreak.

Chowdhury et al. (1994) analysed the pesticide residues in water, fish and plankton in some EUS affected areas and mentioned that though high concentrations of organochlorine pesticides such as BHC, DDT and their metabolites were occasionally detected in water yet no tangible correlation had been found between concentration of pesticides and EUS outbreaks.

Investigations to find out relationship between EUS outbreak and environment in two lakes Laguna and Naujan in Philippines for two years failed to establish a correlation between pesticide concentrations in water and EUS outbreak (Palvisoc and Arator, 1995).

Flooding

Saha et al. (1992) reported that in Sunderbans EUS was detected in many confined waters after a devastating flood. Barua (1994) reported that in Bangladesh a rapid outbreak of EUS took place after a severe flood conditions. Mohan and Shankar (1994) observed that in Karnataka in Couvery river system outbreak of EUS first appeared after

the flood during August-September, 1991. Abdul Hameed (1996) observed that outbreaks of EUS occurred in Karnataka after major flood for four consecutive years (1991-1994).

Signs of the disease

Jhingran and Das (1990) in India reported that the signs and other characteristics of the epizootic ulcerative syndrome were different from other ulcerative condition in fishes. Fishes in the river as well as in ponds exhibited abnormal swimming behavior with head projected out of water. In the primary stage of the disease, the infection generally started in the form of multiple inflammatory red spots on the body causing localized haemorrhage. In case of carps, the infection first took place within scale pockets and in due course the infection spread to a larger area with sloughing of scales with degeneration of epidermal tissue. In advanced stage the ulcers became deep, haemorrhagic and necrotic often with a black melanistic rim. In the final stages or in acute stages deep, haemorrhagic, necrotic ulcers were generally found in all parts of the body of the fish, especially in the head, abdomen and peduncle.

During the initial outbreak of the disease in the plains of North Bengal, Pal and Pradhan (1990) observed keenly a considerable number of EUS affected air-breathing fishes which included 129 *A. testidineus*, 16 *H. fossilis* and 11 *Clarias batrachus*. They reported that the disease first appeared as a red spot on the skin of the fish body. Later ulcers developed in affected areas damaging badly underlying muscle layer. But in scaly fishes, initial damage of mucous layer covering the scales were followed by appearance of red spots and finally sloughing of scale took place with development of ulcers.

Kumar et al. (1991) reported that distribution of severe ulcerative skin lesions varied from species to species. In murrels the ulcerations were mostly pronounced and developed in the head and caudal areas. In advanced stages the tail lesions could erode the affected areas to such an extent that there was total loss of peduncle portion. Sometimes the erosion progressed deep into the body exposing the abdominal cavity.

In *Puntius sp.* dark red haemorrhagic, superficial ulcers area found on either side of the body. But in Indian major carps long striped haemorrhagic lesions were found in the region of caudal peduncle. Pradhan et al. (1991) reported infection of different stages of development in Indian major carps like *Catla catla, Cirrhinus mrigala* and *Labeo rohita*

By using clinical and histological features, Viswanath et al. (1997) classified different types of lesions associated with EUS in India. They observed more than 300 EUS affected fish and characterized three distinct types of EUS lesions:

Type1 lesions were like tiny red spots on the body surface with no observable haemorrhages and ulcerations.

Type2 lesions were large (2-4cm.) and appeared as dark raised discoloured areas on the body surface. Scales and skin were not affected.

Type3 lesions appeared as circular to oval open dermal ulcers extending into skeletal musculature. At these advanced stages haemorrhagic and necrotic open ulcers devoid of epidermis and scales were also found.

Chinabut and Roberts (1999) classified the disease into five types:

Type I It is characterized by a highly distinctive dark red to brown lesion on one or both blanks. The affected fishes had floated the surface of water and grasped air before they expired.

Type II This type was mostly found in top order predator air breathing fish, such as the snakeheads. The infections were characterized with chronic and extensive lesions.

Type III In this case the ulcers were chronic and extensive and at early stage of infection a small red rosette or an ulcerated scale bed were found. It depends on the age and size of fish. This type is also found in snakeheads.

Type IV This type of lesions is found in the grey mullet (*M. cephalus).* It is characterized by small necrotic erosions of one or more scale beds which gradually

extended to produce a shallow ulcer with a red Centrum, white rim surrounded by black edge.

Type V This type is common in some large Indian carp. In this type a single, small, areas of haemorrhagic ulceration on finback was found.

Management of the disease

Conditions like over crowding of fish, poor water quality, sudden fall of temperature interfere with the normal physiological conditions of fishes and the fishes are exposed to stresses which make them more susceptible to infections (Jhingran, 1990). Management of EUS includes both prophylactic measures as well as therapeutic measures.

Potassium Permanganate

Jain (1990) prescribed that bath treatment of EUS affected fishes with potassium permanganate @ 5 ppm. Jhingran (1990) observed that potassium permanganate @ 0·5-2 ppm in water showed a good result in curing ulcers.

Lime

Use of lime in the pond of EUS affected area @ 200-600 kg per hectare was proved to be effective in either checking the outbreak of EUS or in curing the ulcers (Jhingran et al., 1990). Jain (1990) reported that better result was recorded when liming was followed by bath treatment of the infected fish in 5 ppm potassium permanganate solution or common salt at 3% to 4% solution. Jhingran et al. (1990) stated that liming in water was more effective because it raised the pH value of the water, precipitated suspended or soluble organic materials, promoted biological productivity by enhancing the breakdown of organic substances by bacteria and killed most of the undesirable microorganism due to its caustic action.

Antibiotics

Erythromycin, Nalidixic acid, Oxytetracyclin, Terramycin are commonly used for the treatment of EUS affected fishes. The prescribed dose for effective result was @ 60-100 mg per kg of feed for 7 days (Jhingran, 1990).

CIFAX

Application of CIFAX, a drug formulated by CIFA in EUS affected captive water @ 1L/hectare metre of water showed encouraging results (Das & Das, 1993).

Lymphohaemopoietic organs

In fishes, especially in teleosts, spleen and thymus are the two most important lymphomyeloid tissues (Fanage, 1984). Kidney in fish acts as the bone marrow equivalent of vertebrates containing lympho haemopoietic tissue, which is a typical condition in vertebrates that have bone marrow (Zapata, 2001).

There was evidence that the thymus played a vital role in the ontogeny of immunologic competence in fish (Miller, 1961; Good et al., 1962; Cooper and Hildemann, 1965; Cooper, 1973). Beard (1894) suggested that the thymus was the source of lymphoid cells in elashmobranch. In teleosts the head-kidney played definite role of a lymphoid organ (Rasquin, 1951; Smiths et al., 1980).

Morphological and histological studies of lymphoid organs of a teleost, *Tilapia mossambica* showed that organized lymphoid tissues were present in thymus, head kidney and spleen (Sailendri and Muthukarruppan, 1975). They found that the thymus was encapsulated by thin strands of collagen fibers and divided into three regions outer, middle and inner. The head kidney was characterized by the presence of lymphoid follicles, sub capsular sinus, a hilus like area and lymphoid vessels. The spleen was clearly divided into white pulp and red pulp regions. White pulp region harboured only a reticular area without definite lymphoid centers and the red pulp contained predominantly erythrocytes.

Other workers reported that the spleen and head kidney were important in the immunological defense mechanisms and haematopoiesis in fish (Ellis et al., 1976; Ellis, 1980; Secombes and Manning, 1980; Tamer et al., 1984; Lamers, 1986).

Kumar et al. (1991) performed histological examination of kidney of EUS affected *Puntius sp.* along with some other organs and noticed haemotopoietic as well as immunologic role of kidney specially in anterior part (head kidney) where renal function had disappeared. He also noticed some necrotic changes in the kidney of EUS affected *Puntius sp.*

Pradhan (1992) also found a varying degree of degeneration in kidney of EUS affected *Clarias batrachus.* Histopathological studies by Pal and Pradhan (1995) of kidney of EUS affected *Clarias batrachus* also showed similar degenerative changes.

Histological studies of thymus in the dog-fish, *S. canicala* showed that it was covered completely by a connective tissue capsule, containing fibroblasts, macrophages and collagen fibres (Navasro, 1987; Lloyd-Evans, 1993). Collagen fibres projected into the thymic parenchyma by connective tissue trabeculae carrying large blood vessels and nerves and dividing the thymic lobes into several lobules. Its histological structure showed the presence of cell types almost similar to those described in the most vertebrates.

Ultra structural studies or cytological studies

Information on ultra structure and cytology of lymphoid organs of fish was previously reported by other authors (Good et al., 1966; Bielek, 1981; Fange, 1982; 1984, 1987; Zapata, 1981, 1982; Pulsford et al., 1982; Fshizebki et al., 1984; Hart et al., 1988; Powley et al., 1988). They showed the presence of varying numbers of lymphomyeloid and erythroid cells within but did not notice the presence of any trabeculae. Ultra structural and cytological studies of spleen and head kidney in striped Bass showed that different regions of the spleen contained varying numbers of erythrocytes or their precursors, dividing haemotoblasts, promonocytes, lymphoblasts, lymphocytes and

plasma cells etc. (Bodammer et al., 1990). They also observed the presence of reticular cells closely associated with reticular fibres. Head kidney contained almost all cells types found in spleen but appeared to have much more neutrophils and their precursors. The cells were loosely packed compared to the cells in the spleen and this loose packing of cells allowed examining the developmental stages of blood cells more perfectly.

Zapata et al. (1996) studied the ultra structure and cytology of the lymphoid organs in elasmobranches. They observed mainly the presence of lymphocytes, epithelial cells and macrophages in thymus. But alongwith those cells they also noticed the presence of myoid cells in thymus. Except myoid cells, all other cell types found in thymus were also noticed in the spleen.

Ultra structural and cytological studies on thymus of a gobiid fish (*Pscudopocryptes lanceolatus*) showed the presence of plasma cells, cystic cells and macrophages etc. (De and Pal 1998). Transmission electron microscopic study showed the presence of hypertrophied cystic structures in the thymus of Gobiid fish, *Pseudocryptes lanceolatus* (De and Pal, 2002). De and Pal (2004) reported a special type of secretory cell containing numerous membranes bound dense vesicles, granules, multivesicular bodies (MVBs) and nuclei with two polarized noucleoli within the thymus of Gobiid fish, *Pseudocryptes lanceolatus*.

Cytological studies on the developing thymus in a marine teleost, *Diplodus puntazzo* showed the presence of four types of epithelial cells like a) limiting cells, b) medullary and cortical reticular cells, c) nurse cells and d) Hassall-like corpuscles by Romano et al. (1998). Along with four types of epithelial cells, large blast likes lymphoid cells and small lymphocytes were also reported in medulla and cortex of thymus respectively. Further studies on ontogeny of the thymus in a teleost fish, *Cyprinus carpio L* revealed a huge existence of apoptotic cells within the macrophages of cortex of thymus, by Romano et al. (1999). Romano et al. (2002) also studied the cytological organization of head kidney of Antarctic fishes and found almost all cell types found in the thymic micro-environment.

The thymus of mandarin fish, *Siniperca chuasi* showed the presence of cell types containing thymic epithelial cells, limiting epithelial cells (LEC), macrophages, lymphocytes and three types of granulocytes (Xie et al.,2005).

Ultra structural organization of the thymus of juvenile turbot, *Scophthalmus maximus* contained different cells like limiling thymic epithelial cells, dark stellate (TEC) with an electron dense cytoplasm, pale TEC characterized with electron lucent cytoplasm, macrophages, pigment cells (melanophores) lymphocytes and rodlet cells etc.

Haematological studies

Nearly sixty six years ago Duthie (1939) reporting on the region, development and functions of blood cells in certain marine teleost remarked "..... The development from small lymphocytes to erythrocytes is most difficult to follow, though I feel sure that it occurs....."

Mahajan and Dheer (1979a, 1979b) described different types of blood cells in the peripheral blood of *C. punctatus* and found almost all types of mammalian counter parts in fish blood. Mahajan and Dheer (1980) also studied haemopoiesis in *Channa punctatus* using spleen and head kidney imprints. Lewis et al. (1978) working with certain catfish found two types of lymphocytes, small and large.

Mahajan and Dheer (1979) showed that the number of blood corpuscles of fish blood (*C. punctatus)* vary with change of season. Hamers (1994) was able to prove more convincingly the presence of different cell types in fish (*Cyprinus carpio L)* by cytochemical studies. A generalized increase in leucocytes in the gut of goldfish *Carassius auratus (L)* suffering from frunculosis was observed by Mawdesby-Thomas (1969). Pavlidis et al. (2007) reported that the numbers of different leucocyte cell types were not influenced by sex or maturity stage in six Mediterranean fish species.

Immunological studies

The nonspecific defenses available to the fish are essentially those available to higher mammals (Fletcher, 1982). Goblet cells continuously secrete mucus which not only offers mechanical protection and physically removes microorganisms, but also it contains nonspecific anti microbial activity in the form of lysozyme, C - reactive protein and complement and can also provide immune protection in form of secretory immunoglobin (Fletcher, 1973, 1982). In teleost fishes both alternative complement and classical complement pathways have been shown to generate bactericidal activity (Corbel, 1975; Munn et al., 1982). The presence of interferon has been demonstrated in trout following infection by viruses (de Kinkelin et al., 1982).

Macrophages are widespread in tissues of teleosts, including the gills and peritoneum, but are mainly found as reticulo-endothelial cells in the kidney, the spleen and in some fish, the atrium of the heart (Ellis, 1982). These fixed macrophages appear to be very efficient in clearing the bloodstream of bacteria (Munn et al., 1984).

The fish is also able to respond to microbial attack with an immune defense but it is especially less sophisticated in anti body-mediated defense because the fish is restricted to one class of immunoglobulin (Ig) while higher vertebrates have two to five (Ellis, 1982).

But humoral immunity is not the only arm of specific immunity in fish. The growth and multiplication of many pathogens which live and multiply in host cells, especially phagocytes, are stopped by the activation of macrophages and accumulation of phagocytes to infective loci where activation of macrophages and accumulation of phagocytes are made by lymphokines produced by T-lymphocytes (Sissons et al., 1985).

In teleost, especially in carp cell mediated immunity matures rapidly (Manning et al., 1982, 1985).

Lymphocytes from fish immunized against *A. salmonicida* do produce the lymphokine macrophages migration inhibition factor (Smith et al., 1980).

Baba and Imamura (1988) studied the mechanism of protection in carp, *Cyprinus carpio L.* against *Acromonas hydraphila* which is one of the aetiological agents of epizootic ulcerative syndrome (EUS) in fish. The results indicated that the protection shown by carp immunized by dipping in crude LPS or lipopolysaccliaride was dependent on cellular immunity regulated by T-like cell macrophages system.

They further showed that the protection in carp against *Aeromonas hydrophila* by vaccination is not dependent on humoral immunity.

Later Karunasagar et al. (1996) performed experiments to study the effect of thymectomy on the humoral immune response of *Labeo rohita* against *Aeromonas hydrophila.* The results of the experiments showed that antibody titres in thymectomized fingerlings of *Labeo rohita* Ham. were 2-4 times lower than in non-thymectomized control. Not only, were the antibody titres significantly less in thymectomized fingerlings of *Labeo rohita* Ham. compared to the non-thymectomized fingerlings, even in case of the thymectomized fingerlings the protection against *Aeromonas hydrophila* was partially compared to non-thymectomized fingerlings after immunization against *Aeromonas hydrophila.* The results suggested that T-helper cells might be involved in the production of antibody against *Aeromonas hydrophila.*

Thompson and Lilley et al. (1999) examined the immune responses against a fungus *Aphanomyces invadans* which is also one of the aetiological agents of epizootic ulcerative syndrome in fish and found development of nonspecific antibody. The macrophages were able to phagocytose spores in vitro.

Miles et al. (2001) showed that fishes treated with immunostimulants developed an enhanced immune status.

Recent studies by Rao et al. (2006) on effect of *Achyranthes aspera* on the immunity and survival of a major carp, *Labeo rohita* infected with *Aeromonas hydrophila* showed that *Achyranthes aspera* stimulated immunity and increased resistance to infection.

Materials & Methods

3.1. Selection of Ponds

Three ponds which have a previous record of outbreak of EUS in different areas of Darjeeling and Jalpaiguri district, West-Bengal, India were selected for collection of EUS affected fishes. One pond is situated at by the side of North Bengal University campus, Darjeeling district. Two ponds are situated at Sahudanghi and Lataguri of Jalpaiguri district.

The measurement of the ponds at Sahudanghi (Fig.1), Lataguri (Fig.2) and by the side of the North Bengal university campus are 80`×90`, 40`×30` and 60`×40` respectively.

3.2. Collection and maintenances of healthy fish

Healthy *Cirrhinus mrigala* were collected from ponds of Darjeeling and Jalpaiguri districts having no past record of EUS outbreak. The health conditions of the fishes were given the utmost priority while selecting the fishes.

All the fishes were brought to laboratory in such a way so that the fishes were exposed to least possible stress. The healthy fishes were kept in a glass aquarium measuring 90×35×35 cm in which depth of water was 20-25 cm with constant blowing of air through water by air pump in laboratory. The fish were fed regularly with Tokyu fish food. All the fishes were acclimatized under laboratory conditions for at least 15 days before using them for experimental works.

3.3. Collection of diseased fishes

A number of fish species, e.g. *Cirrhinus mrigala, Catla catla, Labeo rohita, Puntius sp.* and *Mystus sp.* (Figs. 3, 4 and 5) were commonly affected in the ponds. Diseased fishes were collected from the infected ponds as well as from local markets.

Fig.1. Picture of the ponds of Sahudangi of district of Jalpaiguri.

Fig.2. Picture of the pond of Lataguri of district of Jalpaiguri.


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Fig. 3: Picture of a EUS affected *Catla catla* where the tail region has been totally lost.

Fig. 4: Picture of a EUS affected *Cirrhinus mrigala* where the ulcer has been so deep that the peritoneal cavity has been exposed.

Fig. 5: Picture of a EUS affected *Labeo rohita* showing ulcer on its body.

Fig. 6: Picture of intra peritoneal injection of a *Cirrhinus mrigala.*


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3.4. Materials for histopathological techniques

The internal organs such as thymus, head kidney and spleen of healthy and diseased fish, *Cirrhinus mrigala* were taken out from the fishes using sterilized dissecting instruments. In case of diseased fish ulcer tissue was also taken out using sterilized dissecting instruments. The collected samples were fixed in Bouin's fixative and preserved in cedar wood oil if necessary.

Bouin's fixative

a) Saturated aqueous picric acid solution – 75 mL

b) Formalin – 25 mL

c) Glaceial acetic acid – 5 mL

3.5. Materials for Tissue-Imprinting technique

Materials for Graham-Knoll Benzidine counterstained with Giemsa-

1. Giemsa solution

   a) Giemsa Powder -1 gm

   b) Glycerine – 66 mL

   c) Methanol – 66 mL

2. Formal Alcohol

   a) 40% Formalin – 5 mL

   b) Rectified spirit – 45 mL

3. Benzidine solution

   a) 90% alcohol – 30 mL

   b) Benzidine powder-1 pinch

   c) H_2O_2 - 7drops

   d) Distilled water – 20 mL

3.6. Materials for Periodic acid Schiff (PAS) staining (de Tomasi, 1936)

1. Solution A

   a) 10% Periodic acid

2. Solution B or Schiff's reagent

   a) Basic Fuchsin -1 gm

   b) Distilled water – 200 mL

   c) Hydrochloride acid (N) – 20 mL

   d) Sodium Metabisulphite -1 gm

   e) Activated charcoal – 2 gms

3.7. Materials for Haematological studies

1. Hayem's Solution

   a) Sodium chloride (NaCl) – 1%

   b) Sodium thiosulphate (NaS_2O_4) – 2.5%

c) Mercuric chloride ($Hgcl_2$) – 0.25%

2. N/10 Hydrochloric acid or HCl

Dissolve 10ml concentrated HCl with 1000ml distilled water.

3.8. Materials for Immunological studies

1. Alsever's Solution

a) Dextrose - 20gm

b) Sodium citrate - 8gm

c) Sodium chloride – 4.1gm

d) Double distilled water – 1000mL

2. Tris – NH_4Cl (0.84%, P_H 7.4)

a) Tris base – 206mg

b) Ammonium chloride (NH_4Cl) – 770mg

c) Distilled water – 100ml

3.9. Materials used for preparation of tissue samples for Transmission Electron Microscopy (TEM)

1. 0.1M Phosphate Buffer Solution (pH 7.2)

a) 0.1M disodium hydrogen phosphate – 72 mL

b) 0.1M sodium dihydrogen phosphate - 28 mL

2. 2% Para formaldehyde solution.

3. 2.5% Gluteraldehyde solution (EM grade).

4. 1% osmiumtetraoxide.

5. Acetone.

6. Toluene.

7. Araldite (CY 212)

8. Dodecenyl succinic anhydride (DDSA).

9. 2, 4, 6 tridimethyl amino methyl phenol (DMP-30).

10. Dibutylopthalate.

11. Uranyl acetate.

12. Lead citrate.

3.10. Histopathological techniques

3.10.1. Sampling and fixation

Live fishes were quickly put into a container of benzocaine solution (25mg/litre) for two minutes. Then the fish was taken out of the solution and pinned on a dissection tray. The external lesions were first excised from the fish body with sterilized scissors and forceps and put in Bouin's fixative. The internal organs were carefully dissected out, cut into small pieces of 3mm to 5mm and placed in fixative as early as possible. The volume of fixative was always at least 20 times of the volumes of the tissues. The tissues were kept in fixative for overnight.

3.10.2. Processing

The fixative was first washed out of the samples by 70% alcohol. Then routine procedure was followed by passing through a graded series of alcohol solutions (70%, 90% and 100%) for dehydration of the samples. (Schäperclaus, 1986)

After dehydration the tissues were then placed in xylene and infiltrated with molten paraffin (melting point 58° -60°C) and allowed to cool and harden. Some times tissues were preserved in cedar wood oil following fixation and dehydration.

3.10.3. Sectioning

The hardened paraffin containing the tissues were trimmed into rectangular blocks and mounted on the microtome and sections of 6μ thickness were cut. The sections were placed on grease free slides with albumin serving as an adhesive. The sections were properly stretched on warm distilled water and the slides were dried by keeping them overnight at room temperature.

3.10.4. Staining

Before staining the stretched and dried sections were completely dewaxed by keeping them in xylene for 5 to 10 minutes.

3.10.5. Procedures for Haematoxylin-Eosin stain

After complete dewaxing the slides with stretched sections were kept in absolute alcohol for 5 min. and passed through descending grades of alcohol e.g. 90%, 70%, 50% and 30% to water. The sections were then stained with haematoxylin and washed thoroughly with water to remove the excess stain. Next the sections were examined under microscope to check the differentiations level. If the differentiation was upto the mark, the sections were then passed through ascending grades of alcohol and stained with alcoholic eosin. The excess eosin was washed off by 90% alcohol and again examined under the microscope to check the differentiation level. If the counter staining was satisfactory the tissues were further dehydrated in absolute alcohol, cleared in xylene and mounted in DPX mountant.

3.11. Haematological techniques

3.11.1. Smear preparation and staining of blood

At first the slides on which blood smears were to be prepared, were made grease free. To make the slides grease free, the slides were first kept in chromic acid for 48 hours after which the slides were thoroughly washed with tap water and dried and then placed in alcohol and benzene (1:1) solution. Then the slides were wiped with a piece of clean cotton cloth and drawn through flame (Schäperclaus, 1986). Now a fresh drop of blood obtained by cutting the caudal region of a fish was placed on a grease free slide. A thin film of the blood was drawn with the help of another slide held at 45°C. The smears were then air dried at room temperature. The smears were then stained with Leishman stain, PAS, Graham-Knoll-benzidine counter stained with Giemsa and Sudan Black-B so that blood cell morphology can be characterized with sheer perfection.

3.11.2. Blood Sampling

Fishes were picked up from aquariums and put in benzocaine solution of 25mg/L for 2 minutes for proper anesthetization. The caudal region of the fish was cut off and the blood was collected in a tube rinsed with EDTA solution (1mg/mL). Leucocyte and erythrocyte counting and measurement of haemoglobin content of blood were done immediately after collection of blood.

3.11.3. Blood studies

Leucocyte count

The clean and dry W.B.C pipette was first rinsed with 1% sodium citrate so that blood clotting can be avoided. Blood was then sucked upto the 0·5 mark and immediately blood was diluted with Hayem's solution (NaCl- 1%; NaS_2O_4- 2·5%; $HgCl_2$- 0·25%) 20 times sucking upto 11 mark. Then the pipette was rotated slowly so that blood could be mixed properly with diluting solution. After discarding the clear fluid in the capillary part, a drop of mixture was added to the edge of a cover slip which was already placed on the platform of the haemocytometer (Fein-optik, Blakenburg, Germany). After three

minutes count was made under a microscope. The leucocytes were recognized by their refractile appearance.

Calculation

$$\text{Number of leucocytes per cubic mm} = \frac{\text{Number of cells counted} \times \text{Dilution} \times 10}{\text{Number of 1 sq.mm counted}}$$

Erythrocyte count

The clean and dry erythrocyte pipette was rinsed with 1% sodium citrate to avoid clotting of blood. Blood was then sucked to the 0·5 mark. The excess blood sticking to the tip of pipette was wiped off. Then the diluting solution was sucked upto 101 marks. The rubber tube was discounted and the either end of the pipette was gripped between forefinger and thumb and was shaken thoroughly for a minute. The rubber tube was reattached to the pipette and unused diluting fluid present in the stem of the pipette was rejected. Now an optimum drop was placed between the crevice of cover slip and the slide of counting chamber. Counting was then done in cells in 16 squares in five different parts of the slide.

Calculation

R.B.C of 80 small squares is multiplied by 10,000.

Determination of Haemoglobin content of blood by Sahli's method

Five drops of N/10 HCl were poured into an absolutely clean Sahli's tube so that the surface of acid reached the 10 mark in the tube. Fish blood was drawn into Sahli's pipette from cut off arterial caudalis upto the 20 mark. After wiping out the blood from the tip of pipette with the help of sterilized cotton, the tip of the pipette was dipped into the Sahli's tube below the surface of the HCl and the blood was carefully expelled. Drop by drop distilled water was added into the tube and was constantly matched with the colour of mixture with the colour of adjacent tubes on either side of the instrument. This was continued until the colour of the mixture exactly matched with the standard colour. Now the end point was recorded.

3.11.4. Quantifying erythropoietic efficiency of haemopoietic organs

Erythropoietic efficiency of haemopoietic organs was studied following the method of Homechowdhury and Jha (2001). The whole head kidney and the thymus were first dissected out from the live fish and excess peripheral blood was removed by using Whatman filter paper. Now both the organs were weighed and transferred to two separate watch glasses containing 1ml Hayemen's solution and were cut into several pieces. Cells were then dispersed by gently holding pieces of tissue with fine forceps in diluting fluid for exactly six minutes. Different cells along with blast cells were freed from the tissue and precipitated in the watch glass. After proper mixing of the cells in diluting fluid, 100mL of mixture was micropipetted into a dry and clean centrifuge tube and centrifuged for 1 minute at 1000 r.p.m. As the blast cells remained in the supernatant and other debris were pelleted. Ten microlitres of supernatant was carefully transferred to a Neubaeurs's improved double haemocytometer for total counting of different blast cells. At this stage special care was taken to avoid the mixing the debris to the testing sample. The temperature during tissue handling process was strictly maintained at 18°c. Presence of different blast cells as for example small lymphoid haemoblasts, basophilic erythroblasts, polychromatophillic erythroblasts, acidophilic erythroblasts and other lineage cells such as young reticulocytes, mature reticulocytes and erythrocytes in the haemocytometer were confirmed by comparing their morphometry with that of the various cell types identified from simultaneous tissue imprints of head kidney and thymus prepared according to Ashley and Smith (1963).

Calculations

The number of blast cells present in four corners of haemocytometer with an area of 1mm and a depth of 1/10 mm. So the volume would be $1/10mm^3$. Summation volume of four corners would be $= 4\times 1/10mm^3$

$$= 0\cdot 4\ mm^3$$

Now the number of blast cells/mm^3 was multiplied by dilution factor 2·5.

3.11.5. Preparation of tissue-imprints of head kidney, thymus and spleen following Ashley and Smith (1963)

The head kidney, thymus and spleen were dissected out. Excess blood associated with the organs was removed by blotting with Whatman filter paper. Next one end of the tissue was held by fine forcep, and with the help of a fine scissor the tissue was cut longitudinally into two parts and the cut was made at right angle to the tissue. Now the surface which was opened due to longitudinal cutting was put on the grease free slide, pressed gently and dragged for a distance resulting a thin film of tissue layered on the slide. The slides were now kept at room temperature for overnight. Then the slides were stained with appropriate staining method.

