HISTOPATHOLOGICAL STUDIES ON ZINC TOXICITY IN MILKFISH *CHANOS CHANOS* FORSSKAL

DISSERTATION SUBMITTED BY
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C E R T I F I C A T E

This is to certify that this Dissertation is a bonafide record of the work done by Shri. K.C. Dinesan, under my supervision and that no part thereof has been presented before for any other degree.

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PREFACE

Aquaculture embraces a wide range of activities from 'Sea-ranching' and management of activities in large bodies of water to intensive culture with fertilization and feeding of fish in small manmade ponds. It is an age old practise in different parts of the world and recently its potential in upgrading rural economy in developing countries has been recognized by International Organizations like FAO.

In countries like India, rich animal protein sources is not within the reach of majority of population. Hence the need for developing aquaculture practices to augment the production of cheap source of animal protein is highly felt. Aquaculture is the best available method to convert the otherwise useless organic waste into high quality animal protein. Further, rapid strides have been made in aquaculture by application of modern scientific methods directed to produce maximum amount of flesh in a minimum volume of water.,

Many countries including India have fertile bays, estuaries and intertidal zones which are ideal locations for taking up aquaculture. In the early part of 20th century most of these areas were not polluted. But unfortunately today they face the major problem of environmental degradation due to the accumulation of industrial waste, agricultural run-off and the waste of increased urbanization (Iverson, 1976; Pillai, 1976).

Like any other developing country we are also facing the problem of industrial pollution especially in our aquatic systems. Among

the industrial pollutants, heavy metals are of a major problem. Zinc is a major effluent from the industries like softdrinks, flavouring, fur dressing and dyeing, fish processing, laundry, etc. (Klein et al., 1974; Nair and Nair, 1986).

Many studies involving heavy metal pollution has been conducted in India and abroad. However these studies were limited to estimation of pollutants in water, its accumulation in aquatic organisms and its lethal effects. Very limited studies are available regarding the effect of pollutants on various vital systems of cultivable organisms at cellular level. A study involving the deleterious effects of pollutants on the tissues and organs of the organisms at various concentrations and extent of its exposure is essential to evaluate the damage caused to the biota. There is very little information regarding the toxic effect of zinc on milkfish (Chanos chanos) - a commonly cultured euryhaline fish. Therefore this study was taken up with a view to investigate the damages caused by zinc at cellular level on various organs of milk fish. This study involved histological examination of various vital organs, such as liver, kidney, brain, gills, skeletal, muscle and spleen.

I express my deep sense of gratitude to Dr. K.C. George, Scientist S-2, Central Marine Fisheries Research Institute, for his valuable guidance and whole-hearted support throughout the period of this study. My thanks are due to Dr. P.S.B.R. James, Director, Central Marine Fisheries Research Institute, for providing facilities to work on this problem. I also thank Dr. S.C. Mukherjee and Dr. George John for their valuable suggestions and help during the study. My heartiest thanks are due to Shri. A. Sait Sahul Hameed, Senior Research Fellow, for his constant help throughout

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INTRODUCTION

The deleterious effects of aquatic pollution is a subject of study by large number of workers. Among the various types of pollutants, heavy metal poisoning in aquatic animals occupies an important position (Forstner and Wittmann, 1977; Munro, 1978) and hence is a matter of concern.

There are reports regarding severe poisoning of mercury, cadmium, zinc, copper in aquatic animals (Irukayama, 1967; Yagamata and Shigamatsu, 1970; Kobayashi, 1971; Natta, 1972). The lethal level of zinc for fishes vary from 330 ppm (Carpenter, 1927) to 0.01 ppm (Affleck, 1952). The median lethal concentration (LC_{50}) values for zinc had been reported to vary depending on species, quality of water etc. (Skidmore, 1964; Taylor, 1981). Eventhough zinc is toxic at higher levels, it is also an essential element in traces. It is necessary for enzyme activity, protein and carbohydrate metabolism and also for growth (Vallee, 1959; Ogino and Yang, 1978; 1979). But when present in excess, it retards the growth of fishes (Crandall and Goodnight, 1962, 1963; Bengston, 1974; Watson and McKeown, 1976; Pierson, 1981).

With regard to heavy metal absorption in fishes, there were some indirect evidences that the zinc absorption in fishes was mainly through gills and not through gut of skin (Saiki and Mori, 1955; Cairns and Scheier, 1957; Joyner, 1961; Skidmore, 1964). Holcombe et al., (1979) studied the concentration of zinc in the experimental fishes where gills showed three times higher concentration than that of

control fishes. Liver and kidney also revealed two times higher concentration where as in brain and muscle there was no accumulation of zinc. Pentreath (1973) and Lovegrove and Eddy (1982) were of the opinion that main sites of heavy metal uptake were the gills and gastrointestinal tract. Extensive studies on biochemical parameters in association with metal poisoning had been done in a number of species of aquatic animals. It was reported that various heavy metals at toxic levels had effect on cellular respiration and glucose metabolism (Hiltibran, 1971; Bilinski and Jonas, 1973; Larsson, 1975; Gill and Pant, 1981).