3.11.6. Staining procedures adopted for blood smears

1) Leishman's stain

1. The air-dried blood slide was kept in a modified petridish meant for staining of slides.

2. The slide was flooded with Leishman's stain, kept at 4°c.

3. The slide was kept for 5 minutes in covered condition.

4. After 5 minutes the same amount of distilled water was added to the slide.

5. The slide was then kept in covered condition for another 10 minutes.

6. The slide was washed with distilled water and air-dried.

7. Then the slide was mounted with D.P.X mountant.

2) Graham's Knoll- benzidine counterstained with Giemsa

1. Slides were first immersed in a cuplinger full of Formal alcohol for only 30 seconds.

2. The slides were kept in running water for 5 minutes.

3. The slides were air-dried.

4. The slides were then kept in Benzidine solution for 10 minutes

5. Then the slides were kept in running water for 10 minutes.

6. The slides were again air-dried.

7. The slides were kept in Giemsa (1mL Giemsa + 49mL distilled water) solution for at least 48 hours at 4°c.

8. Finally the slides were washed with distilled water and dried and mounted with D.P.X mountant.

3) Periodic Acid Schiff stain (De Tomasi, 1936)

1. The blood slides were slowly brought to water following down grades of alcohol (100%, 90%, 70%, and 50%).

2. Then the slides were kept in 10% periodic acid for 5-8 minutes.

3. The slides were washed with running water for 3 minutes.

4. The slides were washed with distilled water for 1 minute.

5. The slides were then kept in Schiff's reagent for 15 minutes in dark.

6. The slides were washed with tap water for 10 minutes and then dried and mounted with Euparol mountant.

4) Sudan Black – B

1. Slides were fixed in formalin vapour for 2-5 minutes.

2. Then slides were immersed in 25% acetic acid for 2 minutes.

3. Slides were washed thoroughly in tap water, then in distilled water.

4. The slides were then dried.

5. The slides were then stained in saturated Sudan Black B in 70% alcohol for 2 hours.

6. Excess stain was removed by washing in 70% alcohol.

7. Slides were blotted to dry and mounted in Glycerin jelly.

3.12. Immunological studies

Healthy *C. mrigala* (60-80gm) were carefully dissected for the location of their lymphoid organs and were placed in a petridish containing phosphate buffer saline (PBS – pH 7·2).

3.12.1. Antigen

Sheep blood was drawn from Juglar vein of sheep with the help of sterilized syringe in Alsever's solution and was washed twice with PBS. The pellet was diluted with PBS to make 25% solution of sheep red blood corpuscles or SRBC. This 25% solution of sheep red blood corpuscles or SRBC was used for immunization for all experiments.

3.12.2. Immunization

Different dosage of 25% SRBC and different routes of administration of SRBC were tried for experiments in search of the optimum dose and most effective route of administration out of three different doses (such as 0.1mL; 0.2mL and 0.4mL) 0·2ml was found to be the optimum and out of three routes of administration (such as intramuscular (i.m), intravenous (i. v) and intraperitoneal (i.p) injection was found most effective. (Fig. 6)

3.12.3. Cell Suspension

Healthy *C. mrigala* (60-80gm) was anesthesized by keeping in benzocaine solution. Lymphoid organs (head kidney, spleen and thymus) were dissected out separately and the

cells were dissociated by pressing the organs against a 200 gauge stainless steel wiremesh as per standard methodology (Chaudhuri, 1983). Before pressing the lymphoid organ against the wiremesh a small amount of PBS was injected into the lymphoid organ. At the time of rubbing, PBS was added drop by drop and the whole solution was collected in a sterilized Petri dish. The cell suspension was transferred to a centrifuge tube with the help of a Pasteur pipette. The centrifuge tube was spinned at 1000 r.p.m for 10 minutes in a centrifuge machine. The supernatant was discarded. Then 5ml chilled Tris-NH_4Cl (0.84%, pH 7.4) was added to the tube and kept for 5 minutes to lyse the red blood cells completely. The cell suspension was washed with PBS twice. The cell suspension was layered over Ficoll Hypaque solution and was spinned at 3000 r.p.m for 5-8 minutes. Lymphocytes were collected from interface and were washed twice with PBS. Finally cell suspension was adjusted to a required concentration with the help of Trypan blue exclusion test.

3.12.4. Haemogglutination Titre Assay

Healthy *C. mrigala* (60-80gm) was immunized by injecting 0.2mL of 25% SRBC intraperitoneally. After a certain day the caudal region of the immunized fish was cut and blood was collected in a small test tube. The blood was allowed to clot. After proper clotting it was centrifuged and the serum was collected. The serum was then heat inactivated for 30 minutes at 56°c to inactivate the complement. Round bottom 96 well microtitre plate (Tarson) was taken and 360µl of PBS was poured in well no.1, and 180µl of PBS was poured of the wells. 20µl heat inactivated serum was added in well no.1 and was mixed thoroughly. Then 200µl of mixed solution from well no.1 was taken and mixed with well no.2 and from well no.2 again 200µl of mixed solution was taken and was mixed with well no.3. This same method was followed upto well no.12 and from 12^{th} well the 200µl was taken and thrown out. Finally, 20µl of 1% SRBC was added to each well. The micro titre plate was kept at 37°C in a moist sterilized condition for overnight.

3.12.5. Plaque Forming Cell (PFC) Assay (Cunningham & Szenberg, 1968)

For PFC assay Cunningham and Szenberg (1968) technique with minor modifications was followed. Lymphocyte cell suspensions from head kidney and thymus on 5th day after immunization with an intraperitoneal injection with 0.2ml of 25% SRBC were prepared The concentration of cell in cell suspension was adjusted to 10^6 cells /mL. Prior to the preparation of cell suspension Plaque Forming Cell Assay (PFC) slides were prepared.

Preparation of PFC assay slides

1. At first the microscopic glass slides were cleaned and made grease free.

2. Clean and dry grease free slides were kept either on a smooth surfaced hard board or on a thick paper.

3. Three double strips of double-sided tape (TOV tape) cut at 7mm width were placed at the two ends and on the middle of the slides.

4. Then the cover of the upper surface of upper layer of the double sided tape was stripped off.

5. Two slides were placed on top of the exposed gum of the tape, matching each slide face to face.

6. Then the slides were pressed with the rubber stopper of a bottle along the taped line to get a set of two slides fixed face to face.

Charging of the PFC assay slide

First 0.1mL of the diluted cell suspension, 0.05mL of rabbit complement and 0·05ml of 10% SRBC were mixed in a small tube with a microtip pipette. Then the mixed solution was introduced into the microchambers of PFC assay slide. The longer edges of the paired slide were dipped into a molten 1:1 paraffin and Vaseline mixture to

seal the edges. The slides were then incubated at 37°C for 3-4 hours in a humidified atmosphere. After 3-4 hours of incubation in a humidified atmosphere haemolytic plaques were appeared as clear circular spaces on the background of intact red blood corpuscles. The plaques were counted under a low power microscope.

3.12.6. Transmission Electron Microscopy (TEM)

Head kidney, spleen and thymus were dissected out from healthy and EUS affected *Cirrhinus mrigala* (150gm) and cut into pieces of 2×1mm size. The pieces were washed in 0.1 (M) phosphate buffer solutions (pH 7.2). The tissues were then fixed in the fixative solutions at room temperature for 20 minutes and then were cut into two pieces after 3 hours of fixation. Total fixation will be for 8-12 hours at 4°C. A change with fresh fixative was given in the middle. Then the tissues were washed with 0.1 (M) phosphate buffer solution (pH 7.2), three times, for one hour duration each at 4°C. The tissues were treated with 1% osmium tetraoxide for 2 hours for post-fixation. After treatment with osmium tetraoxide the tissue pieces were again washed with 0.1 (M) PBS three times. The fixed tissues were then dehydrated with an ascending grade of acetone (30-50-70-80-90-95%) at 4°C and finally kept in dry acetone for 30 minutes at room temperature. After proper dehydration, cleaning was done with toluene twice for 30 minutes followed by infiltration with embedding medium and toluene (1:1). Then embedding was done in pure embedding medium containing araldite (CY212), dodecenyl succinic anhydride (DDSA), 2, 4, 6 tridimethylaminomethyl phenol (DMP-30) and dibutylopthalate. Then polymerization was done by putting the blocks at 50°C for 12 to 14 hours followed by 24 to 48 hours at 60°C. After polymerization 0.5 to 2μm ultra thin sections were cut with ultra microtome. Sections were then placed over copper grids. The sections were stained with uranyl acetate for 10 minutes at dark and then with lead citrate for 10 minutes. The grids were photographed under Transmission electron microscope (Philips CM 10, The Netherlands) at AIIMS, New Delhi.

Observations and Results

4.1. Head kidney of healthy *Cirrhinus mrigala*

4.1.1. Morphological studies

The kidney of *Cirrhinus mrigala* is divided into two distinct parts, an anterior part, the head kidney and a posterior part, the trunk kidney. The two lobes of the head kidney are leaf like and are joined by a bulbous portion to the trunk kidney. Kidney is situated dorsal to the abdominal cavity (Fig. 7).

4.1.2. Histological studies

Routine histological studies under light microscope showed that the head kidney was covered by a thin strand of collagen fibres. It was divisible into two distinct regions, outer cortex and inner medulla. Renal tubules were scanty (Fig. 8).

Histological sections showed presence of erythroid and lymphoid cells in both cortex and medulla of the kidney. The presence of more lymphoid cells and less erythroid cells have been found in the cortex compared to medulla.

4.1.3. Ultra microscopic studies

Cells present in the head kidney were varying numbers of lymphomyeloid and erythroid cells and they are not tightly packed Adjacent to blood capillaries or blood sinusoids lymphoblasts were predominantly found along with granulocytes (melanomacrophages), monocytes, plasma cells and epithelial cells (Fig. 9). Other regions showed presence of heterogenous population of cells consisting of neutrophils, neutrophilic myelocytes, haemoblasts, mature erythrocytes, thrombocytes, erythroblasts, lymphocytes and lymphoblasts etc (Fig. 10).

Occasionally presence of epithelial cells forming a network with a few lymphocytes, thrombocytes was detected. Cystic cavity with pyknotic cells was also found (Fig. 11).

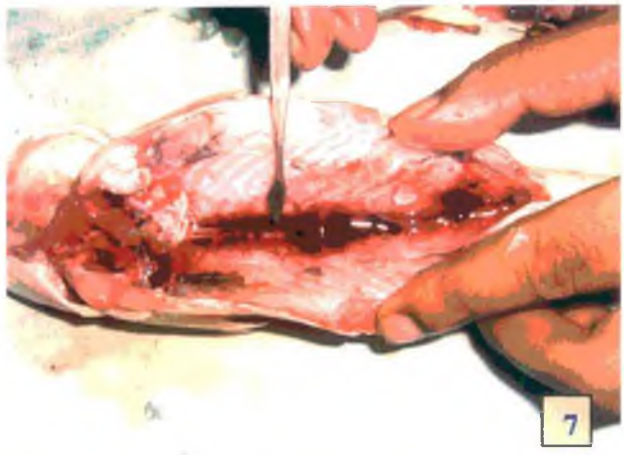
Fig. 7: Head kidney of a healthy *Cirrhinus mrigala.*

Fig. 8: Histological section of head kidney of a healthy *C. mrigala* showing lymphoid and erythroid cells alongwith regional distinction as cortex and medulla.

Fig. 9: Electron microscopic picture (TEM) of head kidney of healthy *C. mrigala* showing different ells, small lymphoid haemoblast (shl), lymphocyte (lym), monocyte, melano macrophages and epithelial cells.

Fig. 10: Electron microscopic picture (TEM) of head kidney of healthy *C. mrigala* showing different cells, neutrophils (N), neutrophilic myelocytes (NM), haemoblasts (Hb), mature erythrocytes (ME), thrombocytes (Th), erythroblasts (EB), lymphocytes (L) and lymphoblasts (LB).

Fig. 11: Electron microscopic picture of head kidney of healthy *C. mrigala* showing the cellular composition predominantly with epithelial cells, forming a network with a few lymphocytes, thrombocytes and cystic cavity with pyknotic cells.



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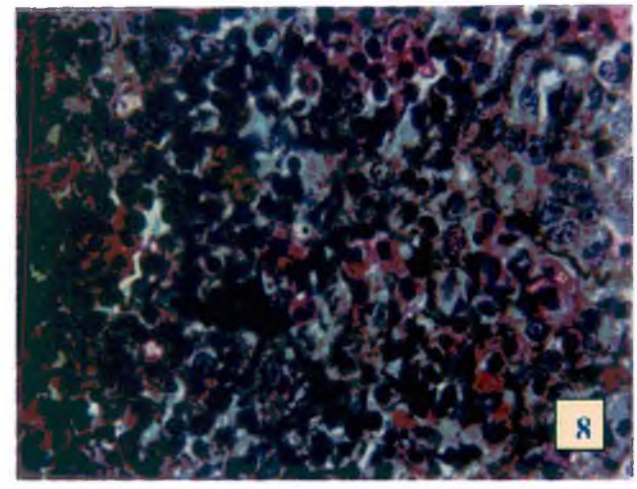

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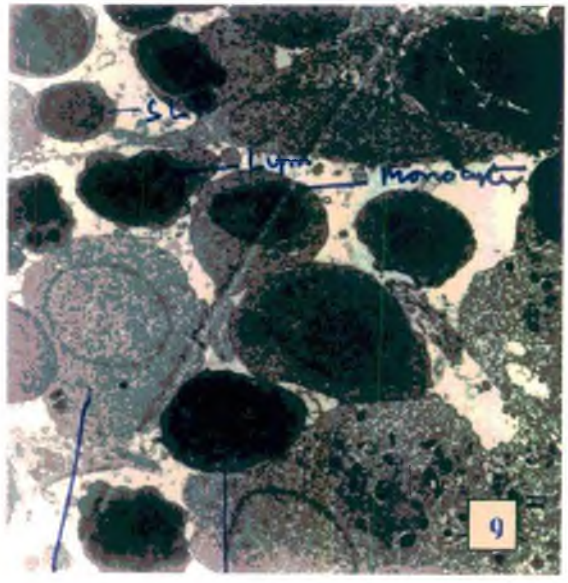

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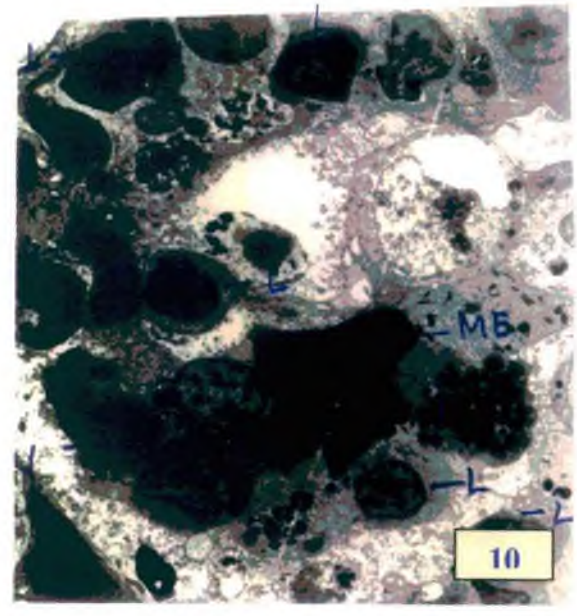

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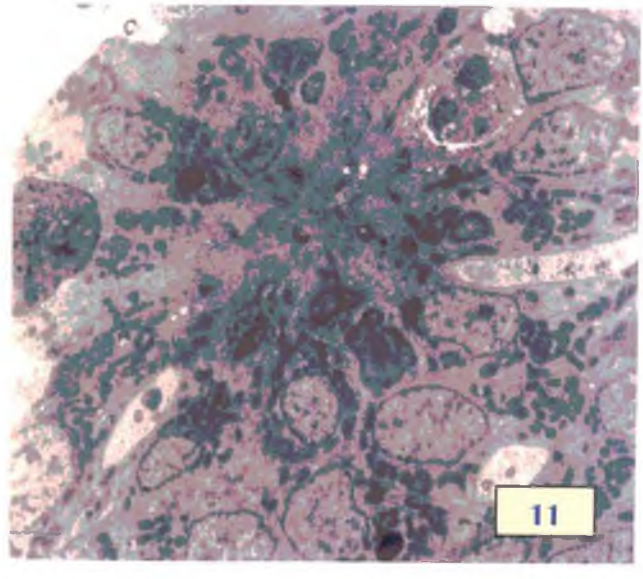

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4.2. Spleen of healthy *Cirrhinus mrigala*

4.2.1. Morphological studies

Spleen of *C. mrigala,* is an irregular structure and dispersed in form. It is located on or between the loops of intestine.

4.2.2. Histological studies

Histological studies of spleen under light microscope showed presence of both red pulp and white pulp regions. White pulp was surrounded by the red pulp. The red pulp was composed of different developmental stages of erythrocytes with a few lymphocytes while the white pulp region was comprised of reticular cells (Fig. 12).

4.2.3. Ultra structural studies

In the spleen the cells were more tightly packed. The cell types found in the spleen were of following types: thrombocytes with electro lucent cytoplasm, lymphocytes, reticular cells, monocytes with prominent cytoplasmic organelles (Fig 13) and neutrophils along with type-I and type-II granules (Figs. 14, 15, 16, 17, 18 and 19).

Presence of cords of erythrocytes was recorded in some regions. The cords were separated by connective tissue and electron lucent reticular cells.

4.3. Thymus of healthy *Cirrhinus mrigala*

4.3.1. Morphological studies

Thymus is a paired organ which is reddish in colour, triangular in shape and occupies the dorsolateral regions of the opercular cavity (Fig. 20).

Fig. 12: Histological section of spleen of healthy *C. mrigala* showing red pulp (rp) and white pulp (wp) regions.

Fig. 13: E/M (TEM) picture of spleen of healthy *C. mrigala* showing different cell types like Young reticulocyte, Mature erythrocyte (ME), Monocyte (M), Neutrophilic myelocyte (NM), Reticulo erythrocyte (RE) etc.

Fig. 14: E/M (TEM) picture of spleen of healthy *C. mrigala* showing a typical thrombocyte electro-lucent cytoplasm and extensive surface connected canalicular system of cytoplasm.

Fig. 15: E/M (TEM) shows the picture of a typical splenic lymphocyte.


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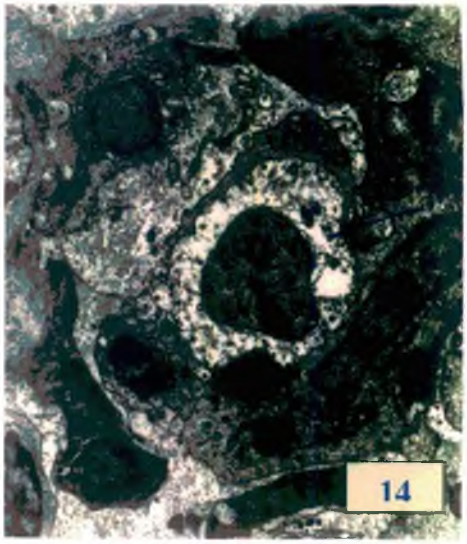
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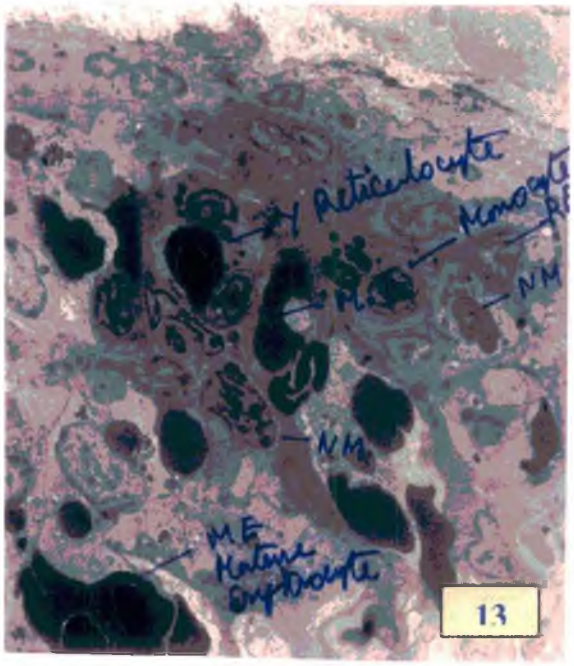
Reticulocyte
NM
NM
M.E
Mature
Erythroblast
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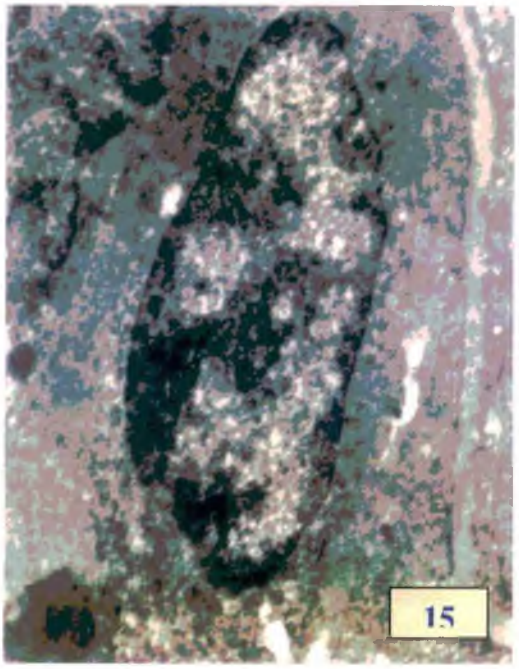
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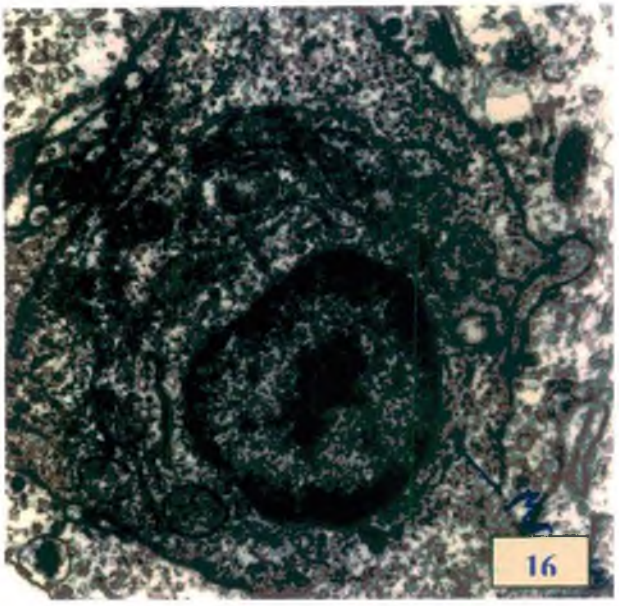
Fig. 16: E/M (TEM) shows the picture of typical cell found in spleen of healthy *C. mrigala.*

Fig. 17: E/M (TEM) shows a neutrophil present in spleen of healthy *C. mrigala* with Type-I and Type-II granules.

Fig. 18: E/M (TEM) shows a typical cell surrounded by red blood cells in spleen of healthy *C. mrigala.*

Fig. 19: E/M (TEM) shows the cords of erythrocytes along with indistinct RT or Reticulo endothelial cells in spleen of healthy *C. mrigala.*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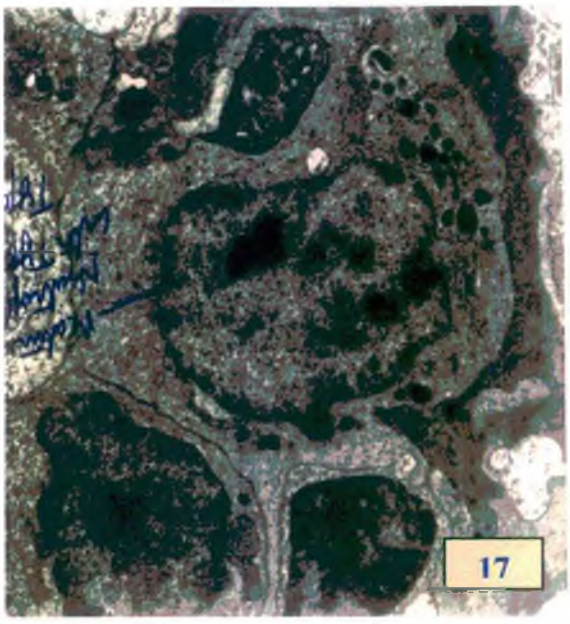
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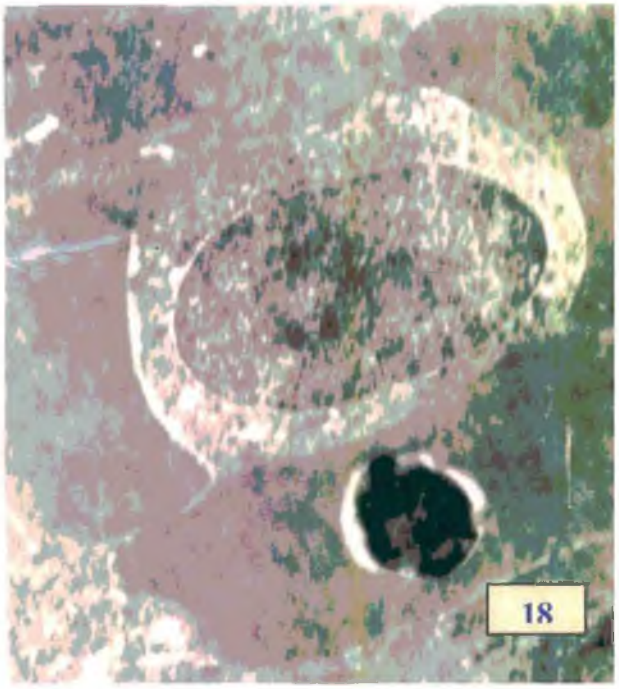
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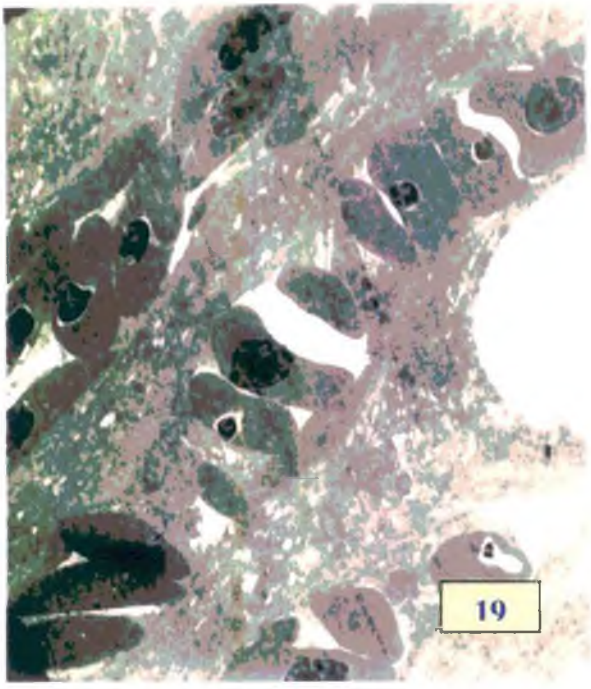
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Table2. The size and weight of the thymus of healthy *C. mrigala*

Weight of fish (gm)	Thymus		
	Length (mm)	Width (mm)	Weight (gm)
72.00±5.40	3.928±0.2177	2.815±0.2404	0.0116±0.00245

Mean±S.D; n=10

The weight of thymus of healthy *C. mrigala* weighing 72.00±5.40gm was 0.0116±0.00245 gm (Table 2). The Fig. 37 shows the comparison between the weights of thymus of healthy and EUS affected *C. mrigala.*

The length and breadth of thymus were 3.928±0.2177mm and 2.815±0.2404 mm respectively.

4.3.2. Histological studies

Histological sections of the thymus of healthy *C. mrigala* showed the presence of a thin capsule which sends trabeculae into the stroma to form lobules. The lobules were not completely separated but interconnected due to discontinuous nature of the trabeculae. The stroma was not differentiated into cortex and medulla. The stroma showed presence of hemopoietic cells arranged in cords (Fig. 21). The cords of hemopoietic cells contained mainly developing blood cells. Reticular epithelial cells were present in the space between the cords of hemopoietic cells.

Hassall's corpuscles like structures consisting of concentric layer of epithelial reticular cells were also detected.

4.3.3. Ultra microscopic studies

Transmission electron microscopic studies showed the presence of cords of erythrocytes separated by cords of cells with secretory granules. Apart from cords of

erythrocytes and cords of cells with secretory granules, a considerable number of lymphocytes, heterogenous epithelial cells, macrophages, pigment cells (melano macrophages), granulocytes, myoid cells were also detected (Figs. 22, 23 and 24).

Heterogenous epithelial cells community mainly constituted four types of epithelial cells: reticular epithelial cells (RT), epithelial cells with semi electron dense secretory granules, large epithelial cells with cystic cavity and limiting epithelial cells along with capsule (Fig. 22).

Reticular epithelial cells were characterized by the large euchromatic nucleus and with electron lucent cytoplasm. Epithelial cells with semi electron dense secretory granules were frequent in between two cords of erythroid cells. Large epithelial cells with cystic cavities were found less frequently in an inner part of thymus. Limiting epithelial cells along with connecting tissue capsule were also found (Fig. 25).

Pigment cells or melano macrophages characterized by a huge number of electron dense granules in the cytoplasm were also detected (Fig. 26). Mature myoid cells mainly occupied the inner region of thymus and were characterized by large round or oval cells with electron dense nucleus and cytoplasm. These cells were mostly found in association with smooth muscles (Fig. 27).

4.4. Head kidney of EUS affected *Cirrhinus mrigala*

4.4.1. Morphological studies

Morphological study of the head kidney of EUS affected *C. mrigala* showed no significant structural changes. Only the colour of head kidney of EUS affected *C. mrigala* appeared pale red compared to the head kidney of healthy fishes.

4.4.2. Histological studies

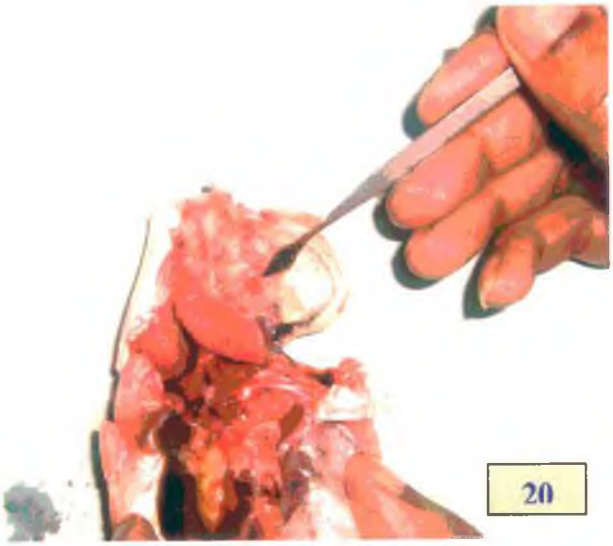
Light microscopic studies of the head kidney of naturally infected *C. mrigala* revealed significant changes in the histological structure of the head kidney.

Fig. 20: Picture shows the position of thymus in the fish.

Fig. 21: Histological section of thymus of healthy *Cirrhinus mrigala* showing inter connected lobules, trabeculae, cords of erythrocytes separated by other cell types.

Fig. 22: E/M (TEM) picture showing the cords of erythrocytes separated by other cell types present in thymus of healthy *C. mrigala.*

Fig. 23: E/M (TEM) picture of thymus of healthy *C. mrigala* showing different cells like, mature erythrocyte (MR), cells with huge secretory granules, Hypertrophied epitheliam (H.ep) etc.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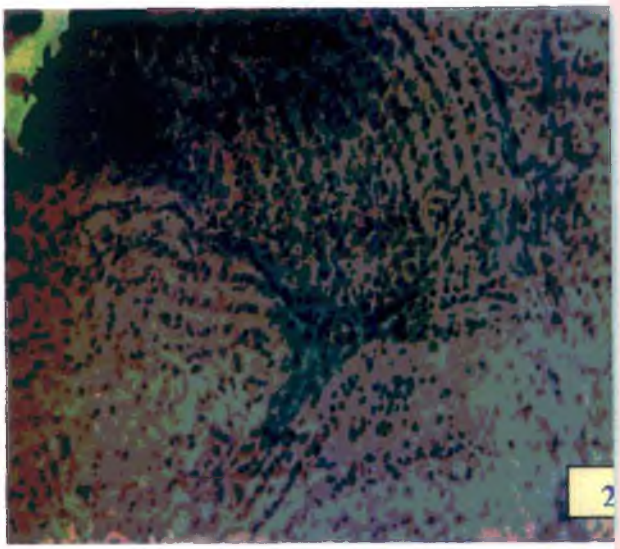
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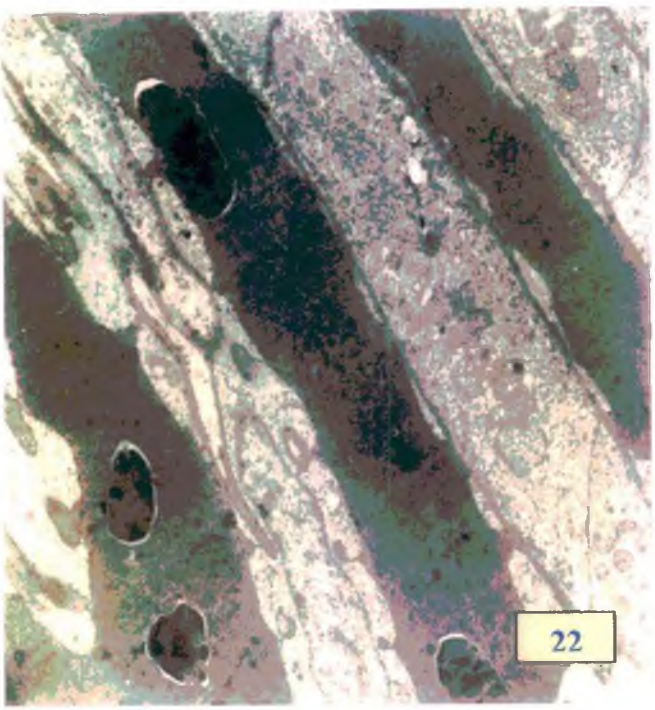
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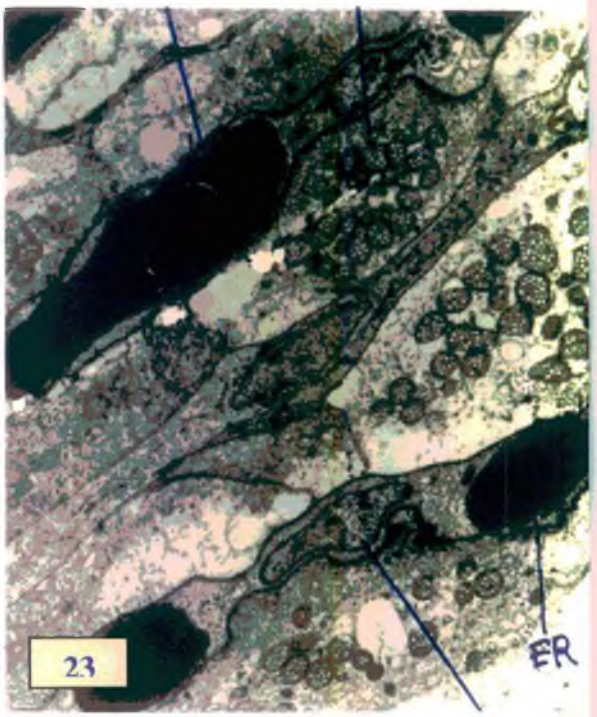
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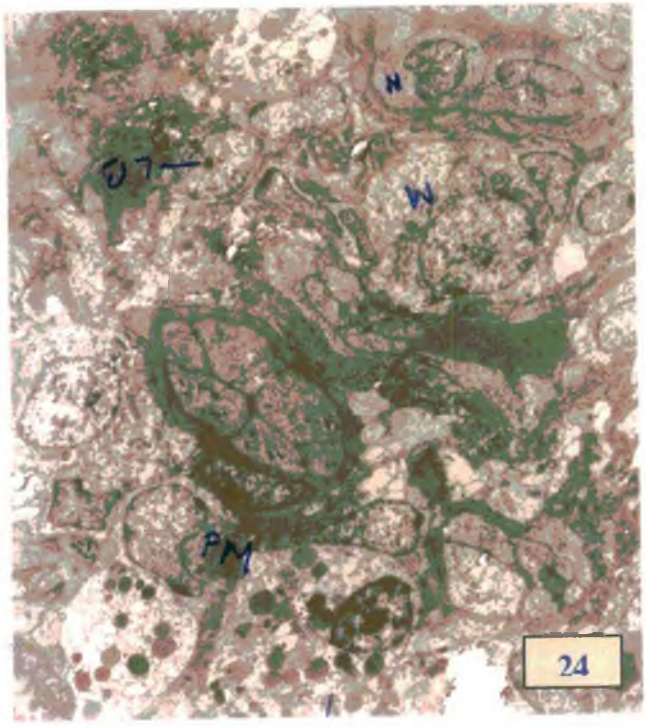
Fig. 24: Picture shows number of lymphoid cells within a network of epithelial cells.

Fig. 25: E/M (TEM) picture of thymus of healthy *C. mrigala* showing different cells like, lymphocytes, heterogeneous epithelial cells, macrophages, pigment cells (melano macrophages), granulocytes and myeloid cells etc.

Fig. 26: E/M (TEM) picture of thymus of healthy *C. mrigala* shows a cell with huge number of electron-dense granules.

Fig. 27: E/M (TEM) of thymus of healthy *C. mrigala* shows a typical myoid cell.



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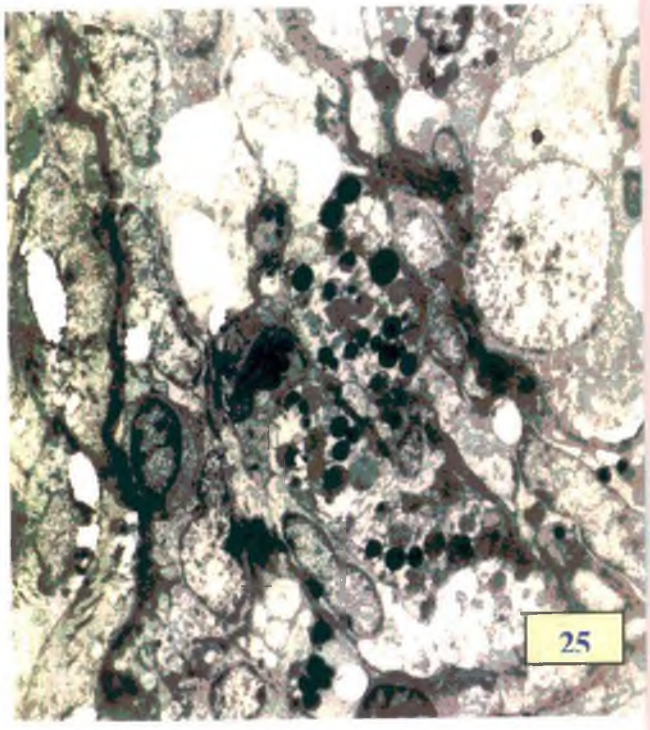
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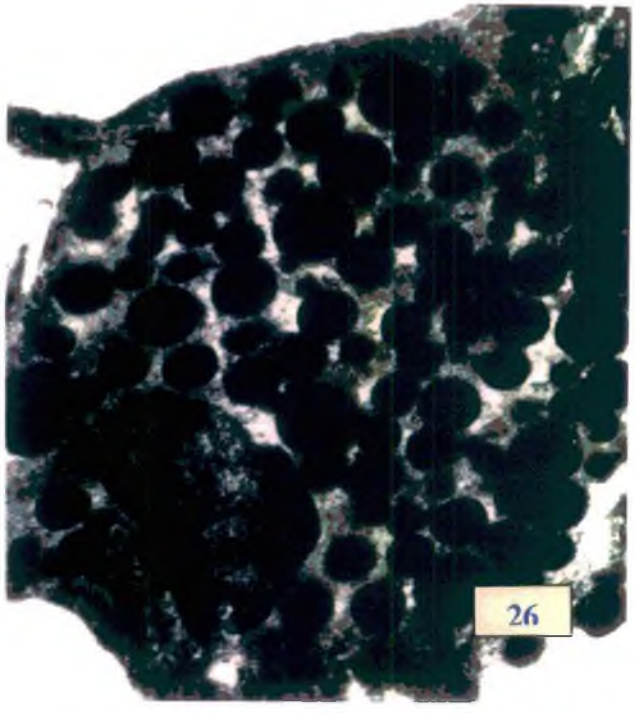
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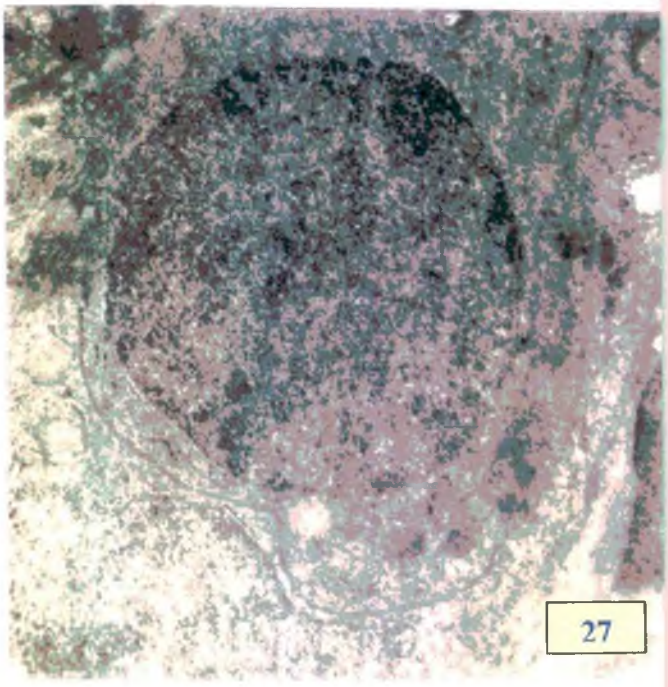
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Haemorrhages in some areas were detected along with the presence of haemosiderin laden macrophages. Necrotic changes were also very common in the head kidney of naturally infected *C. mrigala* (Figs. 28 and 29).

4.4.3. Ultra microscopic studies

Ultra structural studies of head kidney of EUS affected *C. mrigala* showed a considerable change. Presence of bacteria was found throughout the tissue. Along with the presence of bacteria, large areas of renal tissue showed necrotic changes. Melanin depositions and fibrin clumps were also observed (Figs. 30, 31 and 32).

Concentrations of erythrocytes in head kidney were significantly less compared to head kidney of healthy fish.

4.5. Spleen of EUS affected *Cirrhinus mrigala*

4.5.1. Morphological studies

No significant morphological changes of spleen of EUS affected *C. mrigala* were identified.

4.5.2. Histological studies

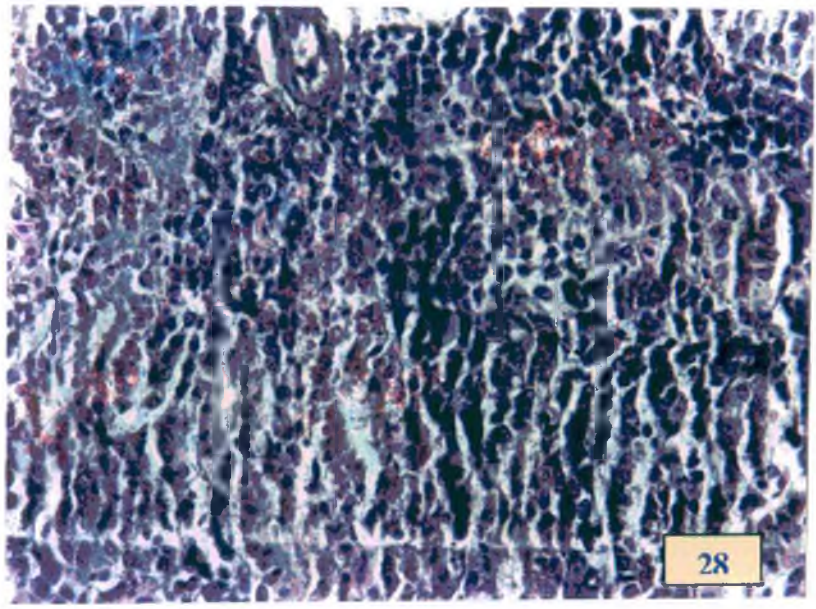
Necrotic changes along with vacuolation in the white pulp regions of spleen of naturally infected *C. mrigala* were noticed. Haemorrhages were also found (Fig. 33).

4.5.3. Ultra microscopic studies

Ultra structural studies of spleen of EUS affected *C. mrigala* showed some pathological changes. Necrotic changes and presence of fibrin clumps, depositions of melanin were noticed. Considerable decrease of erythrocytes was also observed. The splenic tissue of affected fish showed the presence of bacteria. Macrophages laden with bacteria were detected in some areas of the spleen (34, 35 and 36).

Fig. 28: Histological section of head kidney of EUS affected *C. mrigala* shows necrotic changes in the renal tissue.

Fig.29: Histological section of head kidney of EUS affected *C. mrigala* shows necrotic changes in the renal tissue in high power.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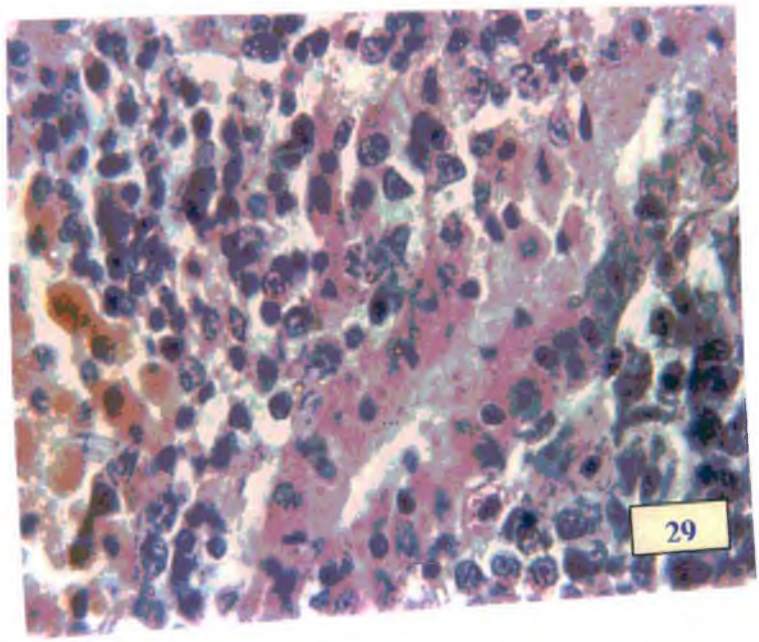
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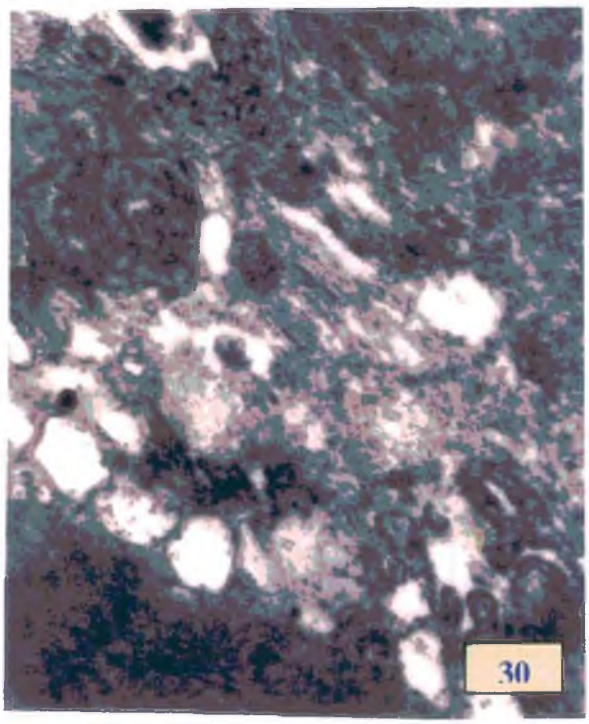
Fig.30: E.M studies of head kidney of EUS affected *C. mrigala* shows the presence of bacteria in renal tissue.

Fig.31: E.M studies of head kidney of EUS affected *C. mrigala* shows the melanin deposition and fibrin clumps around a lymphocyte.

Fig.32: E.M studies of head kidney of EUS affected *C. mrigala* shows the presence of necrotic change in renal tissue.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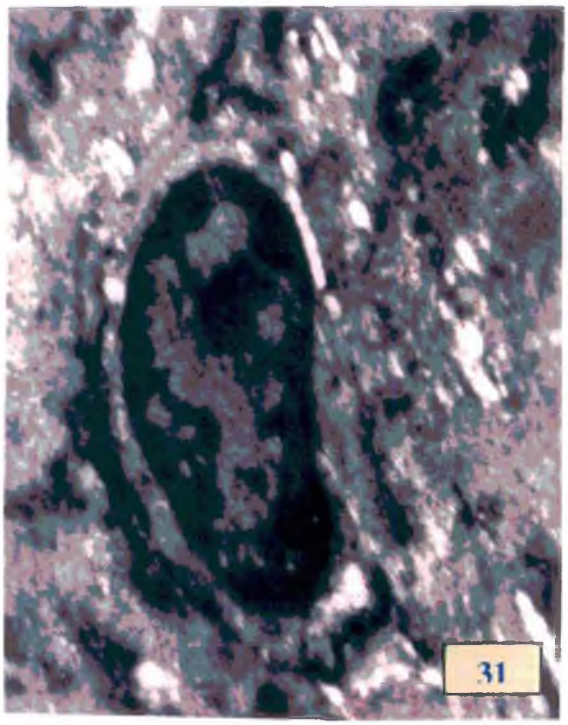
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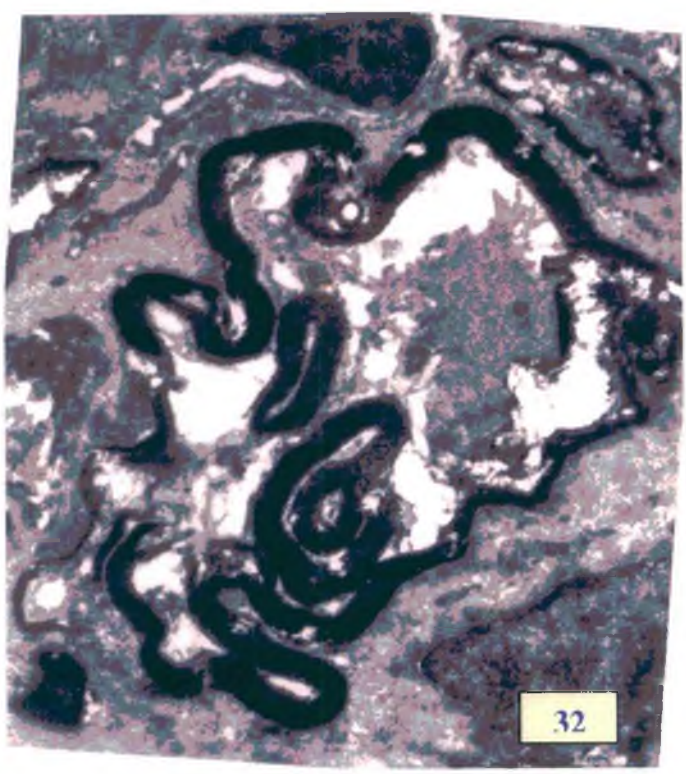
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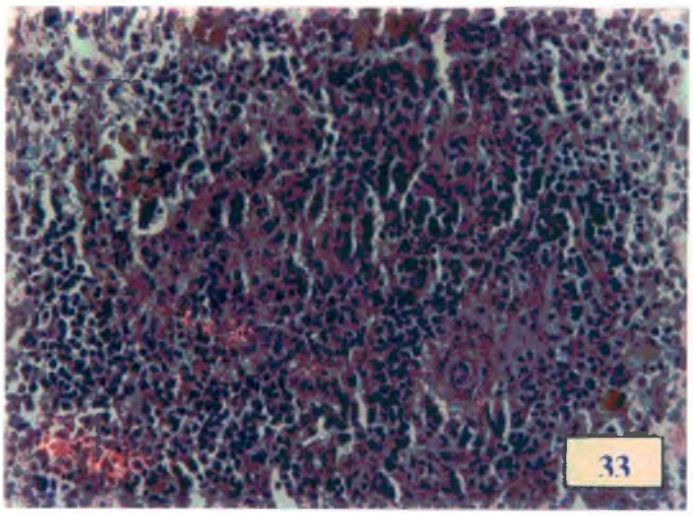
Fig.33: Histological section of spleen of EUS affected *C. mrigala* shows the necrotic changes in splenic tissue.

Fig.34: E.M studies of spleen of EUS affected *C. mrigala* shows the presence of melanin deposition and fibrin clumps.

Fig.35: E.M studies of spleen of EUS affected *C. mrigala* shows the presence of bacteria in between erythroid and lymphoid cells.

Fig.36: E.M studies of spleen of EUS affected *C. mrigala* shows the presence of huge gap between erythrocytes.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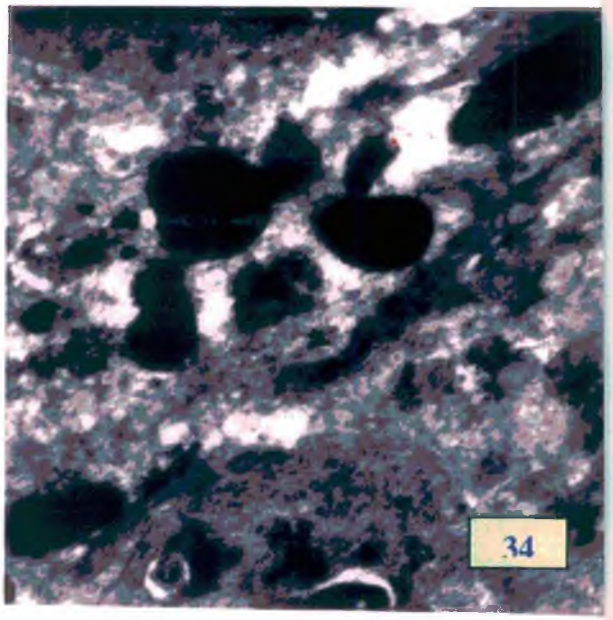
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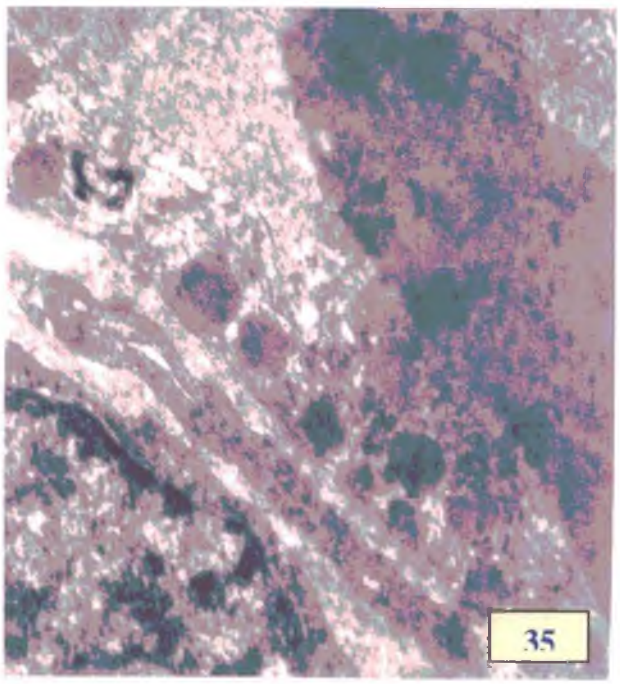
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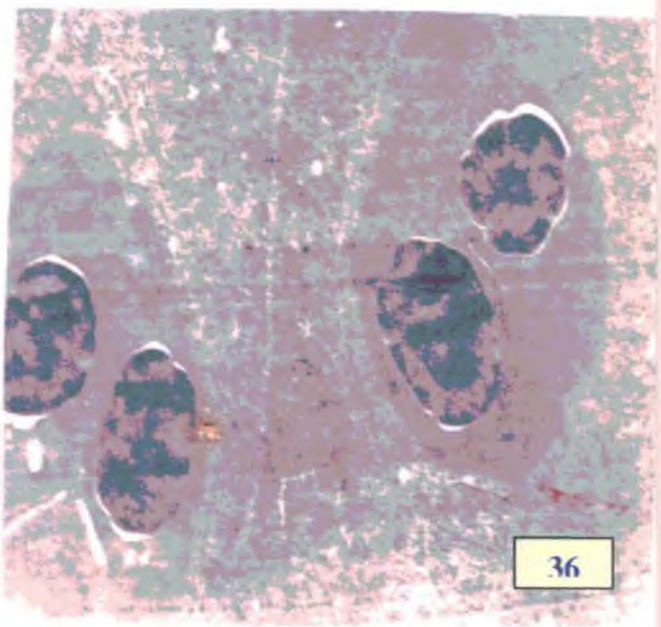
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4.6. Thymus of EUS affected *Cirrhinus mrigala*

4.6.1. Morphological studies

Morphological studies of thymuses of EUS affected *C. mrigala* revealed that thymuses of EUS affected fishes were to some extent enlarged compared to that of the healthy fishes. The colour of thymuses of EUS affected fishes appeared more redish.