The effect of heavy metals on hormones, biotransformation enzyme activities and other enzyme kinetics were studied by Srivastava (1982), Brown et al., (1984), Nemcsok et al., (1984), and Jambulingam, (1988).

The effect of toxic levels of zinc on liver was well documented in fishes. Crandall and Goodnight (1963) reported pathological alterations like vacuolation of hepatocytes, degeneration of hepatic parenchyma when guppies were exposed to different levels of sublethal concentration of zinc, i.e., 1.15 ppm to 2.3 ppm for 55 to 95 days. Severe necrotic changes were obtained in the liver of fishes exposed to lethal concentration of zinc (Kumar and Pant, 1981). Necrosis, vacuolation and other degenerative changes of hepatocytes were observed in liver of fishes in association with heavy metal pollution and other toxic conditions (Koyama and Itazawa, 1977; Roberts, 1978; Kumar and Pant, 1981; Sultan and Khan 1981; Bhattacharya et al., 1985; Forlin et al., 1986; Jambulingam, 1988). In general, heavy metals were observed to have effect on cellular respiratory metabolism of hepatic cells (Hiltibran, 1971; Brown et al., 1984).

Kidneys are very sensitive to poisoning/toxic conditions (Runnells et al., 1965; Jones and Hunt, 1983; Brown et al., 1984). The nephrotoxic effects of heavy metals were characterised by degenerative changes in tubules, particularly in proximal convoluted tubules and glomeruli. Crandall and Goodnight (1963) observed the degenerative changes of kidney. They noticed distension of glomeruli and tubules when the fishes were subjected to sublethal zinc toxicity. Kumar and Pant (1981) noticed vacuolation and necrosis of the epithelial cells lining the renal tubules and dilated glomerular capillaries when fishes were subjected to lethal concentration of zinc. Other heavy metals also produced similar changes in kidneys (Gardner and La Roche, 1973; Trump et al., 1975; Koyama and Itazawa, 1977; Roberts, 1978). Gardner and Yevich (1970), Koyama et al., (1978), Forlin et al., (1986) noticed the maximum damage of the kidney due to heavy metal toxicity was in proximal tubules. Saxena (1981) reported changes like swelling of glomerular capillaries and the increase in Bowman's space in fishes associated with cadmium toxicity.

In the case of higher vertebrates brain is highly susceptible to all types of toxic condition. These changes are characterised by neuronal lysis, satellitosis, neuronophagia and other degenerative changes (Runnells et al., 1965; Jones and Hunt, 1983). However, in fishes very limited work has been done in relation to pathomorphological changes of brain. Vascular congestion of brain was noticed in fishes after exposure to pesticides (Cope et al., 1970; Kennedy et al.,1970). Gardner and La Roche (1973) observed dilation of blood vessel and haemorrhage in brain when fishes were exposed to copper. Hyperemia of brain was reported in the fishes which was exposed to endosulfan and dieldrin (Walsh and Ribelin, 1975).

Ultrastructure studies on brain cells from fish larvae exposed to toxic conditions were carried out by Cameron and Smith (1980) and Somasundaram et al., (1984). They reported swelling of nuclear membrane,changes in rough endoplasmic reticulum, increased in intercellular spaces and shrinkage of mitochondria.

The toxic effect of zinc on the gill has been studied in many species of fishes. Carpenter (1927) and Jones (1938) stated that in acute zinc toxicity, gill damage was caused by excessive secretion and precipitation of mucus. In acute toxic conditions Lloyd (1960), Kumar and Pant (1981) and Kodama et al.,(1982) observed that changes in gills were characterised by swelling of epithelial cells, necrosis of cells, desquamation of epithelial layers and oadema of gill lamellae. Skidmore (1970) observed the death in Salmo gairdneri subjected to an acute zinc treatment was probably caused by an internal hypoxic situations after metal exposure, which was generated by the increase of distance that respiratory gases have to cross between water and blood due to gill damage. This hypothesis was further confirmed by Burton et al., (1972). In 1972, Skidmore and Tovell suggested that initial changes in the gill tissue of rainbow trout exposed to 40 ppm of zinc sulphate solution were characterised by typical acute inflammatory reaction. The epithelium covering the secondary lamallae was desquamated in continuous sheet from the pillar cell system. This increased the diffusion distance from water to blood.

Crandall and Goodnight (1963) did not find any change in gills of fishes which were exposed to sublethal toxicity. According to Skidmore (1964), chronically toxic concentrations of zinc caused no damage to the gills, but lethal concentrations definitely caused severe

cytological damage to the gills. The only exception is that of Tuurala and Soivio (1982)'s experiment. They exposed Salmo gairdneri to sublethal concentrations of zinc and observed the fusion of adjacent secondary lamellae and detachment of the epithelium from the pillar cell system. The cells in the outer layer of the epithelium did not loose their lateral contacts to the adjacent epithelial cells.