The average weight of thymus of EUS affected *C. mrigala* weighing 58±6.7494 gm was 0.01468±0.001349gm and the average length and breadth of thymus were 4.714±0.1646mm and 3.31±0.2671 mm respectively (Table 3). The Fig. 37 shows the comparison between the weights of thymus of healthy and EUS affected *C. mrigala.*

Table3. The size and weight of thymus of EUS affected *C. mrigala*

Weight of fish (gm)	Thymus		
	Length (mm)	Width (mm)	Weight (gm)
58±6.7494	4.714±0.1646	3.31±0.2671	0.01468±0.001349

Mean±S.D; n=10

4.6.2. Histological studies

Histological observations of thymuses of EUS affected fishes showed highly cosinophillic areas near the capsule of the thymus which contained erythroblastic islets, cords of mature erythrocytes and reticular epithelial cells (Figs. 38 and 39). In some areas erythrocytes were replaced by reticular epithelial cells with large nucleolus. Sinuses filled with blood cells were frequently present. Hassall's corpuscles like structures were also detected near the trabeculae in the thymus of EUS affected fishes.

Comparison between the weight of thymus of healthy and EUS affected *C. mrigala*

Fig. 37

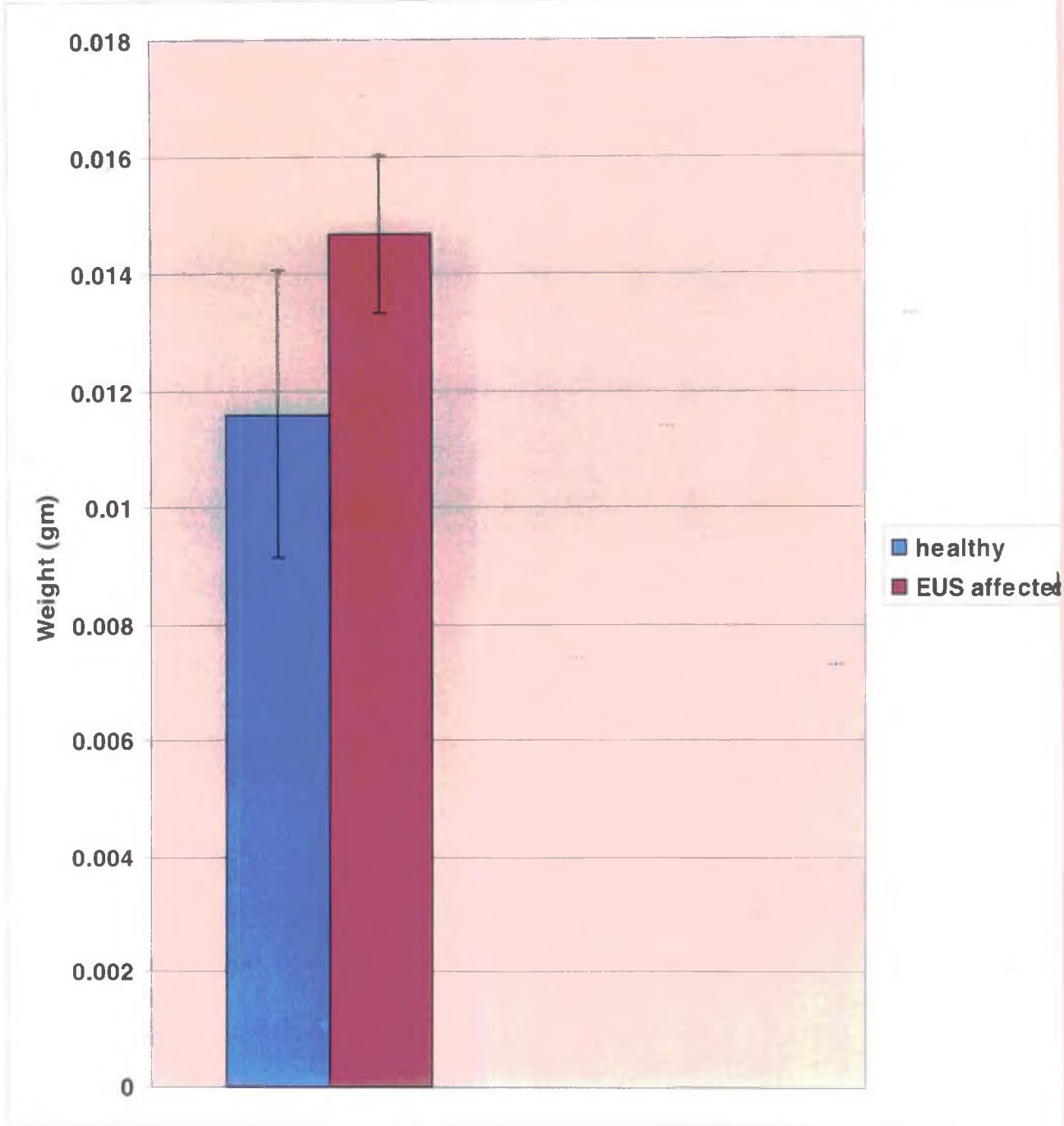
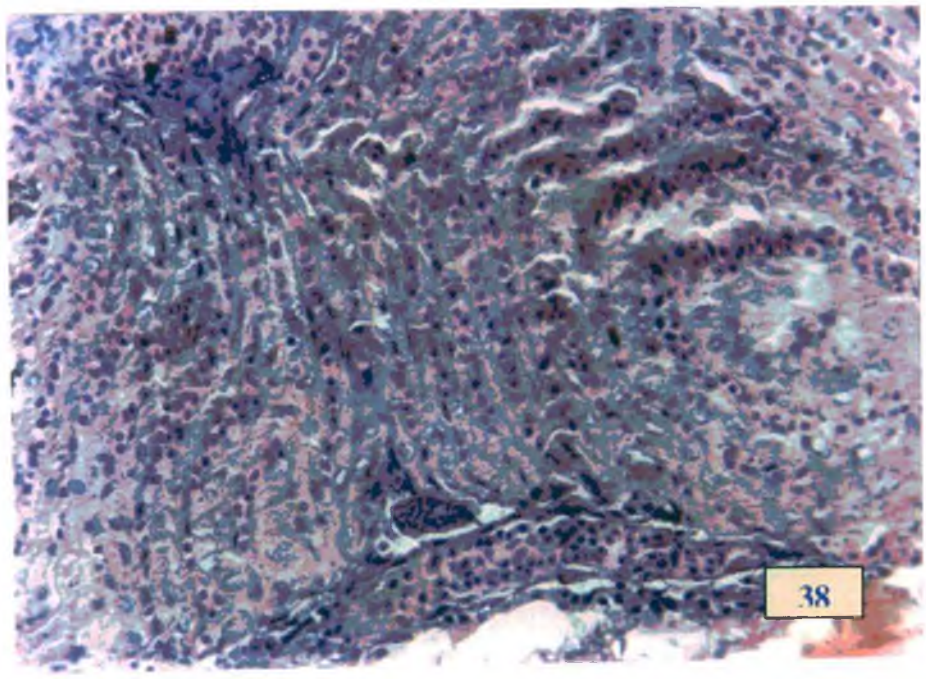


Fig.38: Histological section of thymus of EUS affected *C. mrigala* shows the necrotic changes and presence of erythroid islets.

Fig.39: Histological section of thymus of EUS affected *C. mrigala* shows the necrotic changes along with active macrophages cells in high power.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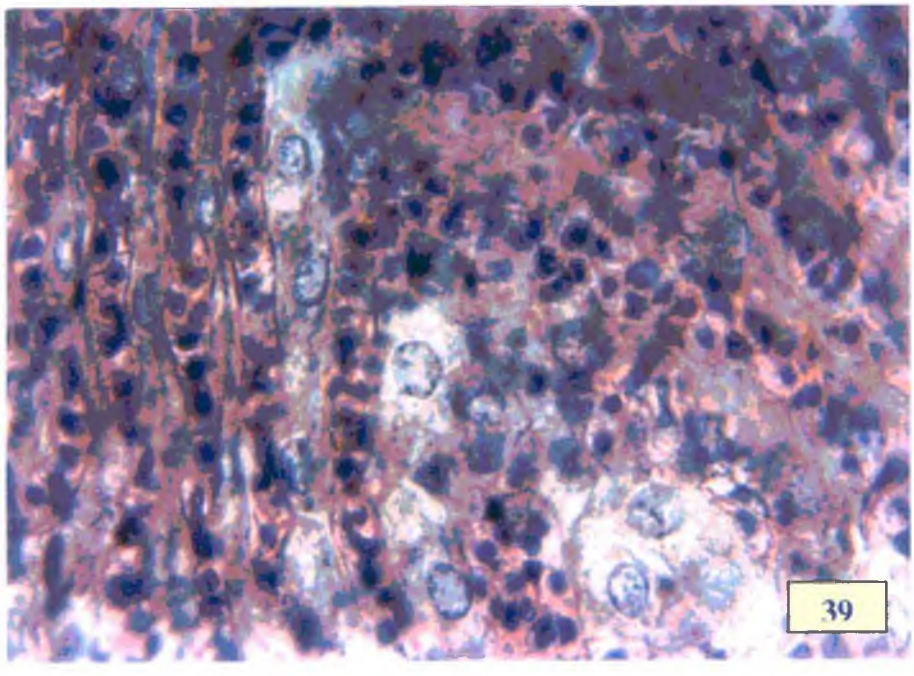
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4.6.3. Ultra microscopic studies

Ultra structure of thymus of EUS affected *C. mrigala* showed the presence of huge number of lymphoid cells which were connected by the desmosomes (Fig. 40). Apart from desmosomes different types of blood cells especially erythroids were also detected but necrotic changes as well as degenerative blood cells were also observed. Bacteria were located extra cellularly and in some cases fibroblasts also appeared to be invaded by bacteria (Fig. 41). Along with the presence of bacteria in the sub capsular and inner zones of thymic parenchyma, necrosis of connective tissue of these regions were also identified. The necrotic changes were established by the disruptions of collagen fibres and of the basal membranes. Pyknotic cells and macrophages with large residuals bodies were also noticed. But pharyngeal epithelium covering thymus showed no significant changes.

4.7. Studies on blood cell profile in healthy *Cirrhinus mrigala*

4.7.1. Morphological and Morphometric studies

Erythrocyte

Erythrocytes of healthy *C. mrigala* under phase contrast microscope appeared either elliptical or oval in form with clearly visible nucleus (Fig. 42). The cytoplasms appeared bluish green when stained with Leishman stain and the nuclei appeared deep magenta in colour. In Sudan Black B stain the cytoplasm of erythrocytes appeared grey in colour and the nuclei appeared dark black. The erythrocytes were PAS negative.

The average cell diameter was 6.0±0.5986 μm while the nuclei diameter was 2.64±0.3375 μm resulting n-c ratio = 1: 2.27.

Leucocytes

Agranulocytes

Lymphocytes

In Leishman stain the lymphocytes appeared almost round in shape. Nucleus took acidophilic stain and appeared deep magenta in colour. The thin rim of cytoplasm encircling the nucleus took basophilic stain and appeared bluish in colour (Figs. 42 and 43).

In small lymphocytes (Fig. 42) the amount of cytoplasm encircling the nucleus are higher in comparison to the cytoplasm present in large lymphocytes (Fig. 43).

The cytoplasm of lymphocytes did not take any stain, and it appeared colourless when stained with Graham-Knoll-benzedine (counterstained with Giemsa). The cytoplasms of lymphocytes showed mild reaction when blood smears were stained with PAS. The nuclei of lymphocytes showed dark colour surrounded by a grey rim of cytoplasm when stained with Sudan Black B (Fig. 44).

The average cell diameter of small lymphocyte was 3.72±0.39 μm while the average nuclei diameter was 2.5±0.64 μm resulting the n-c ratio = 1: 1.48.

Monocytes

The shapes of the nuclei varied from oval to kidney shaped and occupied a peripheral position in the cell.

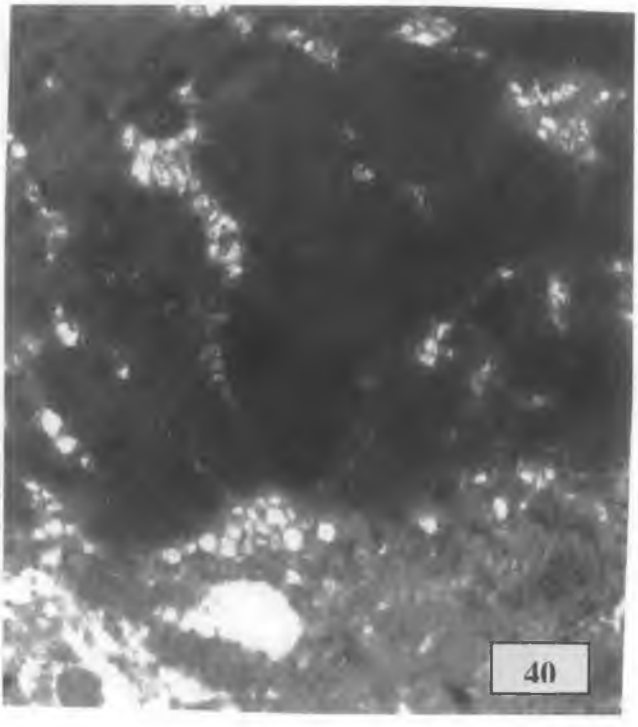
In Leishman stain the nuclei took a very light magenta colour and cytoplasm took a light blue colour. The cytoplasm appeared almost granules free (Fig. 45). The cytoplasms of monocytes showed a slightly stronger reaction for PAS than lymphocytes but like lymphocytes the nuclei of monocytes did not show any PAS reactivity (Fig. 46).

The average diameter of nucleus was 10.33±1.84 μm while the cell diameter was 17.23±2.46 μm resulting the n-c ratio = 1: 1.67.

Granulocytes

Fig.40: E.M studies of thymus of EUS affected *C. mrigala* shows ruptured basement and fibroblast, desmosomes etc.

Fig.41: E.M studies of thymus of EUS affected *C. mrigala* shows the presence of bacteria in thymic tissue.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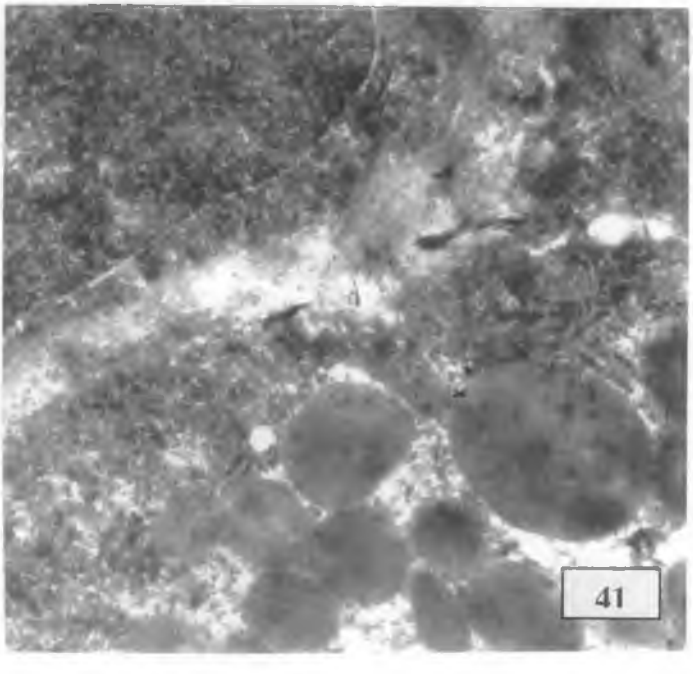
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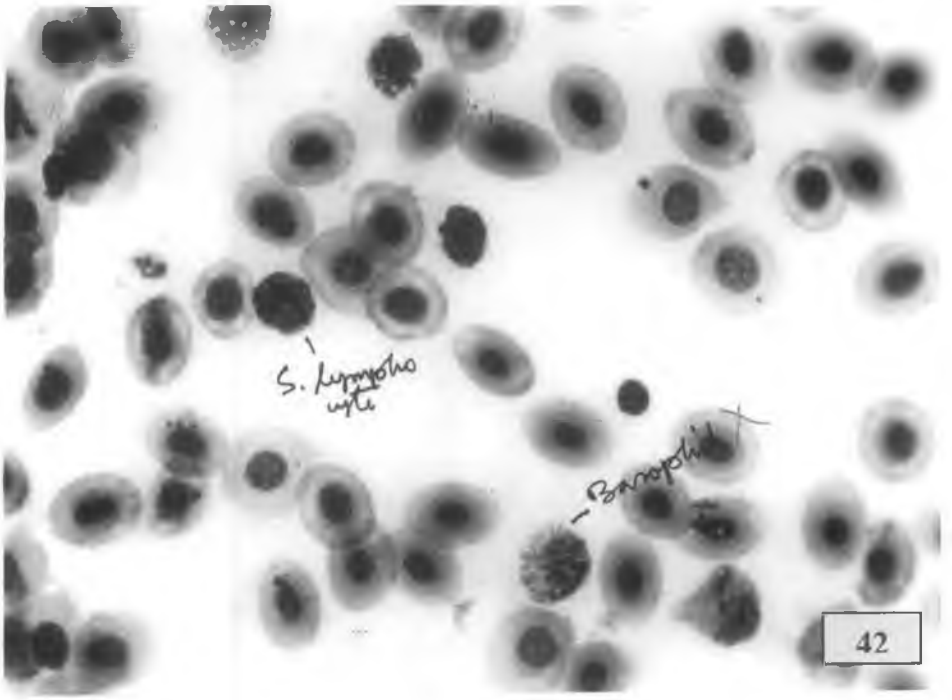
Fig.42: Blood smear of *C. mrigala* stained with Leishman stain shows different blood cells like, erythrocytes (E), small lymphocyte (sl) and basophil (B).

Fig. 43: Blood smear of *C. mrigala* stained with Leishman stain showing the presence of large lymphocyte along with erythrocytes.



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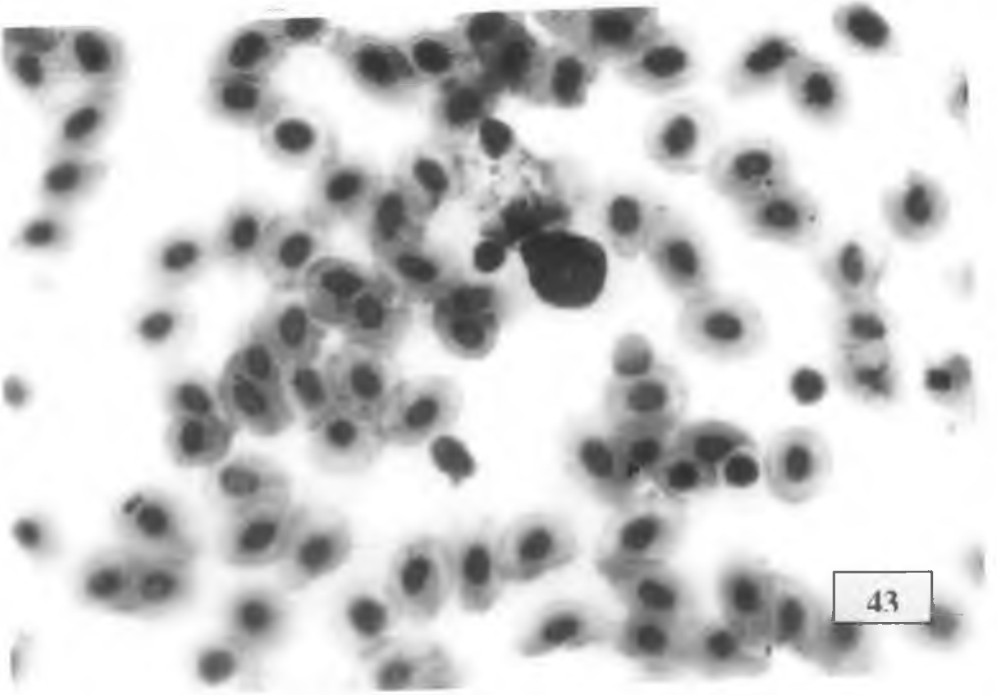

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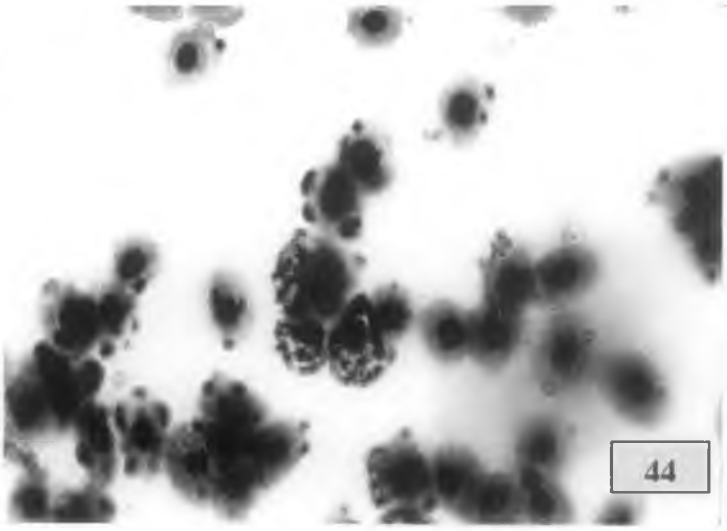
Fig. 44: Blood smear of *C. mrigala* stained with Sudan Black-B showing different blood cells like, erythrocytes, lymphocytes and granulocytes.

Fig.45: Blood smear of *C. mrigala* stained with Leishman stain shows the structure of a typical monocyte and eosinophils.

Fig.46: Blood smear of *C. mrigala* stained with PAS reaction showing the presence of a typical monocyte.

Fig.47: Blood smear of *C. mrigala* stained with Leishman stain shows the presence of a neutrophil.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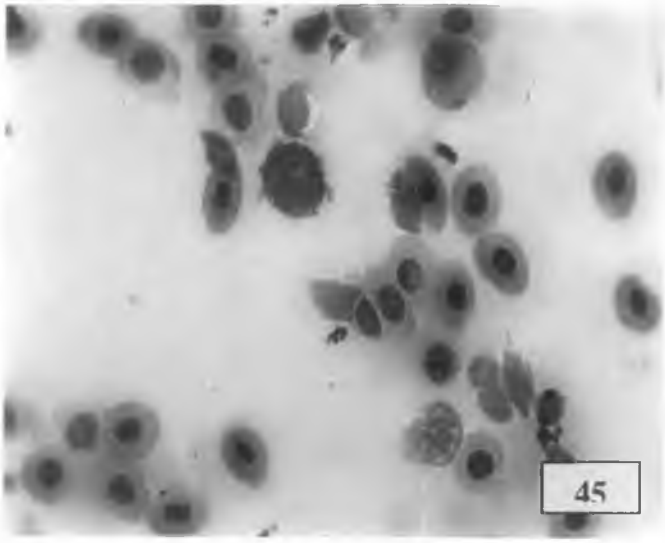
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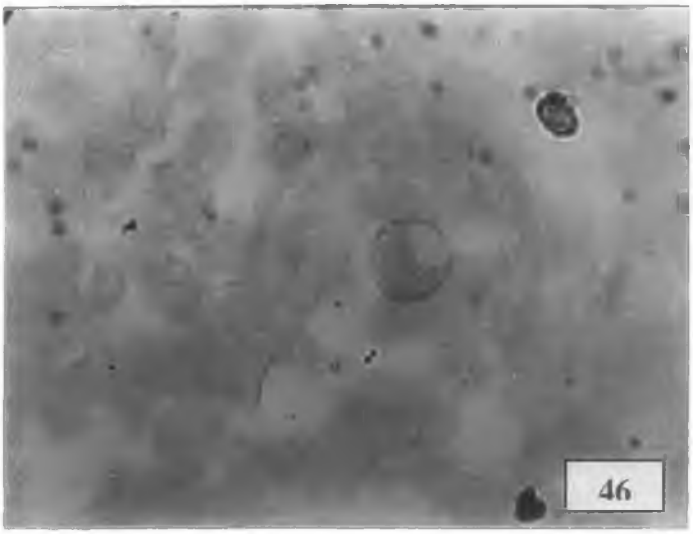
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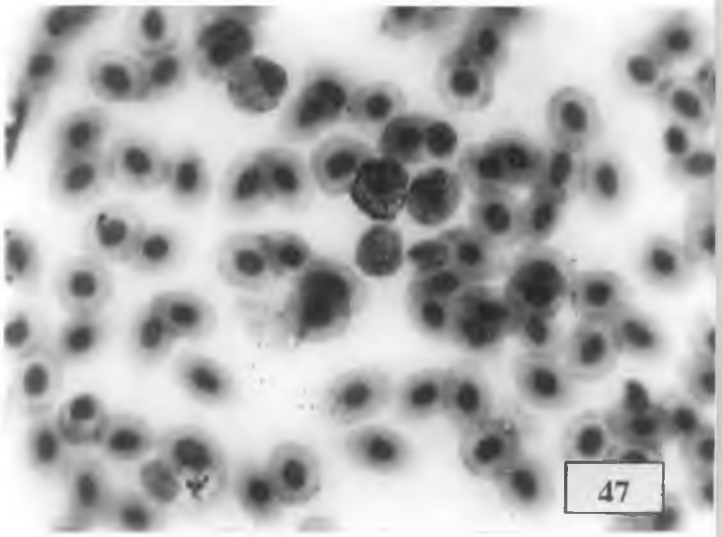
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Neutrophil

The nuclei of neutrophils were oval in shape and rarely did they appear bilobed in structure. The nuclei took light magenta colour while the cytoplasm appeared light blue due to presence of some granular structures in the cytoplasm when stained with Lishman stain (Fig. 47). Presence of lipid droplets were detected in the cytoplasm of neutrophils when the slides were stained with Sudan Black B (Fig. 44).The neutrophils showed strong PAS reactivity. The cytoplasms of the cells showed the strongest reaction while the nuclei showed a very mild reaction (Fig. 48).

The average cell diameter of neutrophils was 15.53±2.27 µm while the average nuclei diameter was 9.6±1.97 µm resulting the n-c ratio = 1: 1.62

Basophils

Basophils were scanty in number. The size of the cells was almost similar to neutrophils but the nuclei were either centrally located or peripherally located. The cytoplasm showed a lot of granules and took bluish colour (Fig. 42). These cells were found slightly PAS positive.

The average cell diameter of the cells was 10.2±0.13 µm and the average nuclei diameter was 6.62±0.12 µm resulting the n-c ratio = 1: 1.54.

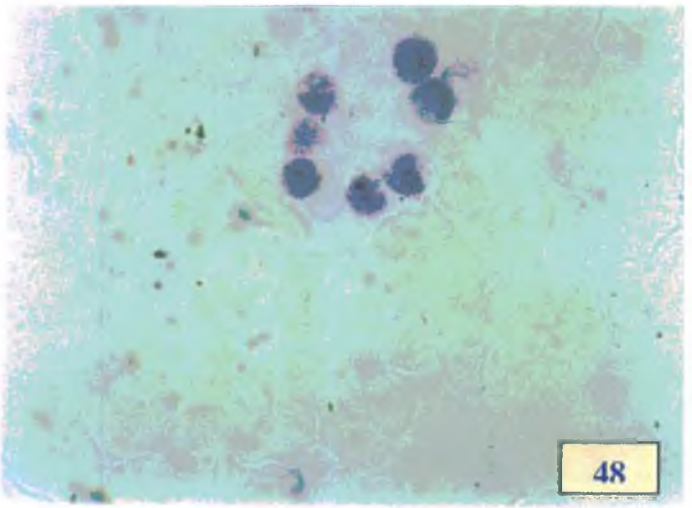
Eosinophils

Eosinophils were very rare in peripheral blood. The shape of the nucleus was irregular and the cytoplasm was full of acidophilic granules (Fig. 45). These cells were found PAS negative.

The average cell diameter of the cells was 17.3±2.31 µm and the average nuclei diameter was10.21 ±1.2 µm resulting the n-c ratio = 1: 1.69.

Fig.48: Blood smear of *C. mrigala* stained with PAS reaction shows the presence of neutrophils showing strong PAS reactivity.

Fig.49: Blood smear of *C. mrigala* stained with Leishman stain shows presence of a Plasma cell.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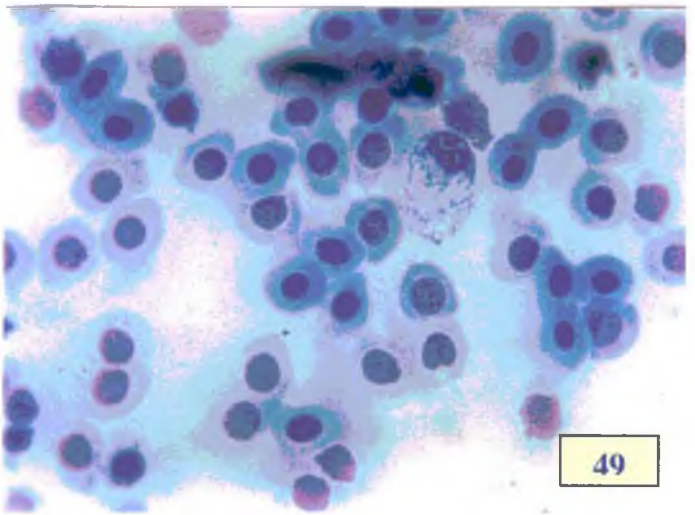
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Thrombocytes

Thrombocytes were seldom found in the peripheral blood of healthy *C. mrigala.* The nucleus was spindle shaped and deep magenta in colour surrounded by very thin cytoplasm which took no stain.

Plasma cells

A very few large cells were found in blood smears of healthy *C. mrigala.* These cells had an eccentric nucleus with a very low n-c ratio. The shape of the cells was oval-elliptical. The cytoplasm did take a very faint stain when stained with Romnowsky's stains. But the cytoplasm showed a fine reticulate structure (Fig. 49).

Table4. Average diameter of nucleus and cell along with nucleus cytoplasm ratio of different blood cells of healthy *C. mrigala.*

	N	Cell	n/c
Erythrocyte	2.64±0.3375	6.0±0.59	1: 2.27
Monocyte	10.33±1.84	17.23±2.46	1: 1.67
Neutrophil	9.6 ±1.97	15.53±2.27	1: 1.62
Lymphocyte			
1. Small	2.5±0.65	3.72±0.39	1: 1.48
2. Large	9.4±0.12	11.2± 0.42	1: 1.19
Basophil	6.62±0.12	10.2±0.13	1: 1.54
Eosinophil	10.21±1.2	17.3±2.31	1: 1.69

Mean ± S.D; n = 50

4.8. Cytochemical studies on erythropoiesis in healthy C. mrigala

The different stages of red blood corpuscle development as well as their morphological features are described below (Table 5).

Small lymphoid haemoblast or slh

The cells were small in size and completely round with a deeply stained nucleus. The nucleus took deep magenta colour in Graham-Knoll Benzidine (counter stained with Giemsa) (Fig. 50). The nucleus was surrounding by a thin rim of cytoplasm. The average diameter of the cells was 3.65±0.15μm and the n-c ratio was 1:1.2.

Basophilic erythroblast or be

These cells represented the next stage of development of erythrocytes. These cells were slightly larger size in comparison to small lymphoid haemoblasts. The average diameter of the cells was 4˙23±0.18μm and the average diameter of the nucleus was 3˙21±0.12μm. The n-c ratio of the cells was 1:1˙31. The nucleus was found less deeply stained compared to small lymphoid haemoblasts or slh.

Polychromatophilic erythroblasts or pe

These cells were characterized by the appearance of acidophilic areas within basophilic cytoplasm. These cells were larger than basophilic erythroblasts (Fig. 51). The average cell diameter was 4˙85±1.5μm and the average diameter of nucleus was 3˙0±0.12μm. The n-c ratio was 1:1˙6.

Acidophilic erythroblasts or ae

These cells represented the fourth stage of development of erythrocytes. The cytoplasm was acidophilic. The size of the cells was larger in comparison to that of other stages. The average diameter of the cells and nuclei were 5.98±1.13μm and 4.18±0.14μm respectively. The n-c ratio was 1:1˙43.

Young reticulocytes

These cells were characterized by the presence of homogenous mass of haemoglobin. The nucleus was oval in shape (Fig. 51). The average nucleus diameter was 2.63±0.13µm and the cell diameter was 7.2±1.2µm resulting the n-c ratio 1: 2.75.

Mature erythrocytes or mr

Mature erythrocytes were larger in size compared to that of young reticulocytes with an oval nucleus (Fig. 51). The average cell diameter was 6.0±0.13 while nucleus diameter was 2.64±0.01

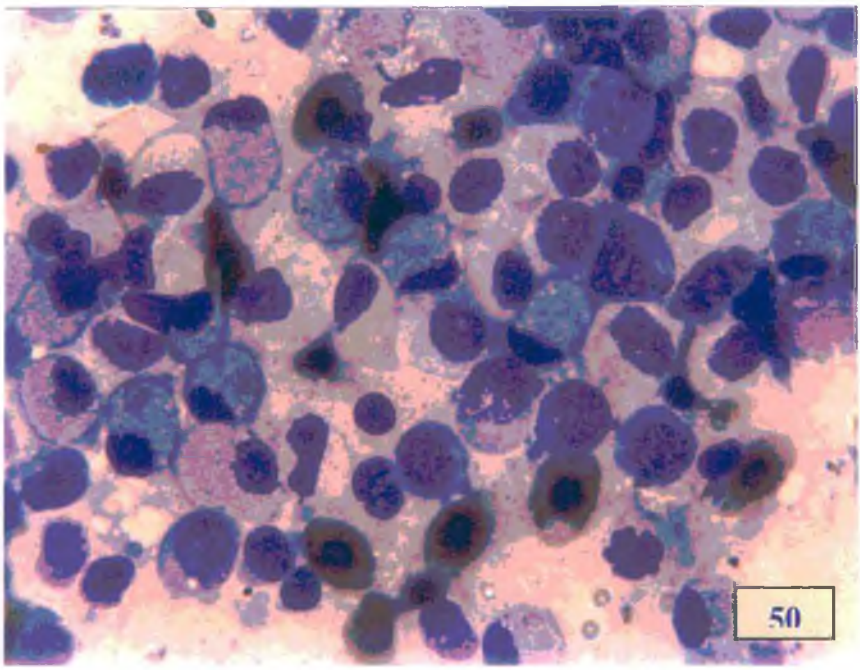
Table5. Measurement of nucleus and cell diameter and n-c ratio of six developmental stages of erythrocytes in healthy *C. mrigala.*

	Nucleus(µm)	**Cell diameter(µm)**	**n-c ratio**
Small lymphoid haemoblast or slh	3.04±0.10	3.65±0.15	1:1.2
Basophilic erythroblast or be	3.21±0.12	4.23±0.18	1:1.31
Polychromatophilic erythroblast or pe	3.0±0.12	4.85±1.5	1:1.6
Acidophilic erythroblast or ae	4.18±0.14	5.98±1.13	1:1.43
Young reticulocytes	2.63±0.13	7.2±1.2	1:2.75
Mature erythrocytes	2.64±0.01	6.0±0.13	1:2.27

Mean±S.D; n= 50

Fig.50: Tissue imprints of head kidney of healthy *C. mrigala* stained with Graham-Knoll Benzidine counter stained with Giemsa show different developmental stages of erythrocytes, like small lymphoid haemoblast or slh.

Fig.51: Tissue imprint of spleen of healthy *C. mrigala* shows the presence polychromatophilic erythroblast or pe, young reticulocytes and mature erythrocytes.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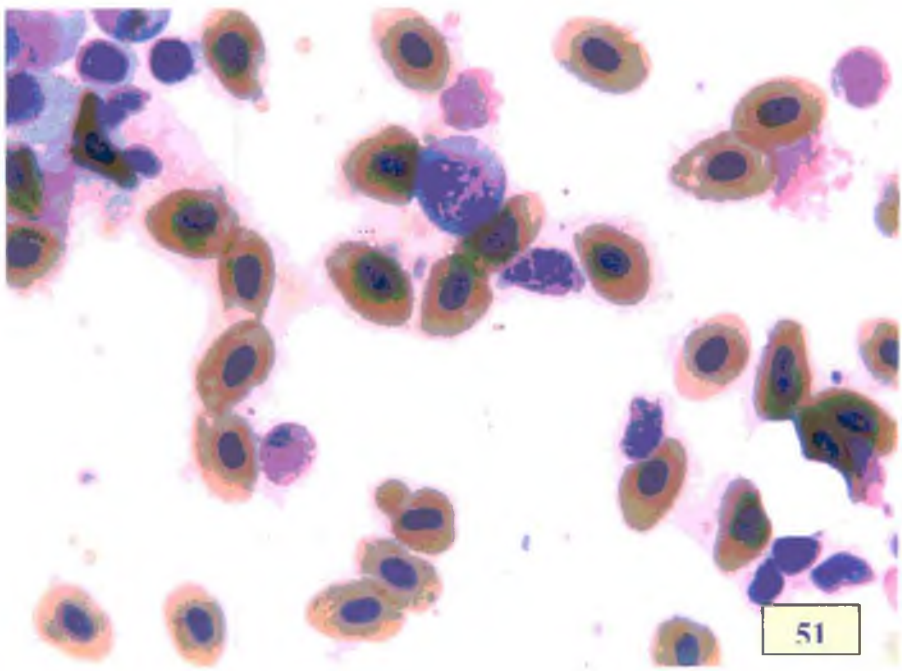

51

4.9. Erythropoietic efficiency of haemopoietic organs of both healthy and EUS affected *Cirrhinus mrigala*

(Table 6) contains the data related with erythropoietic efficiency of head kidney and thymus in both healthy and EUS affected *C. mrigala.*

Healthy *C. mrigala* showed 3291±37.32 blast cells/mm^3 in the head kidney and 3275±66.14 blast cells/mm^3 in the thymus.

But EUS affected *C. mrigala* showed a value of 2695±42.72 blast cells/mm^3 in the head kidney and 2116.67±104.08 blast cels/mm^3 in the thymus.

The results of erythropoietic efficiencies of head kidney and thymus of both healthy and EUS affected *C. mrigala* clearly showed that erythropoietic efficiencies of head kidney and thymus of healthy fish were significantly ($p < 0.001$) higher in comparison to erythropoietic efficiencies of those organs in EUS affected fish.

Figs. 52 and 53 show the comparison among erythropoietic efficiencies of head kidney and thymus of healthy and EUS affected *C. mrigala.*

Table6. Erythropoietic efficiency of head kidney and thymus of healthy and EUS affected *C. mrigala.*

Fish *(Cirrhinus mrigala)*	**Wt. (gm)**	**Wt. of head kidney (gm)**	**No. of blast cells produced (cells/mm^3)**	**Wt. of thymus (gm)**	**No. of blast cells produced (cells/mm^3)**
Healthy	40±2.0	0.0203±0.0005	3291±37.32	0.0079±5.77	3275±66.14
EUS affected	42.167±2.021	0.214±0.001	2695±42.72*	0.0106±0.0012	2116.67±104.08*

Mean±S.D; n==3; * Significant at 0.001% level

Erythropoietic efficiency of head kidney and thymus of healthy and EUS affected *C. mrigala*

Fig. 52

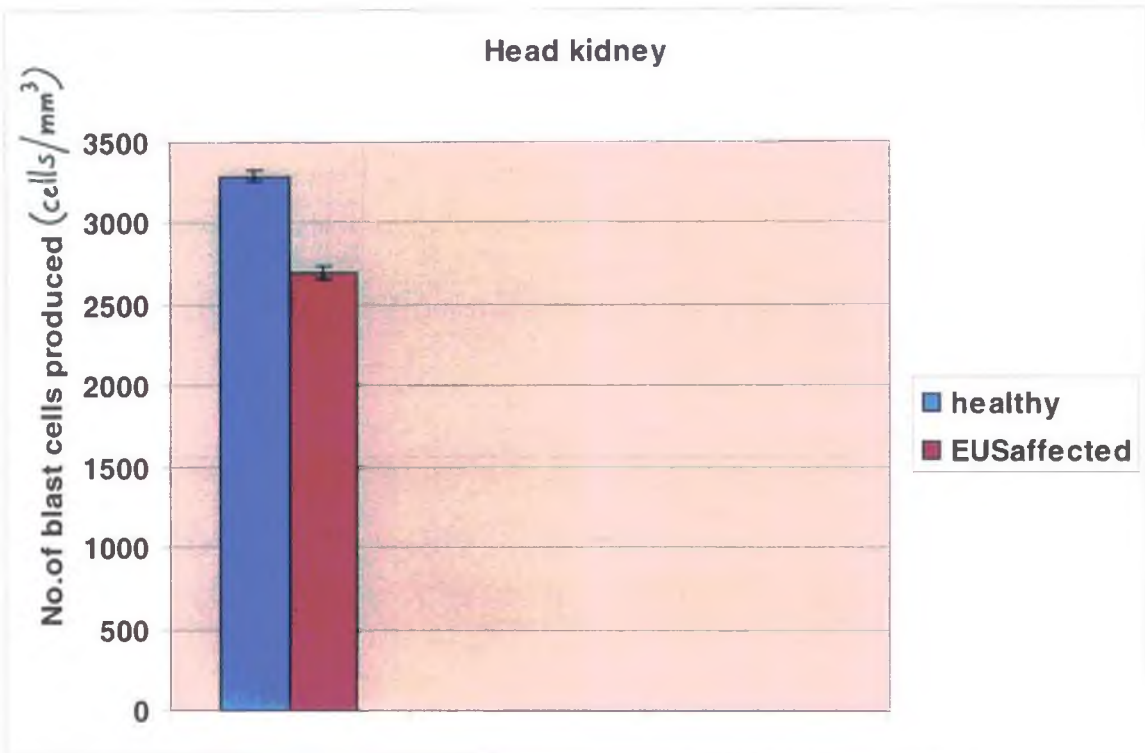
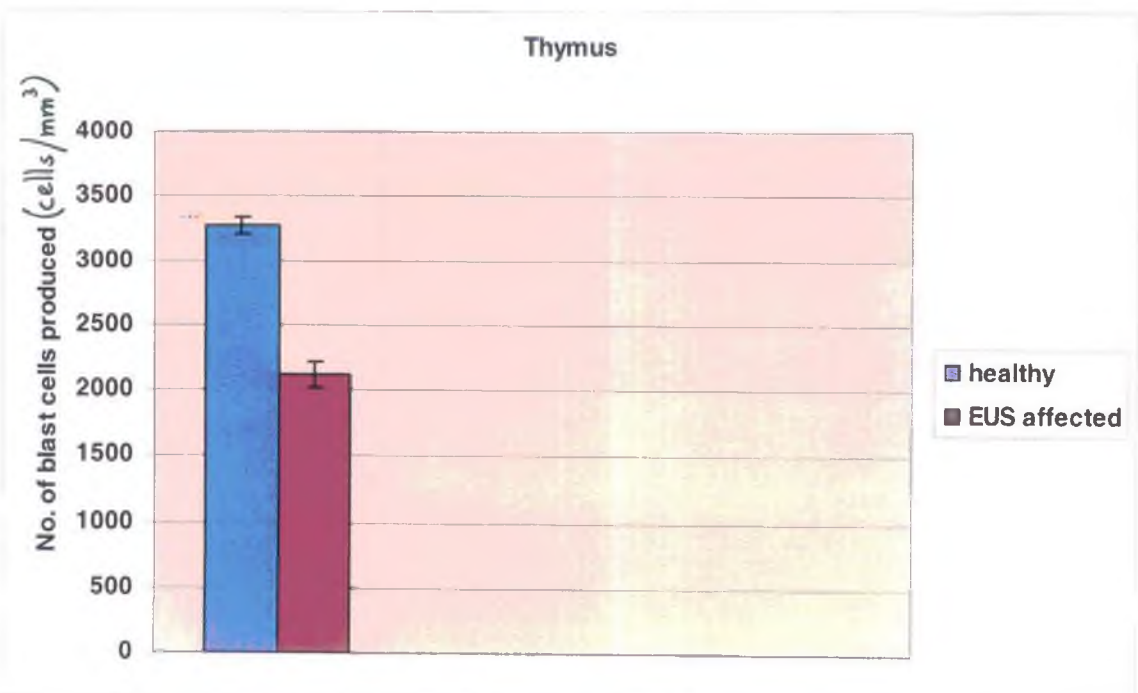


Fig. 53



4.10. Changes in the morphology of erythrocytes of naturally infected fishes

The result of the count of elliptical and oval shaped erythrocytes in the peripheral blood of healthy and EUS affected fishes are shown in Table 7. In peripheral blood of healthy *C. mrigala* the percent of elliptical erythrocytes was 79.6±3.647 while percent of oval erythrocytes was 20.4±3.647.

But in peripheral blood of EUS affected *C. mrigala* the percent of elliptical (Fig. 54) and while oval erythrocytes were 62.2±5.404 and 37.8±5.404 respectively.

The percent of oval (Fig. 55) shaped erythrocytes in peripheral blood of EUS affected *C. mrigala* was significantly higher ($P<0.001$) compared to that in peripheral blood of healthy *C. mrigala.* Fig. 56 shows the comparison between the percentage of elliptical and oval shaped erythrocytes in peripheral blood of healthy and EUS affected *C. mrigala.*

Table7. Count of Elliptical and Oval erythrocytes in peripheral blood of healthy and EUS affected fish

Species ***(Cirrhinus mrigala)***	**Percent of Elliptical**	**Percent of Oval**
Healthy	79.6±3.647	20.4±3.647
EUS affected	62.2±5.404	37.8±5.404*

Mean ±S.D; n=3; *Significant at 0.001% level

Count of elliptical and oval shaped erythrocytes in peripheral blood of healthy and EUS affected *C. mrigala*

Fig. 56

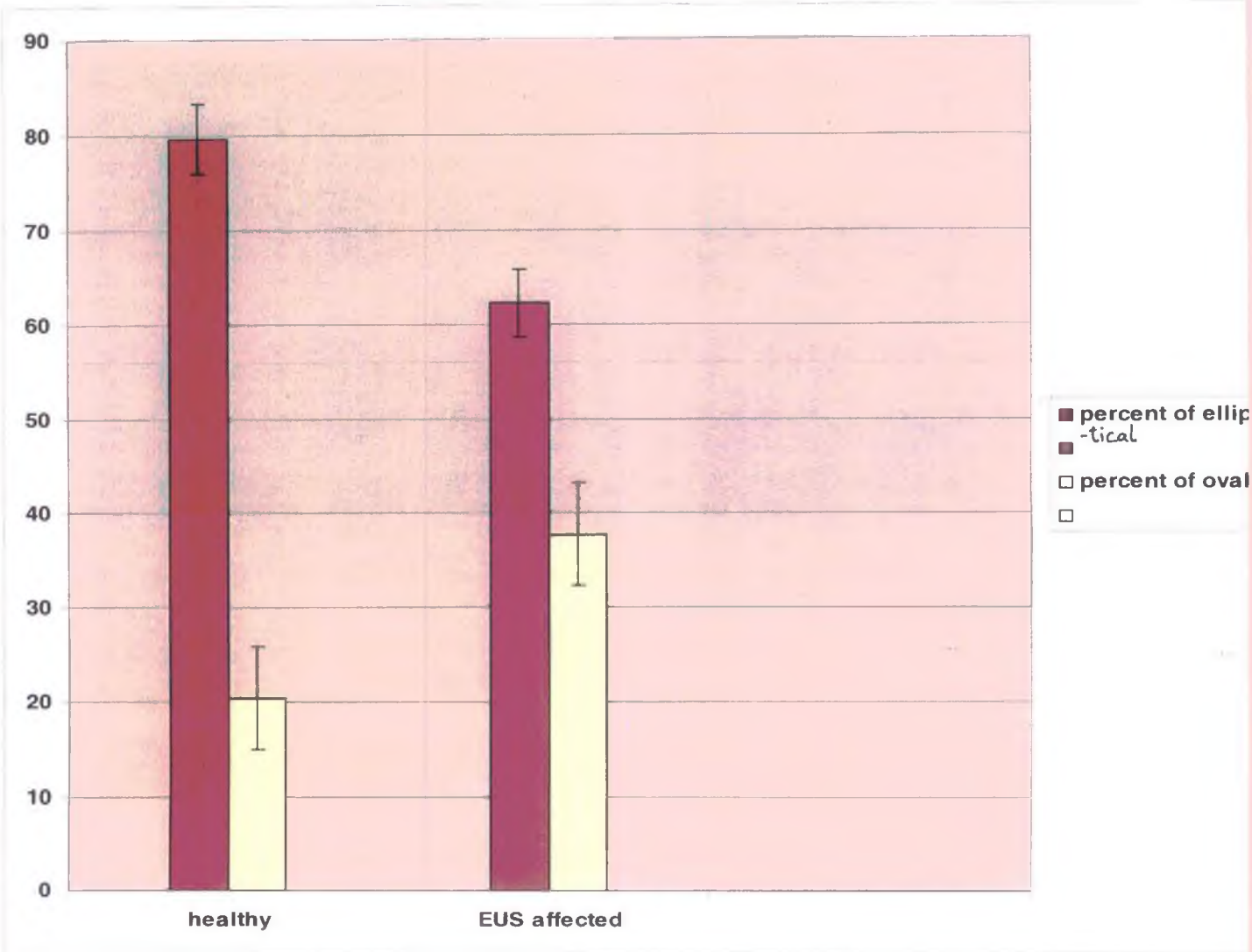
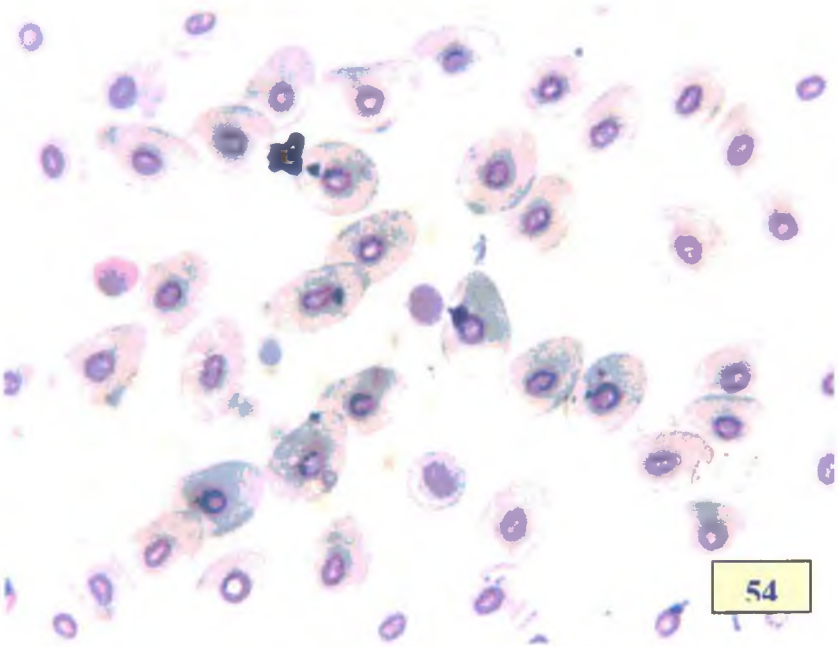


Fig.54: Blood smear of healthy *C. mrigala* stained with Leishman stain shows that almost all the erythrocytes are of mature stage i;e elliptical in shape.

Fig.55: Blood smear of EUS affected *C. mrigala* stained with Leishman stain show the presence of huge number of immature erythrocytes with oval shape.



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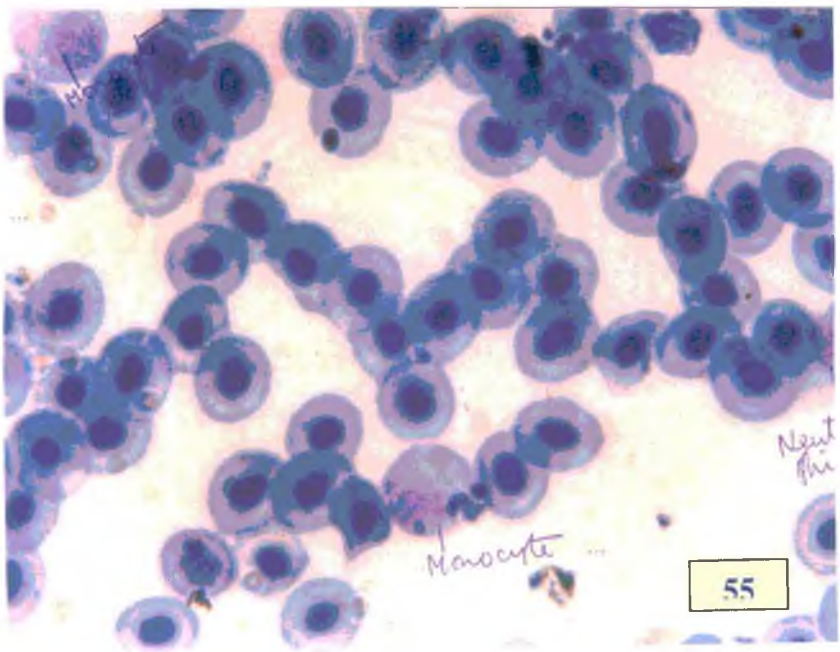

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4.11. Total erythrocyte count in peripheral blood of healthy and EUS affected *Cirrhinus mrigala*

The result of the total erythrocyte count (TEC) in healthy and EUS affected fishes are shown in Table 8.

In peripheral blood of healthy *C. mrigala* the number of total erythrocyte was $4.5\pm0.32\times10^6/mm^3$ while in peripheral blood of EUS affected *C. mrigala* it was only $1.44\pm0.16\times10^6/mm^3$

The TEC in EUS affected fishes was significantly ($p<0.001$) lower in comparison to that of healthy fishes. Fig. 57 shows the comparison of the total erythrocyte count in peripheral blood of healthy and EUS affected *C. mrigala.*

Table8. Total erythrocyte count ($\times10^6/mm^3$) in peripheral blood both healthy and EUS affected *C. mrigala*

Healthy fish	4.5±0.32
EUS affected fish	1.44±0.16*

Mean±S.D; n=10; * Significant at 0.001%

4.12. Total leucocyte count of healthy and EUS affected fishes

Total leucocyte count of healthy and EUS affected fishes are shown in Table 9.

Total leucocyte count in peripheral blood of healthy *C. mrigala* was 26.93±10.13 while total leucocyte count in peripheral blood of EUS affected *C. mrigala* was 90.93±13.86. Fig. 58 shows the comparison of total leucocyte count between healthy and EUS affected *C. mrigala.*

Table9. Total leucocyte count ($\times 10^3/mm^3$) in peripheral blood of both healthy and EUS affected *C. mrigala*

Healthy fish	26.93±10.13
EUS affected fish	90.93±13.86*

Mean ± S.D; n = 10; * Significant at 0.001%

4.13. Haematological studies of healthy *C. mrigala* throughout the year (2007)

Table 10 showed the total amount of haemoglobin, total R.B.C. count or (TRC), total lymphocyte count or (TLC) and differential count of three subpopulations of leucocytes namely monocytes, neutrophils and lymphocytes in peripheral blood of healthy *C. mrigala* of an average weight 88.75±21.11 gm.

Haemoglobin content in peripheral blood of healthy *C. mrigala* varied from 5.16±0.15 to 6.23±0.25 gm/100mL blood throughout the year. From January to May (2007) the haemoglobin content in peripheral blood was between 6.11±0.10 and 6.2±0.10 (Fig. 59). From June to September it showed a trend of decrease and finally in September it went down to 5.16±0.29 gm/100mL level. From October onwards the haemoglobin content started showing an increase in amount and reached upto 6.23±0.25 gm/100mL during December.

Total R.B.C count in peripheral blood varied from 3.5±0.2 $\times 10^6/mm^3$ to 5.1±0.1 $\times 10^6/mm^3$ throughout the year. Like haemoglobin content from January to May (2007) TEC was 4.4±0.26 to 4.63±0.15 $\times 10^6/mm^3$ (Fig. 60). Similarly from June to September it showed a trend of decrease and went down to 3.5±0.2 gm/100mL during August. From

Total erythrocyte and leucocyte count in peripheral blood of both healthy and EUS affected *C. mrigala*

Fig. 57

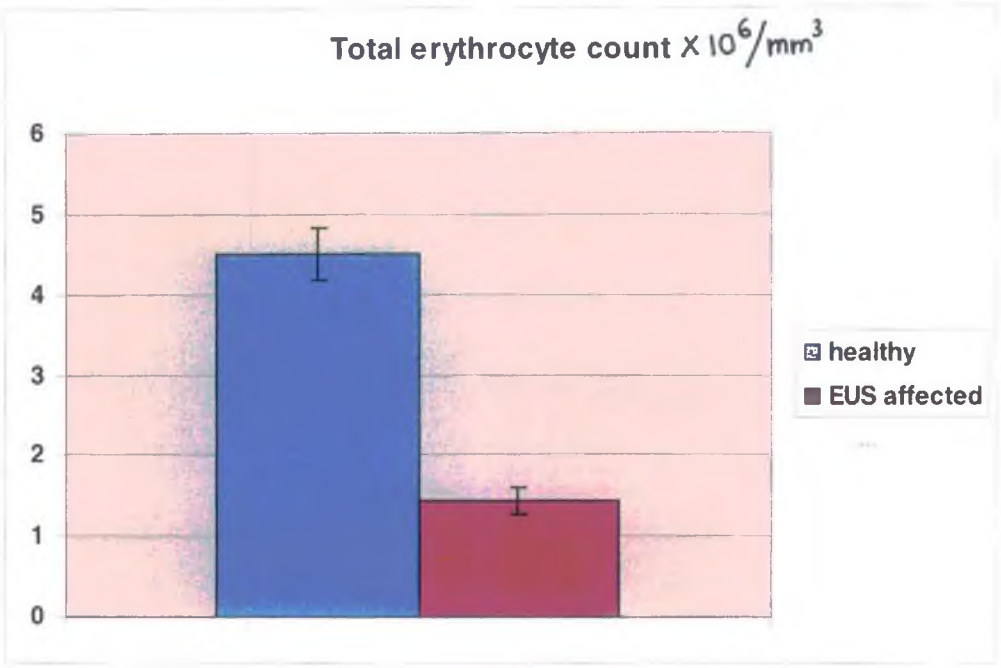


Fig. 58

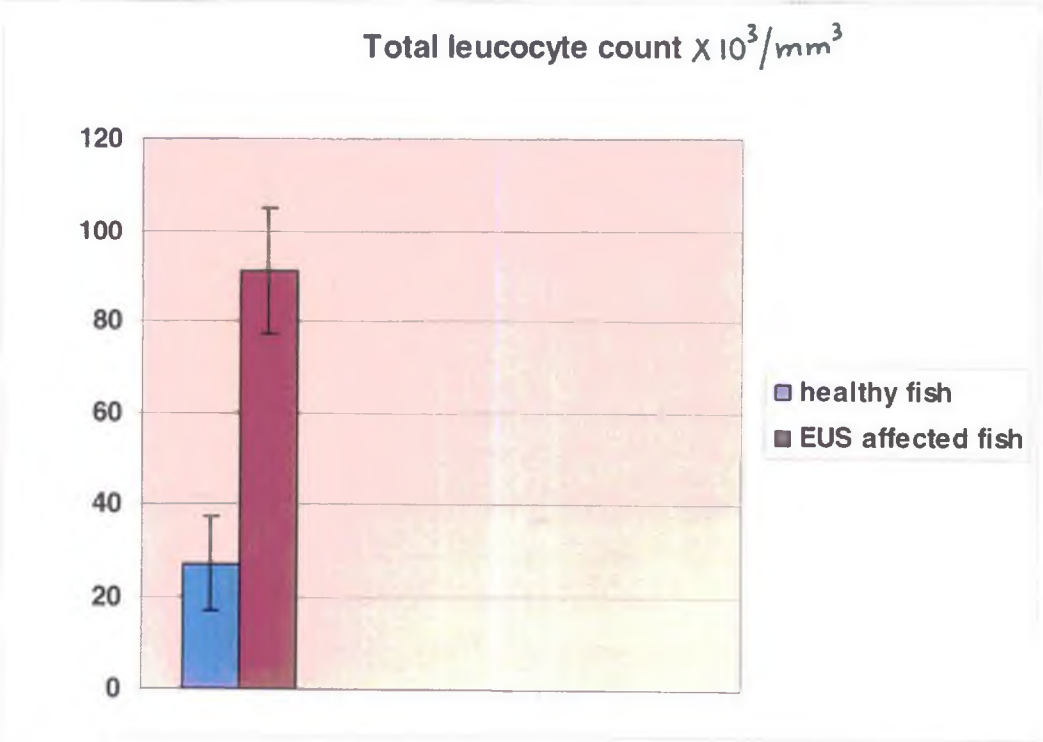


Table10. Hb content, total erythrocyte count (TEC), total leucocyte count (TLC) and differential count of lymphocytes, neutrophils, monocytes in peripheral blood

Month	Water temp. (°C)	Avg. wt (gm)	Hb content (gm/100mL)	TEC ($\times 10^6/mm^3$)	TLC ($\times 10^3/mm^3$)	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)
Jan	16	60±5.00	6.11±0.10	4.63±0.15	22±4.58	59.66±1.53	35.33±1.53	5±1.0
Feb	19	65±5.00	6.2±0.10	4.4±0.26	24.66±3.93	55.3±2.57	35.33±3.05	9±4.0
Mar	24	70±8.89	6.2±0.20	4.6±0.36	23.93±3.58	51.66±2.52	35.33±3.51	13±1.15
April	27	80±20.0	6.1±0.10	4.4±0.2	24.46±4.21	47.66±1.53	36±1.0	16±3.5
May	30	70±12.12	6.16±0.15	4.2±0.2	23.9±4.53	41.33±1.53	38.66±1.53	20±1.15
June	30	80±15.00	5.96±0.15	4.0±0.2	22.4±3.84	44.6±1.64	36.03±1.53	19.33±1.15
July	31	90±20.00	5.5±0.30	3.8±0.2	23.06±3.72	45±1.53	30.33±2.52	25±2.65
Aug	30	100±8.66	5.16±0.15	3.5±0.2	23.5±4.24	60±3	20±1.0	20±1.0
Sept.	25	110±13.23	5.16±0.29	3.8±0.21	22.73±4.71	58±2.0	25±5.29	17±2.0
Oct	25	100±9.66	6.0±0.21	4.4±0.1	22.73±3.58	56.33±2.08	30±2.0	14±2.65
Nov	20	120±20.00	6.1±0.10	4.9±0.1	22.1±3.93	54.33±2.52	33.66±1.53	12±1.0
Dec	17	120±26.46	6.23±0.25	5.1±0.1	21.53±3.46	56±2.0	33±2.52	11±1.73

Data for each index are mean of three (n = 3)

Haematological studies of healthy *C. mrigala* throughout the year (2007)

Fig. 59

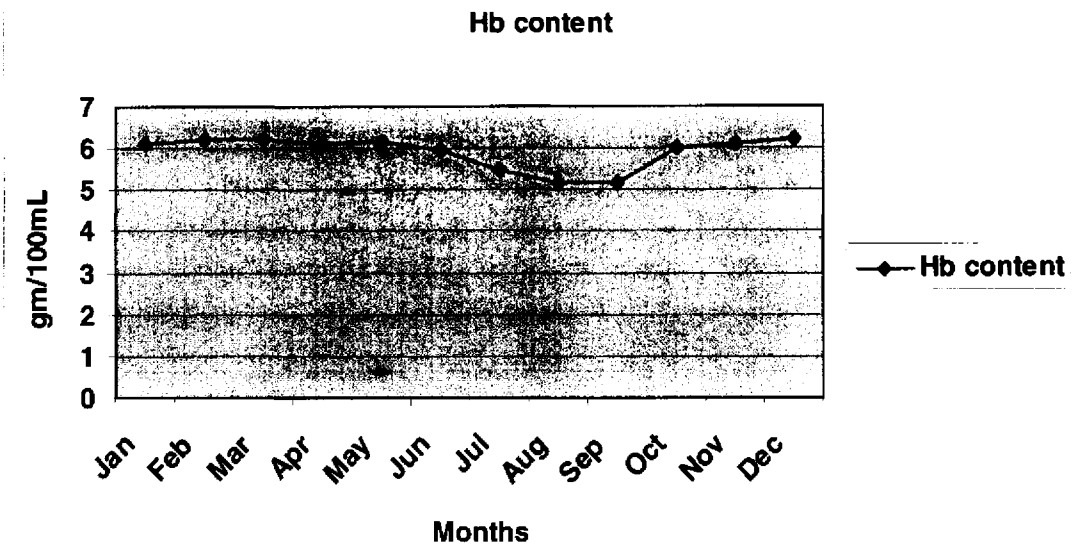


Fig. 60

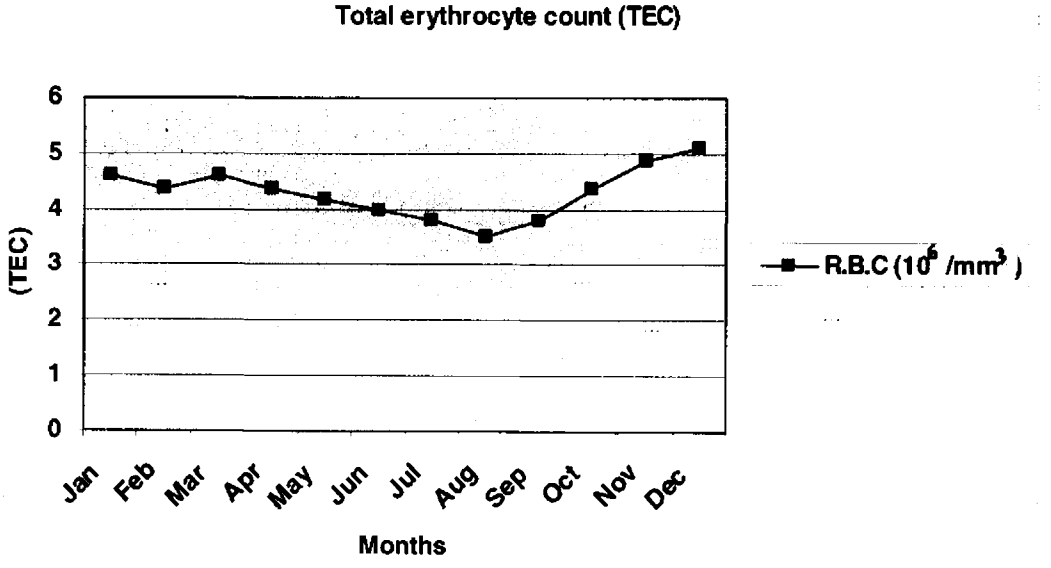


Fig. 61

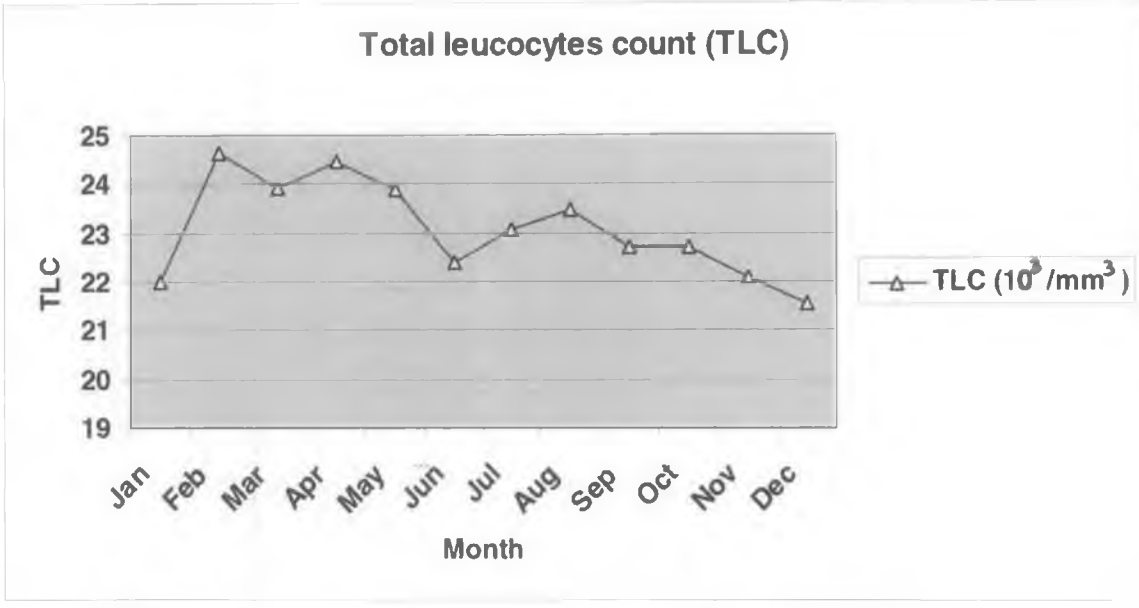
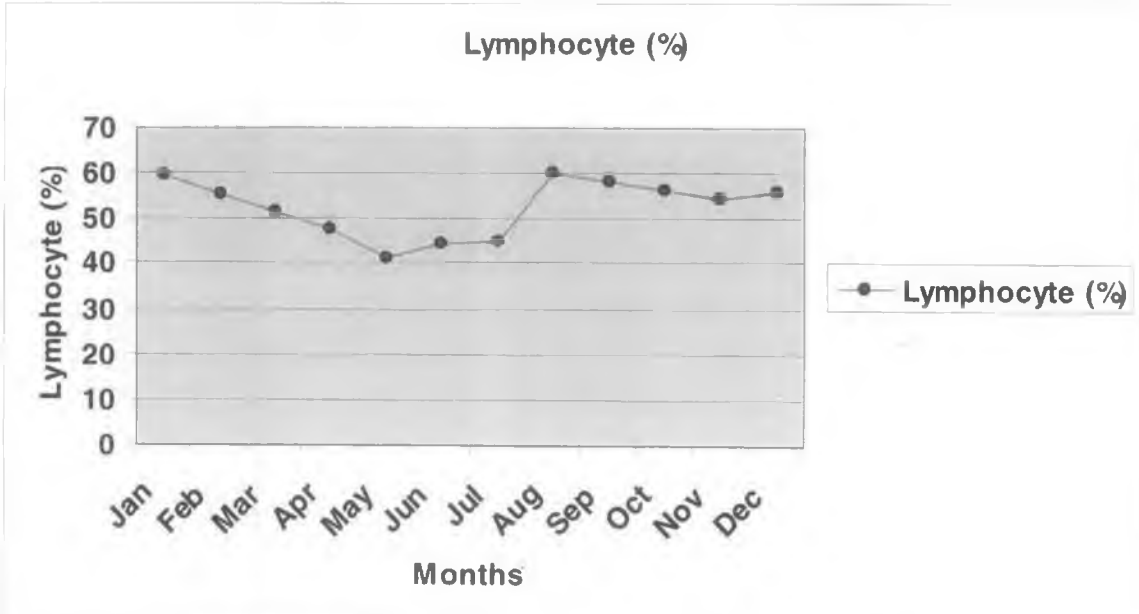

Total leucocytes count (TLC)
TLC
25
24
23
22
21
20
19
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month
TLC (10³ /mm³)

Fig. 62


Lymphocyte (%)
Lymphocyte (%)
70
60
50
40
30
20
10
0
Jan
Feb
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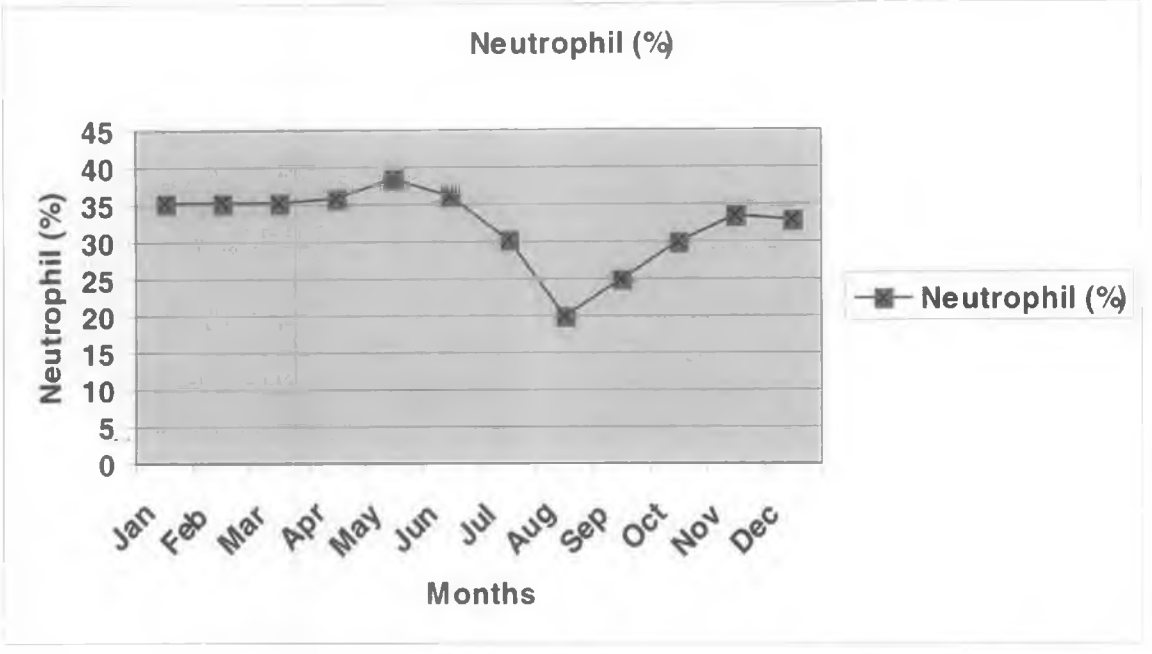
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Months
Lymphocyte (%)

Fig. 63


Neutrophil (%)
Neutrophil (%)
45
40
35
30
25
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15
10
5
0
Jan
Feb
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May
Jun
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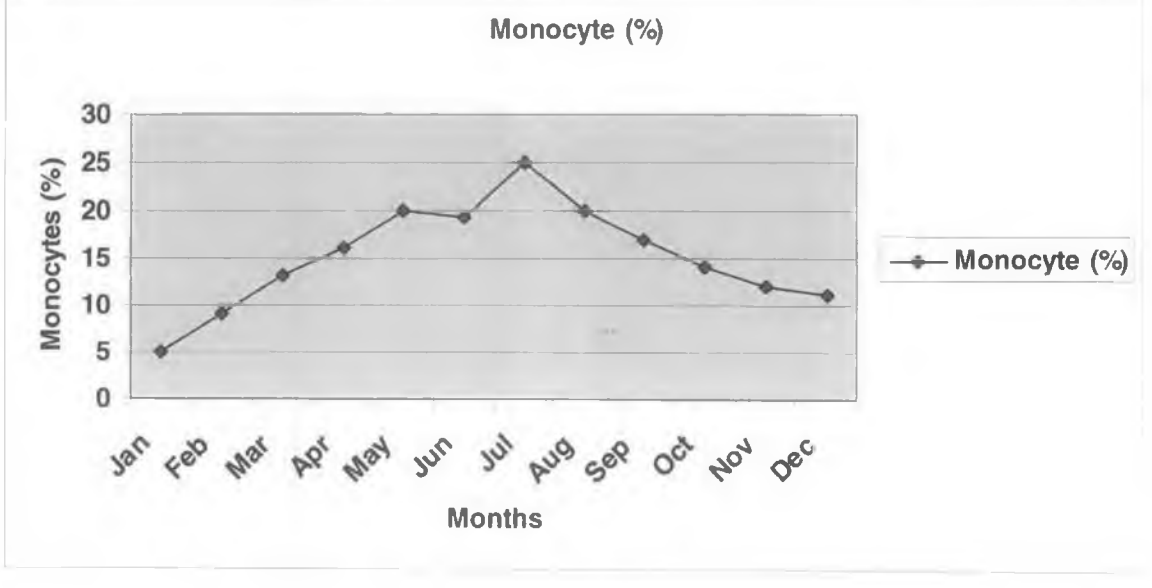
Aug
Sep
Oct
Nov
Dec
Months
Neutrophil (%)

Fig. 64


Monocyte (%)
Monocytes (%)
30
25
20
15
10
5
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Months
Monocyte (%)

October it started showing a tendency to increase. Total leucocyte count in peripheral blood of healthy *C. mrigala* varied between $21.53\pm3.46\times10^3/mm^3$ and $24.66\pm3.93\times10^3/mm^3$ year round (Fig. 61).

Differential count of lymphocytes varied from 41.33±1.53% to 60±3% (Fig. 62). From January to June the percentage started to decline. From July onwards the percentage started increasing and varied between 45±1.53% to 60±3%.

Differential count of neutrophils varied between 20±1.0% and 38.66±1.33% (Fig. 63). From January to March, the differential count was almost unaltered. From April to June the differential count increased marginally. From July to August the differential count decreased being the lowest in August in 20±1.0%. But during November to December it increased to 33±2%.

Differential count of monocytes varied from 5 to 25% in peripheral blood of healthy *C. mrigala*. From January to July it started showing a steady increase then from August to December it showed a steady decline in percentage (Fig. 64).

4.14. Differential count of leucocytes in tissue imprints prepared from three lympho-heamopoietic organs of EUS affected *Cirrhinus mrigala*

Deferential count of different subpopulations of leucocytes in tissue imprints of three lymphohaemopoietic organs of EUS affected *C. mrigala* are shown in Table11.

In the tissue imprints of head kidney, spleen and thymus of EUS affected *C. mrigala* the number of basophils were very rare. In head kidney it was 1% while in spleen and thymus it was 0.66% and 1.33% respectively (Fig. 65).

The monocytes were the highest in head kidney (11±4.58%) while in spleen and thymus the percentage was 8±5% and 7.5±3.5% respectively.

Lymphocytes were found highest in number (Fig. 66). In head kidney it was 54±1.0 percent and in spleen it was 51.35±3.51 percent while in thymus it was 59.29±8.62 percent (Fig. 67).

Neutrophil was 34.66±3.21%; in head kidney 39±4.21% in spleen and 30.24±6.08% in thymus (Fig. 68).

Eosinophils were 1.66±1.0%; 2.33±0.5% and 1.33±0.33% in head kidney, spleen and thymus respectively (Fig. 69).

There was no significant difference in the differential count of subpopulations of leucocytes of three lymphohaemopoietic organs of EUS affected *C. mrigala.*

Table11. The differential count of different subpopulations of leucocytes in three lympho-heamopoietic organs of EUS affected *C. mrigala*

Different leucocytes	**Basophil**	**Monocyte**	**Lymphocyte**	**Neutrophil**	**Eosinophil**
Head kidney	01	11±4.58	54±1.0	34.66±3.21	1.66±1.0
Spleen	0.66	8±5	51.35±3.511	39±4.51	2.33±0.5
Thymus	1.33	7.5±3.51	59.29±8.62	30.24±6.08	1.33±0.33

Mean±S.D; n= 3.

4.15. Differential count of different subpopulations of leucocyte in peripheral blood of EUS affected *Cirrhinus mrigala*

The differential counts of subpopulation of leucocytes in the peripheral blood of EUS affected *C.* mrigala are shown in Table 12.

The differential count of different subpopulations of leucocytes in three lympho-haemopoietic organs of EUS affected *C. mrigala*

Fig. 65

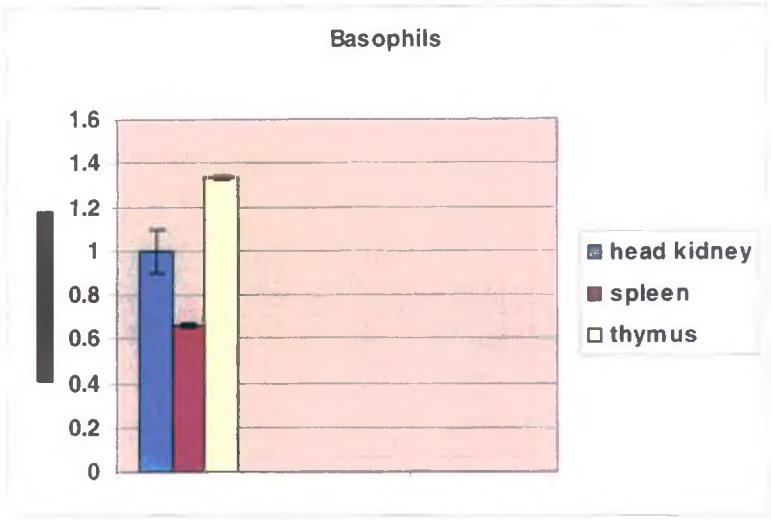
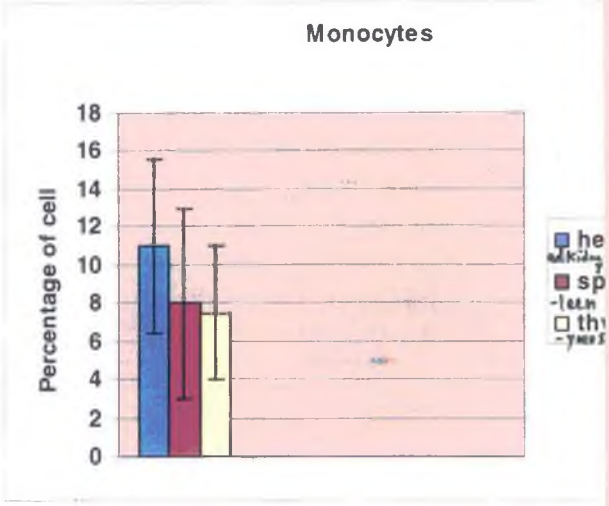


Fig. 66



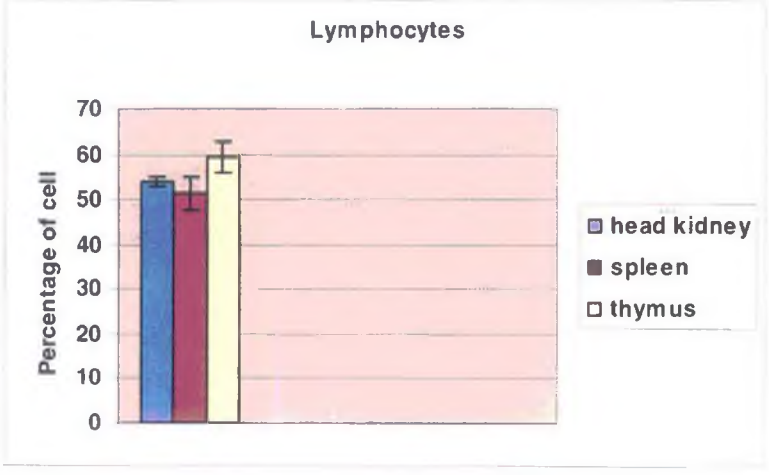


Fig. 67

Fig. 68

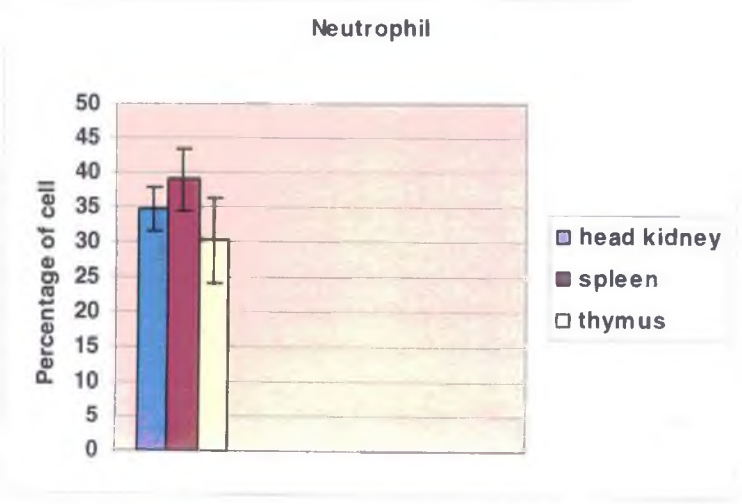
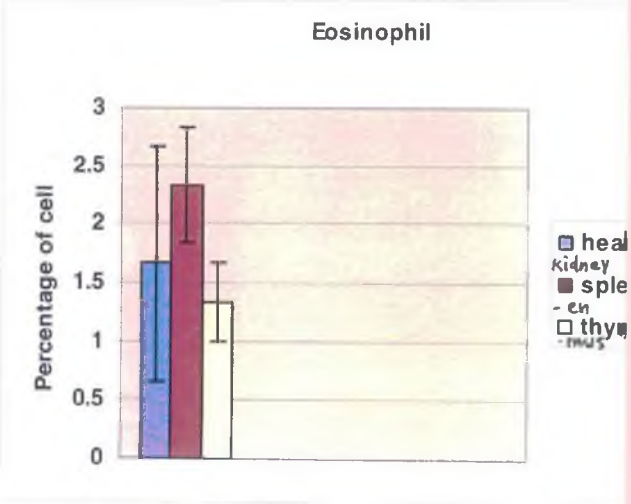


Fig. 69



Lymphocytes were present in peripheral blood of EUS affected *C. mrigala* at the highest percentage i; e 65.0±9.47 but neutrophils were present in only 20.43±6.37 percent. Monocytes were present in 12.17±5.33 percent and basophils and eosinophils both were present in 1±0% (Fig. 70).

Table12. Differential count of different subpopulations of leucocyte in peripheral blood of EUS affected *C. mrigala*

Eosinophil	Monocyte	Lymphocyte	Neutrophil	Basophil
1±0	12.17±5.33	65.0±9.47	20.43±6.37	1±0

Mean±S.D; n=10

4.16. Studies on immunological status of healthy *C. mrigala* year round

Table13 shows the number of plaques/10^6 W.B.C produced from cell suspension of two lymphohaemopoietic organs, head kidney and thymus during three seasons.

During summer number of plaques produced in head kidney was 689±8.54 while in thymus it was 482.33±10.78.

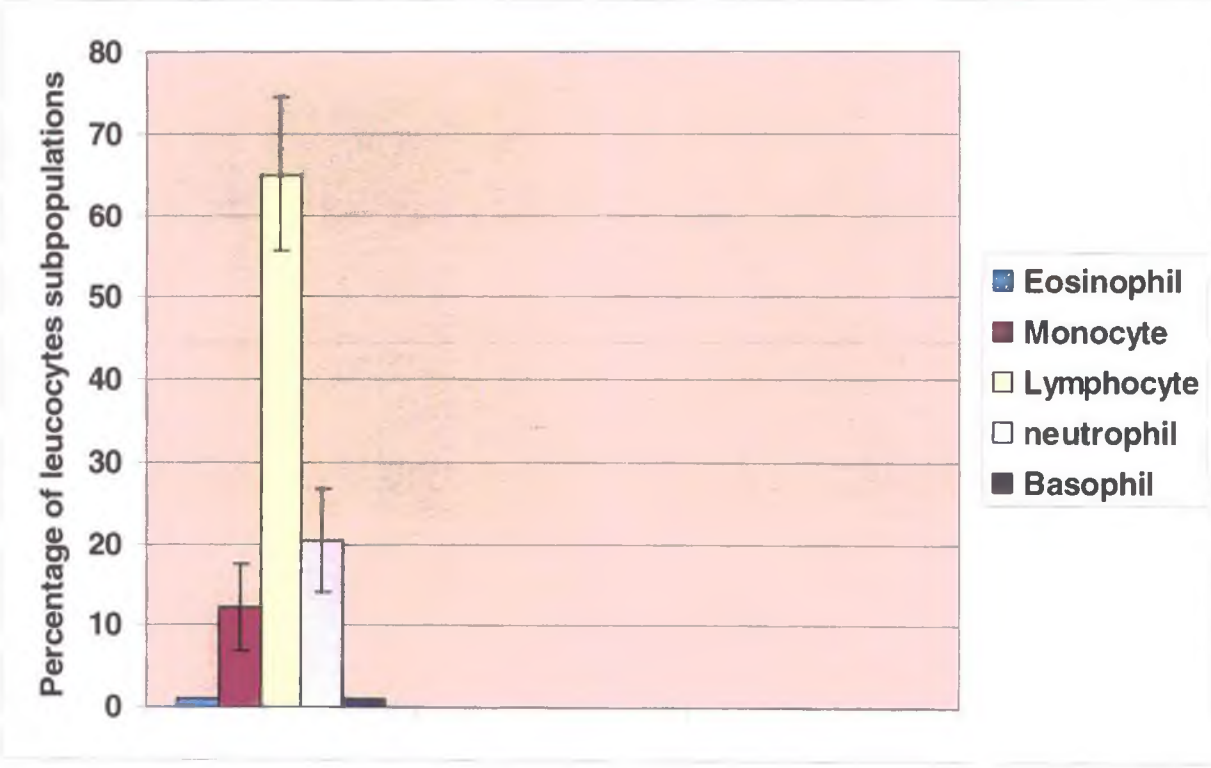
During rainy season, the number of plaques produced in head kidney was 905.88±8.87 while in thymus it was 592.88±43.86.

During winter, the number of plaques produced in head kidney was 530.33±19.19 while in thymus was 410.33±20.75 (Fig. 71).

In case of plaque forming cells assay, it was also observed that during winter months i; e from December to February when water temperature dropped significantly

Differential count of different subpopulations of leucocyte in peripheral blood of EUS affected *C. mrigala*

Fig. 70



the number of antibody secreting cells also dropped drastically. But during rainy season, though water temperature dropped slightly compared to the summer but number of antibody secreting cells increased.

The agglutinating antibodies produced after injection of antigen, SRBC or sheep red blood cells were found 2- mercaptoethanol sensitive, indicating that they were equivalent to the IgM of mammalian species.

Table13. The number of plaques/10^6 W.B.C produced from cell suspension of two lymphohaemopoietic organs, head kidney and thymus in three seasons

Season	Water temperature (°C)	Head kidney	Thymus
Summer	24°C-28°C	689±8.54	482.33±10.78
Rainy	22°C-28°C	905.88±8.87	592.88±43.86
Winter	16°C-22°C	530.33±19.19	410.33±20.75

* Data for each index are mean of three fishes. WBC= White Blood Cells. n=3

4.17. Measurement of amount of antibody produced throughout the year (2007) against (0.2 ml) Sheep Red blood cells or SRBC

Table 14 shows the amount of antibody produced against a fixed amount of antigen, Sheep Red Blood Cells measured by haemagglutination titre test (HA) on 5^{th} day and 10^{th} day after injection throughout the year (Figs. 72, 73, 74 and 75).

The number of plaques/10^6 W.B.C produced from cell suspension of two lympho-haemopoietic organs, head kidney and thymus in three seasons

Fig. 71

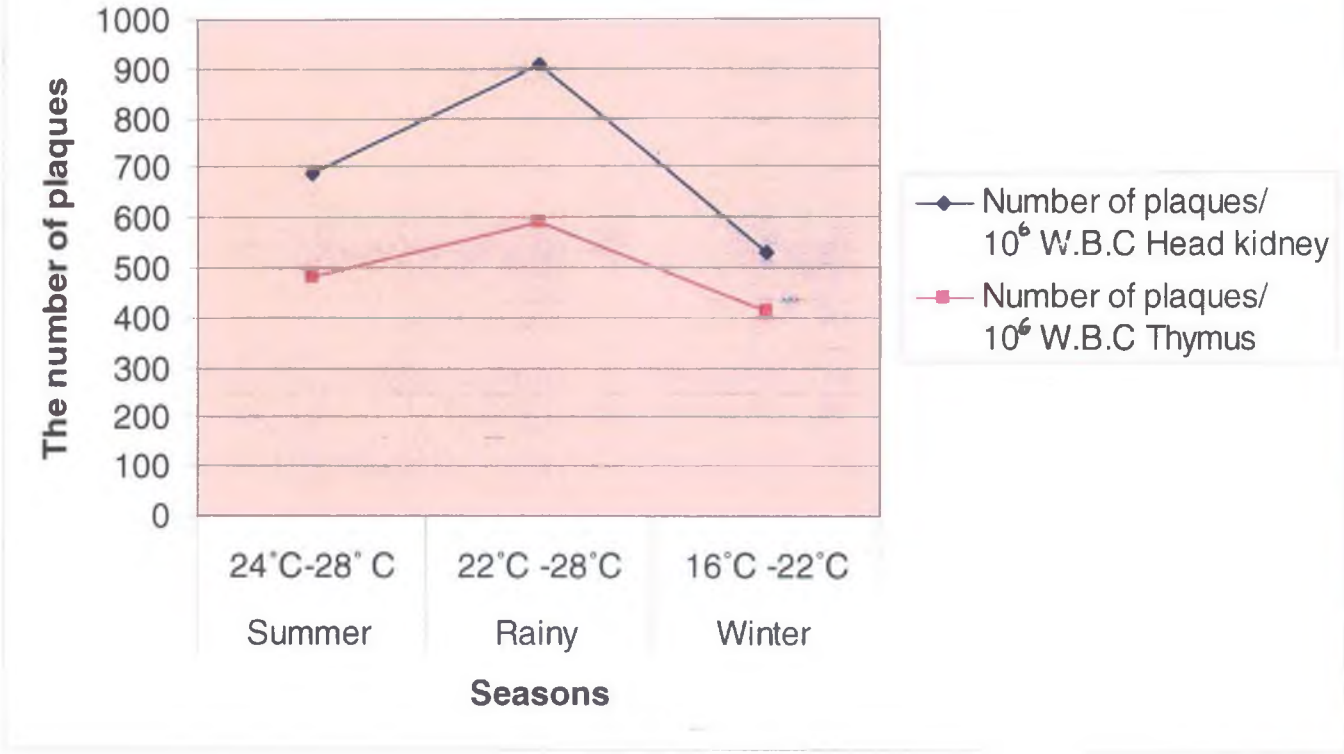
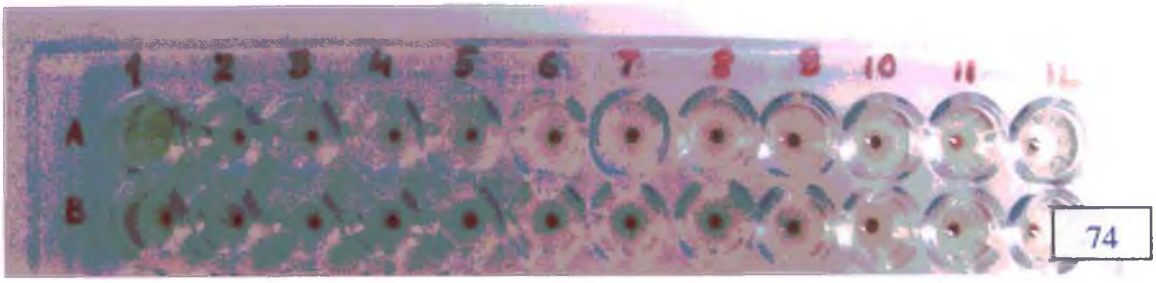
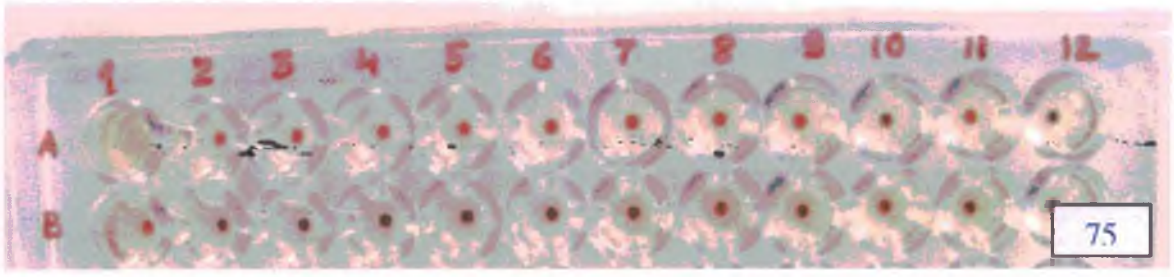


Fig.74: Series 'A' showing reaction in 1st well but series 'B' showing no reaction as 2-Merceptoethanol was added.

Fig.75: Series 'A' showing reaction in 1st well but series 'B' showing no reaction as 2-Merceptoethanol was added.

Fig.76: Series 'A' showing reaction upto 4th well while series B showing reaction upto 5th well.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
74


1 2 3 4 5 6 7 8 9 10 11 12
A
B
75


76

During January when the water temperature was the lowest (16-18°C) the amount of antibody production was also the lowest showing the reaction only in the 1st well in both occasions.

But in February, when the temperature increased (22-24°C) the amount of antibody production also increased showing reaction in 1st and 2nd wells on 5th day after injection and 1st, 2nd and 3rd wells on 10th day after injection .

During July and August the amount of antibody production was highest showing reaction upto 3rd well on 5th day after injection and upto 5th well on 10th day after injection and the water temperature was highest (28-30°C). Then with the decrease of water temperature the amount of antibody production also decreased being the lowest during December.

The results obtained from H.A tests showed clearly that as the temperature of water during winter season dropped the amount of antibody produced also dropped suggesting a partial suppression of immune response of fish.

The amount of antibody production by healthy *C. mrigala* against 0.2 mL of 25% SRBC throughout the year on 5th and 10th day after injection

Fig. 72

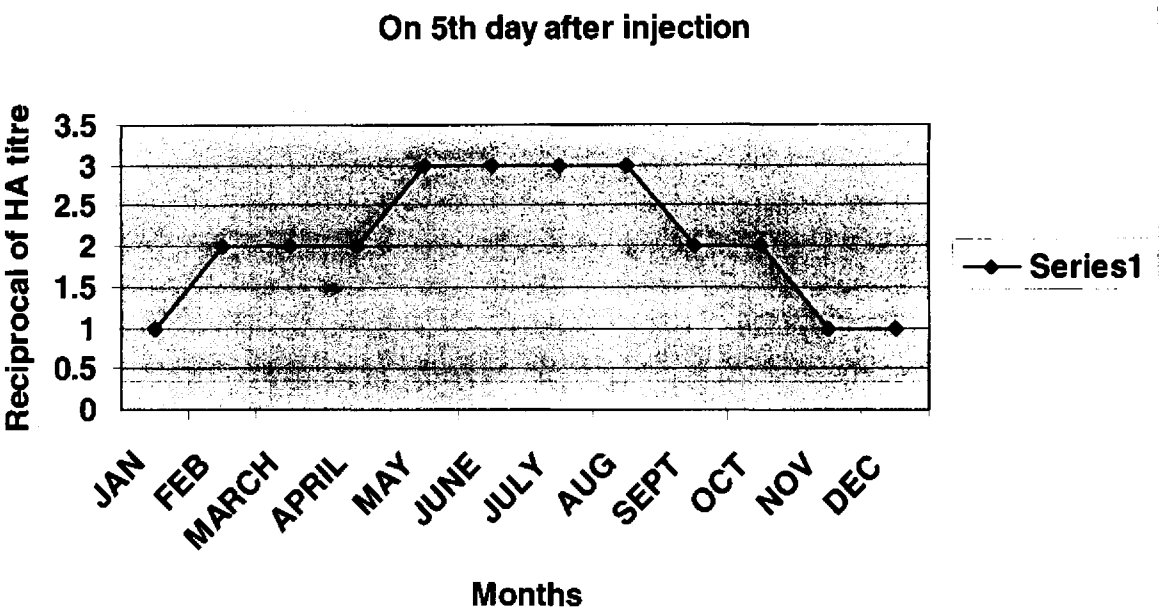


Fig. 73

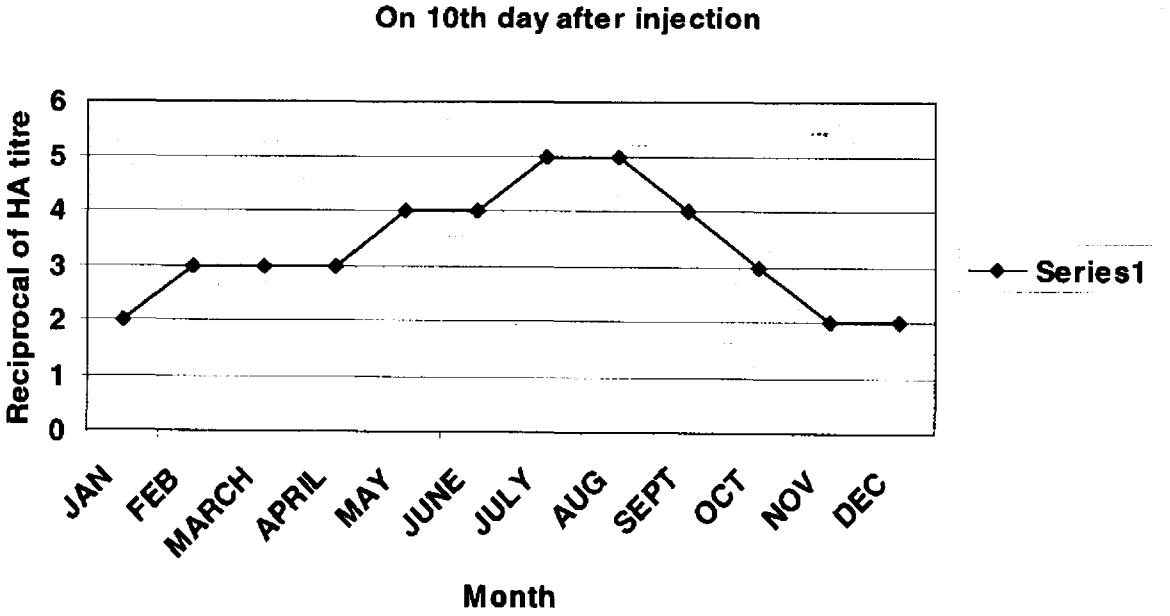


Table14. The amount of antibody production by healthy *C. mrigala* against 0.2 ml of 25% SRBC throughout the year (2007) on 5th day and 10th day after injection.

Month	Water Temp. (°c)	On 5th day after injection	10th day after injection
Jan	16-18	O	OO
Feb	20-22	OO	OOO
March	24-26	OO	OOO
April	24-26	OO	OOO
May	24-26	OOO	OOOO
June	26-28	OOO	OOOO
July	28-30	OOO	OOOOO
Aug	28-30	OOO	OOOOO
Sept	26-28	OO	OOOO
Oct	22-24	OO	OOO
Nov	20-21	O	OO
Dec	19-21	O	OO

Data for each index are mean of three fishes (n=3)

O = 1st well; OO = 1st and 2nd well; OOO = 1st to 3rd well; OOOO = 1st to 4th well; OOOOO = 1st to 5th well.

Discussion

Epizootic ulcerative syndrome (EUS) broke out for the first time in India in 1988 in some regions of the north-east India. Subsequently, it affected West Bengal. Gradually the disease spread to almost all the states of India except, Jammu Kashmir, Himachal Pradesh, Punjab and Gujarat by 1993. At present the disease is under control in most of the states of India but in some areas of North Bengal, the disease has been occurring every year especially during winter months (Roy, 2003; Routh, 2006).

Immune system of animals is the defense machinery to fight against the diseases and the disease state occurs through interaction of the host defense and pathogen. However, the host parasite relationship in fishes is highly infiuenced by the environment. As the fishes are ectothermic in nature and they live in aquatic habitats, they are more susceptible to environmental stress than the homeothermic animals (Trust, 1986).Therefore, studies on the immune system of fishes have been imperative for better management of diseases. Head kidney, spleen and thymus are important in immunologic defense mechanism and haemopoiesis in teleost fish (Ellis et al., 1976; Ellis, 1980; Secombes and Manning 1980; Turner et al., 1984).

Morphological studies of kidney of healthy *Cirrhinus mrigala* showed that it was divided into an anterior part, the head kidney, and a posterior part, the trunk kidney. Routine histological studies of head kidney under light microscope showed the presence of erythropoietic cells as well as lymphoid cells in the cortex and medulla (Fig.8). Ultra structure of head kidney confirmed the presence of lymphomyeloid and erythroid cells. Erythrocytes, immature and mature, lymphocytes, monocytes, neutrophilic myelocytes, neutrophils with type I and type II granules, thrombocytes and macrophages were detected in ultrastructural studies (Figs. 9 and 10).

Morphological studies of spleen in healthy *C. mrigala* showed that it was a dispersed structure found in between the loops of intestine. Histological studies showed presence of red pulp and white pulp regions where white pulp region is surrounded by the red pulp (Fig. 12). Ultra structural studies of spleen showed the presence of almost all types cells found in head kidney. But here the cells are loosely bound in comparison to head kidney.

Morphological studies of thymus showed that it was a paired small organ present dorsolaterally and triangular in shape (Fig. 20). Histological study showed the presence of erythroid cells arranged in cords and presence of trabeculae which divided the organ into incompletely separated lobules. Along with the presence of erythroid cells epithelial cells and some other cell types were also observed. The thymus was not divided into cortex and medulla and Hassall's corpuscles like structure were detected (Fig. 21). Ultra structural studies showed the presence of cell types already found in head kidney and spleen. But cells like hypertrophied epithelial cells and a particular type of cells with a huge number of granules were also found. A number of cells with secretory granules were also found (Figs. 22, 23 and 24).

Sailendri and Muthukarruppan (1975) reported two distinct zones, a deeply stained lymphoid zone and non – lymphoid zone within head kidney of *Tilapia mossumbica.* They detected lymphocytes of all sizes, monocytes, plasma cells, granulocytes, and erythrocytes in the head kidney. Tatner and Manning (1983) reported that head kidney of Rainbow trout was packed with many lymphocytes along with erythrocytes and suggested that head kidney was a lympho-haemopoietic organ.

Sailendri and Muthukarruppan (1975) reported that spleen in *Tilapia mossmbica* was an elongated and flattered structure and was situated along the left side of the stomach. They mentioned that histologically spleen was divided into white pulp and red pulp regions. The red pulp region was mainly erythroid with a few lymphocytes while white pulp region contained reticular centers. Various cells types like lymphocytes, plasma cells, granulocytes, monocytes and erythrocytes were also found by them. Tatner and Manning (1983) observed that the spleen contained both erythrocytes and lymphocytes. They could not detect red pulp and white pulp regions within spleen. Ultra structural studies by Bodammer et al. (1990) in striped Bass showed that the spleen was composed of tightly packed lymphomyeloid cells and erythroid cells.

Sailendri and Muthukarruppan (1975) showed that the thymus in *Tilapia mossumbica* was encapsulated by a thin strand of collagen fibers and consisted of outer, middle and inner zones. Outer zone consisted of predominantly of thymocytes while the

inner zone consisted of predominantly of lymphocytes. The gland was found highly vascularized. Zapata (1981) studied the histological structure of the thymus of a teleost fish, *Rutilus rutilus* and found that the thymus contained a huge number of lymphoblasts and thymocytes within a reticular network, formed by epithelial cells. He also reported presence of macrophages, secretory like cells and epithelial cells with cysts but no Hassall's corpuscles like structure. De and Pal (1998) examined the histological and ultramicroscopic structure of thymus of a Gobiid fish, *Pseudopocryptes lanceolatus*. They showed that histologically the gland lacked cortex, medulla and Hassall's corpuscles. But they found the existence of a huge number of lymphoidal, epithelial and erythroidal cells. Besides those cells, macrophages and cystic cells were also detected. Romano et al. (1999) also studied the histological and cytological structures of thymus of sharpsnout seabream, *Diplodus putazzo* and showed that the thymus was divided into cortex and medulla. The cortical region mainly contained lymphocytes while blast-like lymphoid cell were localized in medulla. Cytological studies by Romano et al. (1999) identified four types of epithelial cells like i) limiting, adjacent to the connective tissue ii) medullary and cortical reticular cells iii) nurse cells iv) Hassall's corpuscle and huge number of erythroid cells. Xie et al. (2006) also found thymic epithelial cells (TEC) nurse – like cells and different types of leucocytes like lymphocytes, granulocytes and macrophages but no Hassall's corpuscles were detected. Along with different leucocytes, erythrocytes of different developmental stages were also detected by them.

From above discussion it can be concluded that head kidney, spleen and thymus are lympho–haemopoietic organs in *C. mrigala*. Consistent reports by different authors regarding the presence of different leucocytes and different developmental stages of erythroid in histological section and ultra microscopic structure of the three organs have strongly established the lympho haemopoietic role of these three organs.

Concentrations of different types of leucocytes and different developmental stages of erythrocytes in these three organs indicated that the head kidney played the most active haemopoietic role among these three organs in the teleost, *C. mrigala*.

Histological studies and ultra microscopic studies of head kidney in different teleost by different researchers have showed almost similar observations. Observations of most of the researchers are in conformity with our observations. Like head kidney, results of histological studies and ultramicroscopic studies of spleen of different researchers showed more or less uniform result.

Histological studies of thymus in healthy C. *mrigala* and thymus in other teleost by different workers like Tatner and Manning (1983), Zapata (1981),.De and Pal (1998) and Romano et al. (1999) showed that it was covered by epithelial membrane containing mucous cells. Epithelial membrane covering thymus provides a biological interface between thymus gland and aquatic environment. The mucous cells secrete mucous which can prevent microbial colonization and infection in the thymus (Ottesen and Olafsen, 1977). Macrophages and granulocytes present in thymus may have roles in the deletion of self reactive thymocytes.

Different types of epithelial cells, especially epithelial cells with secretory granules may be related with hormones secreted from thymus, other epithelial cells and myoid cells are not only unknown in fishes but also are still unknown in higher vertebrates (Zapata, 1996). They probably play an important role in creating the milieu, within T lymphocytes as occurs in birds (Kendall, 1991) and mammals (Ritter and Crispe, 1992).

In case of EUS affected C. *mrigala* morphological structures of head kidney and spleen did not show any significant change. But the thymus showed an increase in size ($p < 0.001$) in EUS affected fish compared to healthy fish.

There were evidences that thymus played a vital role in the ontogeny of immunological competence (Miller, 1961; Good et al., 1962; Cooper 1973). Beard (1894) suggested that in elasmobranches the thymus was the source of lymphoid cells. Naturally in EUS affected C. *mrigala*, the thymus was more active than in healthy fishes.

Though morphology of head kidney of EUS affected C. *mrigala* did not show any significant change, but histological structure of head kidney in EUS affected fishes

showed changes like haemorrhages, presence of haemosiderin laden macrophages and necrosis (Figs. 28 and 29).

Ultra Structural studies of head kidney of EUS affected fishes showed some changes. Presence of bacteria throughout the tissue was found (Fig. 30). A considerable degree of necrosis in some regions was also detected. Fibrin clumps were noticed along with the presence of melanin granules (Fig. 31). Apart from above mentioned changes significant decrease in the number of erythrocytes was the feature of head kidney of EUS affected fishes (Fig. 32).

The nature of histopathological changes has some similarities with the observations reported by various authors on histopathological changes caused by bacteria in infected fishes. In Japanese eel *(Anguilla japonica)* affected with red spot disease atrophy of haemopoietic tissue in the head kidney was detected by Miyazaki and Egusa (1977). Miyazaki (1980) observed the degeneration of renal tubules, and atrophy of renal haemopoietic tissue in *Anguilla japonica* affected by *Aeromonas hydrophila.* Later Miyazaki and Kaige (1985) observed the deposition of haemosiderin in the renal tissue of fishes in which experimentally bacteria were introduced. Saha (1998) observed the almost similar histopathological changes in head kidney of EUS affected *Channa punctatus.*

Roy (2003) reported histopathological changes in the haemopoietic organs of different EUS affected fishes.

Flano et al., (1996) studied the histopathological changes in the renal and splenic tissues of Coho salmon (*Oncorhynchus kisutch*) experientially infected with *Renibacterium solmoninarum* and observed following changes. In both the organs presence of bacteria was detected but in spleen bacteria was detected at much earlier stage of infection compared to thymus.

Histological studies of spleen of EUS affected C. *mrigala* showed necrotic changes along with vacuolation in the white pulp regions (Fig. 33). Hemorrhages were also noticed. Ultra microscopic studies of spleen also showed more or less similar changes found in head kidney of affected fish i;e presence of bacteria in the tissue with a considerable degree of necrosis. Fibrin clumps and melanin depositions were also noticed (Fig. 34). Like head kidney in spleen also considerable decrease in number of erythrocytes were also detected (Fig. 35). Bacteria are quite common in the spleen tissue. Melano macrophages with melanin depositions were also found. Fibrin clumps were also found in splenic tissue (Fig. 36).

Miyazaki (1980, 1985) reported similar changes in the splenic tissue of Japanese eel (*Anguilla japonica)* infected artificially with pathogenic bacteria.

Histological and ultramicroscopic studies of splenic tissue of Coho salmon (*Oncorhynchus Kistuch*) infected experimentally with *salmoninarum* showed fibrin clumps and melanin deposition along with presence of bacteria (Flano et al., 1996).

Roy (2003) studied the histopathological changes of spleen of different EUS affected fishes like *Channa gachua, Puntius* sp. and *Catla catla.* In all species of EUS affected fishes, he observed the vacuolation, haemorrhages and necrotic changes.

In the present study histological structure of thymus of EUS affected C. *mrigala* showed some highly eosinophillic areas, containing erythroblastic islets, cords of mature erythrocytes and reticular epithelial cells (Figs. 38 and 39). Sinuses filled with blood vessels were also found. Hassall's corpuscle like structures was also detected. Ultra microscopic studies of thymus of EUS affected fish showed the presence of bacteria in the outer part of thymus (Fig. 41) but fibroblasts cells were also found invaded with bacteria. The necrosis of the connective tissue was also noticed. Macrophages containing bacteria in their cytoplasm were also detected in thymic parenchyma.

Flano et al. (1996) studied histological and ultra microscopic changes in the thymus of Coho salmon experimentally infected with bacteria and found presence of bacteria in

thymic parenchyma, macrophages containing bacteria and necrotic changes in some areas.

Many workers have opined the need for establishment of normal haematological values in fish with a view to the diagnosis of disease (Hesser, 1960; Snieszko, 1960; Larsen, 1961; Summerfelt, 1967).

By applying different staining procedures and techniques, three types of blood cells such as erythrocytes, leucocytes and thrombocytes were identified. The erythrocytes appeared either elliptical or oval in form with clearly visible nucleus (Fig. 42). The erythrocytes were found PAS negative. All the leucocyte subpopulations analogous to the mammalian leucocyte subpopulations were also identified in peripheral blood of healthy *C. mrigala.*

Seasonal variations of the total erythrocyte count in the peripheral blood of healthy *C. mrigala* were noticed (Fig. 60). The total erythrocyte count in peripheral blood of healthy *C. mrigala* dropped significantly during July to September (3.5 to 3.8 $\times 10^6/mm^3$) and then slowly increased being the highest during December (5.1 $\times 10^6/mm^3$). From January onwards the TEC or total erythrocyte count started decreasing slowly till the end of spawning season. In EUS affected fishes total erythrocyte count dropped drastically ($1.44 \pm 0.16 \times 10^6/mm^3$) compared to that of healthy fishes ($4.5 \pm 0.32 \times 10^6/mm^3$) (Fig. 57).

Mott (1957) mentioned that total erythrocyte counts in fishes were low compared with those of mammals. Knoll (1957) noticed that total erythrocyte count in fish varied considerably with species. Mahajan and Dheer (1979) showed that total erythrocyte count in peripheral blood of healthy *Channa panctatus* varied from 2.90 to 3.18 $\times 10^6/m.m^3$. They also showed that TEC varied with seasons. Das and Mukherjee (2000) reported that the total erythrocyte count in the fingerlings of healthy *Labeo rohita* varied from 2.1 to 2.15 $\times$ $10^6/mm^3$. Martins et al. (2004) observed that total erythrocyte count in the peripheral blood of healthy *Leoporinus macrocephalus* was $1.772 \times 10^6/\mu l$.

Kori–Siakpere et al. (2005) showed that mean total erythrocyte count in peripheral blood of African snake head, *Parachanna obscura* was $1.67 \times 10^{12}/L$.

Das et al. (2006) reported that in *Catla catla, Labeo rohita* and *Cirrhinus mrigala* total erythrocyte count varied from 4.0 to 6.0 $\times 10^6/mm^3$, and the total erythrocyte count in healthy *C. mrigala* was the highest 6.0 $\times$ $10^6/mm^3$.

Drastic fall in total erythrocyte count of EUS affected *C. mrigala* may be due to continuous loss of blood through ulcers developed on the body and the degeneration of red blood cells. Seasonal variations of total erythrocyte count may be the function of more than one factor such as temperature, food and metabolic state etc.

In peripheral blood the lymphocytes are the most common. The number varies from 41 to 60% in peripheral blood of healthy *C. mrigala.* According to the size two types of lymphocytes were identified, small and large.

The average diameter of small lymphocyte was 3.72±0.39µm and nuclei diameter was 2.5±0.64µm. The nucleus was round shaped and was surrounded by a thin film of cytoplasm (Figs.42, 43 and 44).

The average diameter of large lymphocyte was 11.2±0.42 and diameter of the nucleus was 9.4±0.12. The nucleus was almost round in shape and was encircled by a thin film of cytoplasm. These lymphocytes were slightly PAS positive. The number of lymphocytes also varied with seasons.

It was noticed that in EUS affected fishes total leucocyte count or TLC was much higher (90.93±13.86) $\times 10^3/mm^3$ in comparison to that of healthy fishes (26.93± 10.13) $\times 10^3/mm^3$ (Fig. 58).

Ellis (1977) noticed two types of lymphocytes large and small in fish and mentioned that the lymphocytes were slightly PAS positive. Andrew (1965) mentioned that the total leucocytes count (TLC) in fish was higher than that of other vertebrates. The total leucocytes count in fish varied from species, 23,000/mm^3 in roach (Catton, 1951), more than 100,000/mm^3 in some bony fishes (Pukhkov, 1964). Weinreb and Weinreb (1969) found variations in differential counts of lymphocytes in teleosts.

Mahajan and Dheer (1979) during their studies on cell types present in peripheral blood of *Channa punctatus* found two types of lymphocytes, small and large. Total number of leucocytes varied from 60 to 62 × 10^3/mm^3.

Das and Mukherjee (2000) during their studies on sublethal effect of Quinolphos on selected blood parameters of *Labeo rohita* found that total leucocyte count in healthy fish was 20 × 10^3/mm^3.

Kori-Siakpere (2005) reported that the TLC in peripheral blood of African snake head, *Parachanna obscura* was 19.07 × 10^3/mm^3.

Das et al. (2006) also observed that the total leucocyte count in peripheral blood of healthy *Cirrhinus mrigala* at normal pH varied from 20 to 22× 10^3/mm^3.

The total leucocyte count decreased during winter (January and February) with a sharp increase during March and April. This increase is followed by a decrease during May and June and finally increased during breeding season i; e July, August. After the end of breeding season the total leucocyte cocent of showed a tendency of decrease till the March of next year.

Mahajan and Dheer (1979) also noticed the similar nature of change in total leucocyte number in peripheral blood of healthy *C. punctatus.*

Among the leucocytes, monocytes are the biggest in size. The shapes of nuclei varied from oval-round to kidney shaped and occupied a peripheral position in the cell (Fig. 45). The monocytes were found PAS positive (Fig. 46). The average n-c ratio of monocytes is 1: 1.67

The differential counts of monocytes in blood smear of healthy *C. mrigala* showed that it constituted 5% to 25% of the total leucocyte population of peripheral blood.

Ellis (1977) described that morphologically fish monocytes were not only similar to mammalian monocytes, even they were also PAS positive like mammalian monocytes. The morphology of monocytes described by Mahajan and Dheer (1979) is almost similar to the morphology found in healthy *C. mrigala.*

Hammers et al. (1995) described the structure of monocytes in common carp *Cyprinus carpio* and mentioned that the monocytes were the largest leucocytes with basophilic cytoplasm and were PAS positive.

Pavlidis (2007) described the morphology of monocytes in six Mediterranean species of fish. The monocytes are the largest in size and cell diameter varied from 16.65± 0.14µm to 20.33± 0.19µm.

Neutrophils are the most numerous among different granulocytes. The nuclei of neutrophils appeared oval to round in shape and seldom had the nuclei appeared bilobed in structure (Fig. 47). The neutrophils showed the strongest PAS reaction (Fig. 48). Neutrophils stained with Sudan Black- B showed presence of granules in the cytoplasm of the cells (Fig. 44).

The probable cause of the change in number of monocytes in peripheral blood of *C. mrigala* is to compensate the opposite change in number of neutrophil in peripheral blood, so that cell mediated immunological status of fish remains intacstaint.

The average cell diameter of neutrophils of healthy *C. mrigala* is 15.53±0.27 µm while the average nuclei diameter is 9.6±1.97 µm making the n-c ratio 1: 1.62. Neutrophils constituted the 20% to 38% of total leucocyte population in healthy *C. mrigala.*

Ellis (1977) suggested that unlike mammalian neutrophils, the nuclei of fish neutrophils either oval or round in appearance. Ellis (1977) also suggested that neutrophils were PAS positive and granules of cytoplasm appeared dark grey in colour when stained with Sudan Black- B. Mahajan and Dheer (1979) also suggested that neutrophils in peripheral blood of *C. punctatus* were large cells with average cell diameter 10.5 µm (±0.14) and that of nucleus was 6.0 µm (±0.31). The nuclei of neutrophils were either round or oval in shape. Hammers et al. (1995) mentioned that neutrophils in common carp, *C. carpio* were strongly PAS positive and round in shape with eccentrically placed nucleus of different shapes ranging from round or oval to bi-lobed and tri- lobed in structure. Palvidis et al. (2007) during their studies on different

blood cells in six species of Mediterranean fish showed that neutrophils were round in shape with an eccentric nucleus. The shape of the nuclei varied from round to oval, to the shape of human kidney. In some rare occasions, it appeared bi- lobed or tri- lobed in structure. The cell diameter varied from 12.24 ±0.3 µm to 15.12 ±0.13 µm.

Literatures containing reports of neutrophils in the peripheral blood of fish especially in teleosts are very few in number.

Mahajan and Deer (1978) during their studies on seasonal variation of cell types in peripheral blood showed that like monocytes the number of neutrophils also changed throughout the year but just in opposite in manner to the monocytes.

The reciprocal change in number of neutrophils and monocytes in peripheral blood of healthy *C. mrigala* during this study may be due to a kind of compensating function so that immune status of fish remains unaltered.

Eosinophils were very rare in peripheral blood of healthy *C. mrigala.* The shape of nuclei appeared irregular and the cytoplasm was found full of acidophilic granules (Fig. 45). Cells were PAS negative. It constituted only 1.5% - 2% of total leucocyte population.

Presence of eosinophil was reported in the blood of many species of fish and in cyclostomes (Jordan, 1938) and in elasmobranches (Fange, 1968). Ellis (1977) reported that the cytoplasm of eosinophils in fish was loaded with acidophilic granules which appeared bright red with Romnowsky's stain. Mahajan and Dheer (1979) also noticed the presence of eosinophils in the peripheral blood of *C. punctatus.* The number varied from 3% - 6% of total leucocyte population. Hammers (1995) showed that eosinophils of peripheral blood in common carp, *C. carpio* were PAS negative.

Palvidis et al. (2007) also found the presence of eosinophils in the peripheral blood of some Mediterranean species.

Basophils were found very rare in peripheral blood of healthy *C. mrigala.* The size of the cells was almost equal to the size of neutrophils but the nuclei were either centrally located or peripherally located (Fig. 42). They were PAS positive.

Haider (1968) reported presence of basophils in carp, but Hines and Yashouv (1970) failed to find basophils in the Israeli strain of this species.

Duthie (1939) and Catton (1951) found basophil in *Triglida* sp. but more in *Ctenolabrus* sp. Basophils have been reported also from blood of brown trout (Blaxhall and Daisley, 1973). The description of the basophil in gold fish stained with Romnowsky's dyes by Watson et al. (1963) and Weinreb (1963) was typical. According to them the nucleus of a basophil was large and eccentric and the cytoplasm contained basophilic large granules. Pitombeira and Martins (1970) claimed that basophils in Spanish mackerel were PAS positive.

Mahajan and Dheer (1979) mentioned that basophils in peripheral blood of *C. punctatus* could be easily identified by the presence of bluish cytoplasm. Nuclei were either rounded or showed considerable variations. Hammers (1995) showed that basophils of *C. carpio* were PAS negative.

Thrombocytes were seldom found in the peripheral blood of healthy *C. mrigala.* Shapes of thrombocytes vary greatly from spindle shaped to irregular structures. Thrombocytes were PAS negative.

Mahajan and Dheer (1979) described thrombocytes as oval cells with an ellipsoid nucleus in peripheral blood of *C. punctatus.* They also reported the presence of a considerable number of thrombocytes of irregular shapes. Palvidis et al. (2007) described thrombocytes as round, oval, elongate and spindle shaped in peripheral blood of six Mediterranean species.

Six distinct developmental stages of erythrocytes were identified, namely small lymphoid haemoblast or slh, basophilic erythroblasts or be, polychromatophilic erythroblasts or pe, acidophilic erythroblasts or ae, young reticulocytes and mature erythrocytes or mr in the tissue imprints of head kidney, spleen and thymus (Figs. 50 and

51). Tissue imprints of three lympho-haemopoietic organs showed that presence of developmental stages of erythrocytes was the highest in the imprints of head kidney and the lowest in thymus.

Sailendri and Muthukkaruppan (1975) identified different developmental stages of erythrocytes in the tissue imprints of three lympho-haemopoietic organs of *T. mossambica*. Mahajan and Dheer (1980) studied the process of erythropoiesis in the head kidney and spleen of healthy *C. punctatus* by preparing tissue imprints as well as using autoradiography techniques and identified six distinct developmental stages of erythrocytes.

There are two views regarding the development of blood cells in fish. One is known as diphyletic view propounded by Catton (1951) and the other is monophyletic view advocated by Jordan and Speidel (1924) and Duthie (1939).

The major changes noticed during present investigation on erythropoiesis were as follows

i. Cytoplasm was primarily basophilic and slowly became acidophilic passing through an intermediate stage.

ii. Finally the acidophilic cytoplasm was slowly replaced by haemoglobin resulting the change in shape and size of erythrocytes.

iii. Mature erythrocytes appeared smaller in size compared to the immature erythrocytes.

iv. Immature erythrocytes possess mitochondria, golgibodies and other cell organelles, but mature erythrocytes do not possess them. May be for this loss of cell organelles, shrinkage has taken place resulting the decrease in size.

Differential count of different subpopulations of leucocytes in peripheral blood of EUS affected *C. mrigala* showed a considerable increase in number of lymphocytes (65.0 ±9.47) compared to healthy fishes (52.49±2.03).

The number of monocytes in EUS affected *C. mrigala* also showed a considerable increase compared to healthy fishes.

Unlike lymphocytes and monocytes, neutrophils in EUS affected *C. mrigala* showed a significant decrease in number in comparison to that of healthy fishes. The number of neutrophils in peripheral blood of EUS affected fishes was 20.43±6.37 while average per cent of neutrophil in peripheral blood of healthy *C. mrigala* was 32.39±8.08.

Eosinophils and Basophils did not show any significant change in number.

Comparison of erythropoietic efficiency of head kidney and thymus of healthy fish was performed. The results showed that head kidney was more erythropoietically efficient than thymus which was consistent with the results of tissue imprints where developmental stages of erythrocytes were more in head kidney than in thymus (Figs. 52 and 53).

The results on erythropoietic efficiency of EUS affected fishes revealed that erythropoietic efficiencies of head kidney, spleen and thymus in EUS affected fishes were significantly less compared to that of healthy fishes.

Literatures dealing with erythropoietic efficiency of lympho-haemopoietic organs of fishes and infected fishes or diseased fishes are extremely rare. The significant decline in erythropoietic efficiency of lympho-haemopoietic organs in EUS affected fish may be explained by the presence of bacteria in those organs and the necrotic changes done by the bacteria.

The loss of blood from ulcers of EUS affected fishes along with decline of erythropoietic efficiency of lympho-haemopoietic organs might have led the acute anaemic condition in EUS affected fish as reported by various authors.

In peripheral blood of both healthy and EUS affected fishes per cent of round shaped erythrocytes representing immature stages and elliptical shaped erythrocytes representing mature erythrocytes differed significantly. (Table no.) In healthy fish per

cent of elliptical erythrocytes was 79.6±3.647 while in EUS affected fish it was 62.2±5.404.

The per cent of oval or immature erythrocytes in healthy fish was 20.4±3.647 and in EUS affected fish it increased to 37.8±5.404 (Figs. 54, 55 and 56).

Das et al. (2006) also showed that the shape of erythrocytes was changed when the fish was exposed to stressful conditions. In the peripheral blood of fishes exposed to either 5.5 pH or 9.0 pH of water, number of oval shaped or round shaped erythrocytes increased significantly.

As EUS affected fishes face an acute anaemic condition, resulting low oxygen carrying capacity which was reflected by the abnormal behaviour shown by EUS affected fishes, production of huge number of immature erythrocytes may be compensating mechanism against anaemic conditions.

Results of haemoglobin measurement of healthy fish year round showed a similar mode of decrease and increase with that of total number of erythrocytes which contain haemoglobin in blood (Fig. 60).

Mahajan and Dheer (1979) also found the similar results during their studies on *Channa punctatus*.

Above discussion and result clearly state that change of total erythrocytes count is the cause and change of haemoglobin in blood.

As every year during winter months out break of EUS takes place in the districts of North Bengal, the kinetics of the primary immune response of fish (*C. mrigala)* to particulate antigen, sheep red blood cells (SRBC), was investigated in terms of antibody-secreting cells by a plaque forming cell (PFC) assay and haemagglutination (HA) tests.

Plaque Forming Cell (PFC) assay was performed from two lymphoid organs head kidney and thymus of healthy *C. mrigala* during three seasons such as summer, rainy season and winter. Haemagglutination titre test was performed throughout the year against the particulate antigen sheep red blood cells (SRBC). Plaque Forming Cell (PFC)

assay was performed on 5th day after injections collecting cells from head kidney and thymus. But haemagglutination titre test was performed on 5th and 10th day after injection.

The results showed that during winter months specially from December to January when water temperature dropped significantly, the number of plaques i; e antibody secreting 'B' cells and amount of antibody production also dropped significantly compared to the number of plaques produced and amount of antibody produced during summer and the rainy season (Figs. 71, 72 and 73) suggesting a partial suppression to immune response of *C. mrigala*. Along with the seasonal suppression of immune response of *C. mrigala*, it was also identified that number of plaques produced from thymus is always significantly less than the number of plaques produced from head kidney. The antibody produced against sheep red blood cells was found completely 2-merceptoethanol sensitive.

Seasonal variations, particularly environmental temperature, greatly influence the immune system of fish (Zapata et al. 1992). Higher environmental temperatures enhance immune responses, while lower temperature adversely affects their expression (Daggfeldt et al. 1993; Lobb et al. 1984). Many fish species show lower blood lymphocyte count and suppressed immune response to an antigen in winter (Avtalion, 1969; Rijkers et al. 1980; Wishkovosky and Avtalion, 1987). In gold fish *Carassices auratus*, plasma IgM, level show annual changes, high in summer and low in winter (Suzuki et al. 1996). Also in rainbow trout *Oncorhyncleus myeniss*, the IgM levels declined in winter (Sanchez et al. 1993). In *Clarions batrachus*, an air-breathing teleost, amount of antibody production and number of plaques decreased significantly during winter (Sinha and Chakravarty, 1997). On the other hand, no correlations between water temperature and IgM levels were found in channel catfish, *Ictalurus punctatus* (Klesius, 1990). Bly and Clem (1991) reported that functions of B and T cell were affected by temperature.

Though, head kidney is the major lympho-haemopoietic organs in *C. mrigala* as in may other fish, yet a notable feature was the presence of Plaque Forming Cells in the

thymus of immunized fish, although this was low in comparison to the PFC level in the head kidney.

Similar results were obtained by Sinha and Chakravarty (1997) during their studies on immunological response in an air-breathing teleost, *C. batrachus*. Using MAb (monoclonal antibody), several investigators have reported the presence of small number of SIg^+ cells in thymus of fish (Egberts et al., 1983; Miller et al., 1987; Ellsaesser et al., 1988).

These findings indicated that thymus of fish might have contributed antibody production to some extent. 2-ME sensitive antibody in *C. mrigala* suggested that the antibody produced in *C. mrigala* was equivalent to mammalian IgM class. The works of Rijkers (1981) and Sinha and Chakravarty (1997) also support this view.

The production of low number of PFC and less amount of antibody during winter months clearly indicated that during winter months immune response in *C. mrigala* was partially suppressed making them more susceptible to disease during this period in every year.

Summary

The present study dealt with "Haematological and immunological studies of healthy and epizootic ulcerative syndrome affected freshwater teleost, *Cirrhinus mrigala*" consisting of

1. Histological studies of lympho-haemopoietic organs, head kidney, spleen and thymus of both healthy and EUS affected *C. mrigala*. Electron microscopic studies of lympho-haemopoietic organs of healthy and EUS affected fish *C. mrigala*.

2. Studies on blood cell profile in healthy *C. mrigala*.

3. Cytochemical studies on erythropoiesis in healthy *C. mrigala*.

4. Studies on erythropoietic efficiency of head kidney and thymus of healthy and EUS affected *C. mrigala*.

5. Comparison of percentage of mature and immature erythrocytes in peripheral blood of healthy and EUS affected *C. mrigala*.

6. Total count of erythrocytes and leucocytes in healthy *C. mrigala* and EUS affected fishes.

7. Studies on haematological parameters like total erythrocyte count (TEC), total leucocyte count (TLC), total haemoglobin (Hb) content, differential count of three subpopulations of leucocyte, neutrophil, lymphocyte and monocyte in peripheral blood of the healthy fish throughout the year.

8. Studies on the humoral immunity of the fish *C. mrigala*.

9. Count of antibody secreting 'B' cells in healthy *C. mrigala*.

Since the first appearance of the epizootic ulcerative syndrome (EUS) in India, in May, 1988, in various north eastern states, it was distinct by its destructive nature and capacity of affecting a wide variety of fish species in both wild and cultured waters. Subsequently, it spread to West Bengal. By 1993 the disease spread all over India except, Jammu and Kashmir, Himachal Pradesh, Punjab and Gujarat.

The histological studies on head kidney, spleen and thymus of healthy *C. mrigala* showed the presence of erythroid cells and lymphoid cells in the three organs. The sections also showed that head kidney was divided into cortex and medulla while the spleen was divided into red pulp and white pulp regions. Histological sections of the thymus showed presence of a thin capsule surrounding the gland. The stroma was divided into lobules by trabaculae originating from the capsule. Lobules were not completely separated. The stroma was not divided into cortex and medulla. It showed presence of heamopoietic cells arranged in cords having epithelial cells. Hassall's corpuscles like structures were also found.

The histological sections of three lymphoid organs of EUS affected *C. mrigala* showed the necrotic changes in three organs. Haemosiderin laden cells were also found in the spleen of EUS affected *C. mrigala.* Haemorrhages were also noticed in the sections of head kidney of EUS affected *C. mrigala.* EUS affected fishes, thymus showed highly eosinophilic areas along with erythroblastic islets, cords of mature erythrocytes and reticular epithelial cells.

Electron microscopic studies on the three lympho-haemopoietic organs showed the presence of different cell types such as, erythrocytes, erythroblasts, haemoblasts, neutrophilic myelocytes, macrophages, thrombocytes, small lymphoid haemoblasts and lymphocytes. All these cell types were more or less common in these organs but varied in number. Hypertrophied epithelial cells and secretory cells were exclusively found in thymus.

The Electron microscopic studies of the three organs in EUS affected *C. mrigala* showed the presence of bacteria along with fibrin clumps and melanin deposition.

Studies were also conducted on the erythropoiesis of *C. mrigala* and different developmental stages of erythrocytes e.g., small lymphoid haemoblasts or shl, basophilic erythroblasts or be, polychromatophilic erythroblasts or pe, acidophilic erythroblasts or ae, young reticulocyte or ye and mature erythrocytes or me were detected.

Studies on blood cell profile in peripheral blood of healthy *C. mrigala* showed the presence of erythrocytes, agranulocytes like lymphocytes and monocytes, granulocytes like, neutrophils, eosinophils and basophils. Along with these, plasma cells were also identified. Erythrocytes were found PAS negative while neutrophils were strongly PAS positive but lymphocytes and monocytes were slightly PAS positive.

The cell diameter of each type of blood cells and nuclei diameter were measured. The n-c ratio of each type was also calculated.

Erythropoietic efficiency of head kidney and thymus in both healthy and EUS affected *C. mrigala* showed that erythropoietic efficiencies of those organs in EUS affected *C. mrigala* were significantly lower compared to that of healthy *C. mrigala.*

Total count of erythrocytes and leucocytes in peripheral blood of both healthy and EUS affected *C. mrigala* showed that in EUS affected *C. mrigala* the total erythrocyte count was significantly lower while total count of leucocytes was found significantly higher compared to healthy *C. mrigala.*

Differential count of leucocytes in peripheral blood of healthy ad EUS affected *C. mrigala* showed that percent of lymphocytes and monocytes were significantly higher in EUS affected *C. mrigala.*

Studies on haematological parameters in healthy *C. mrigala* throughout the year showed that the haemoglobin content, total erythrocyte count, total leucocyte count and differential count of three subpopulations of leucocytes varied with season. Total erythrocyte count or TEC significantly decreased during July and August followed by an increase during October to December. Total leucocyte count increased during February and March followed by decrease during April to August and again decreased during winter months.

The experiments related with immunological studies of healthy *C. mrigala* throughout the year showed that during winter season when every year EUS outbreak takes place in the ponds of the plains of North Bengal, total antibody production and

number of antibody secreting 'B' cells decreased significantly. The antibodies produced were found 2-ME sensitive.

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TOXIC EFFECTS OF SELECTED PESTICIDES ON *CHANNA PUNCTATUS* (BLOCH) MAINTAINED IN AQUARIA: A STUDY BASED ON BIOCHEMICAL PROFILES

SYNOPSIS

Thesis submitted for the

Degree of Doctor of Philosophy (Science)

BY

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SYNOPSIS

Although pesticides produce good many results in the control of pests, their harmful effects on the non-target animals cannot be ruled out. Organophosphate pesticides though less persistent in the environment leave residues in water and mud even several days after their spray in the adjacent crop field. This poses a constant threat to the non-target organism especially to the fishes. The air breathing fishes are the worst sufferers of this situation. Hence, organophosphates may also cause health risk to mankind through food chain. These pesticides are powerful neurotoxic chemicals. These chemicals are also cytotoxic, mutagenic, teratogenic and carcinogenic. In view of importance of organophosphate contamination on freshwater ecosystem and public health and hygiene an attempt was done to evaluate precisely the toxic effects of two organophosphate pesticides, dimecron and quinalphos (most widely used in crop field and tea plantation respectively) on fish which are of great economic importance to man.

Experimentally acute toxicity determined through biochemical investigations in liver, muscle and kidney of fresh water teleost, *Channa punctatus* was done with dimecron and quinalphos exposure. The dimecron and quinalphos were added @1.8 mg/l, 0.36 mg/l and @ 2.5 mg/l, @ 0.50 mg/l respectively to the medium (tap water) of aquaria. About 25-30 acclimatized fish were introduced in each aquarium. Simultaneously, an aquarium was set up for the study of control animals. A minimum of five fish was sacrificed each time from treated as well as control aquaria. All the experiments were repeated three times for each biochemical estimation. The analysis was performed at 10, 20 and 30 days of exposure.

Both dimecron and quinalphos have been found to cause injury in different organs although the degree of injury vary in different organs. The apparent loss of body weight, organ weight and loss in total protein, DNA and RNA content in all the exposed groups were noticed. It was also dose dependent. On the gel electrophoretic protein band profiles of the experimental and control fish, a critical analysis of the band comparison revealed that certain bands present in control fish were found to be missing and a few unknown protein bands originated, more number of bands were missing with addition of a few new protein fractions in higher dose exposed group.

It may be inferred from this studies that some of the genes involved in the synthesis were switched off resulting in the disappearance of some of the proteins. The appearance of some new proteins may be due to the switching on of some genes. Stress proteins are a group of proteins whose synthesis is induced by a wide variety of physical conditions and chemical agents: heavy metals, xenobiotics, oxidative stress, anoxia, salinity stress, teratogens, and hepatocarcinogens. Some of these proteins are believed to play a role in protecting the cell from damage that can result from environmental perturbations. Others are involved in the regulation of various genes. Stress results in a dramatic redirection of metabolism; a suite of stress proteins is rapidly synthesized and production of cellular proteins is repressed.

In the present study the changes in protein sub-fractions and concomitant changes in DNA and RNA contents would not only confirm the protein loss (due to dimecron and quinalphos exposure) in a more powerful and precise manner, but would again strongly support the cellular protective response against organophosphate toxicity through possible expression of certain genes encoding stress proteins though characterization of those proteins need to be studied.

The changes in the enzymatic activities are also very significant. Increased acid phosphatase, alkaline phosphatase, lipid peroxidation, Cytochrome P450 and concomitant decrease in glutathione level indicate that both dimecron and quinalphos are cytotoxic and also caused oxidative stress. The toxic effect on different tissues has been reflected in the loss of body weight and gain in organ weight along with concomitant behavioral changes of fishes. In conclusion, it can be stated that dimecron and quinalphos are highly toxic chemicals.

The outcome of this present study is that even at sub lethal concentrations, pesticides have deleterious effect on fish, affecting the value of the fish. Therefore it may be suggested that necessary care may be taken to avoid contamination of fresh water bodies while spraying pesticides in adjacent crop field.

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