Other organs and tissues like heart, spleen, intestine and skeletal muscles were also reported to have undergone pathological alterations due to heavy metal poisoning (Crandall and Goodnight, 1963; Gardner and Yevich, 1970; Jambulingam, 1988). In view of this, the present study was taken upto find out the histopathological changes caused by zinc in the vital organs such as liver, Kidney, brain, heart, gills, skeletal muscle and spleen of milkfish (Chanos chanos).

MATERIALS AND METHODS

Milkfish (Chanos chanos) of two size groups i.e, fry (3.0 to 3.5 cm length; 0.3 to 0.45 gm weight) and subadults (13 to 15 cm length; 16 to 20 gm weight) were selected for the experiments. Fishes were collected from Kerala Agriculture University Farm, Puduvypu and Matsyafed Farm, Narakkal. Fry were transported to the laboratory in oxygen filled polythene transportation bags. Subadults were transported by large plastic bins. Transportation were done in morning hours to avoid heat stress to the animal. Care was taken to avoid mechanical injury during collection and transportation.

Fishes were acclimatized for two weeks before commencement of the experiment. During the period of acclimatization, fishes were held in 400 litre fibre glass tanks which contained diluted seawater of salinity 20 ppt, pH 7.2 and temperature 28 ± 2°C. The tanks were provided with biological filters. Same quality of water was used for experiment also. Water was aerated with aquarium aerators. Fishes were fed ad libitum with artificially prepared wet feed. The waste materials were siphoned out daily from the tank.

Exposure to zinc:

Zinc was added in the form of $ZnSO_4.7H_20$ throughout the experiment. The LC_{50} values of zinc for fry was approximately calculated by the method of Finney (1952) for exposing the experimental animals to sublethal and lethal concentrations.

To study the histopathological changes due to the sublethal and lethal concentrations of zinc, experiments were conducted on fry (sublethal and lethal) and subadults (sublethal only). The fishes were grouped into 5 categories. The first group served as the control, while the second, third and fourth were subjected to 2.5 ppm, 5 ppm and 7.5 ppm of zinc respectively. The fifth group consisted of fry was subjected to a zinc concentration of 30 ppm. All the experiments were done in duplicates taking 10 animals in each group. The fishes were limited to one per two litre for fry and one per ten litre for subadults. In order to maintain the concentration of zinc and reduce the build-up of waste products, water was changed once in every 48 hours. Aeration was done throughout the experiment.

Experimental fishes were sacrificed on 10th, 20th and 30th day post exposure. Vital organs like liver, kidney, brain, heart, gills, muscle and spleen were dissected out from subadult fishes and fixed in 10 percent neutral buffered formalin. In the case of fry, incisions were made in the cranium and muscle; abdomen was opened by a longitudinal midventral incision for proper fixation and was fixed in neutral buffered formalin. Fishes from fifth group were fixed immediately after death.

After proper fixation, tissues were processed by routine histological techniques (Bullouck, 1978). Paraffin embedded sections were cut at 4 to 6 μ thickness in mannual rotary microtome. (Weswox optik model MT - 1090A). The sections were stained with Harris haematoxylin and counterstained with alcoholic eosin (H & E). Frozen sections of liver were cut at 15 - 20 μ in cryostat (American opticals) and stained with Sudan black B to demonstrate lipid (Casselman, 1959). Special

staining for polysaccharides of PAS techniques (Himes and Moriber, 1956) was employed for kidney sections. All the sections were examined under light microscope (Olympus) and Photomicrographs were taken whereever necessary.

RESULTS

Median lethal concentration:

The median lethal concentration of zinc in milkfish (Chanos chanos) was found to be approximately 15 ppm for 72 hours.

Histopathological studies:

Vital organs/tissues from five groups of fishes, sacrificed on 10th, 20th and 30th day after exposure were subjected to detailed histopathological studies. Salient histopathological observations are described for each organ. The findings are also summerised in Table 1 and 2. Changes observed in fry and subadults were not significantly different.

Liver:

Group - i:

Liver of Group-i which formed the control did not reveal any major alterations (Fig. 1). Normal architecture of parenchyma was altered very little. The hepatocytes were polyhedral in shape having a central vesicular nucleus. The hepatocytes formed irregular cords which were separated by sinusoids lined with endothelial cells.

Group - ii:

The liver tissue after 10 days exposure revealed vacuolation of hepatocytes and condensation of nuclear chromatin. The samples collected on 20th day showed intravascular coagulation of blood and focal necrosis of hepatic parenchyma in addition to vacuolar changes. Same changes with more severity was observed in the liver samples collected on 30th day.

Group - iii:

In this group, hepatic cells had undergone similar vacuolar changes by 10th day as in Group - ii. The nuclei were either swollen or pyknotic. Many small areas of coagulative necrosis with infiltration of mononuclear cells were noticed in the perinecrotic areas. The intravascular coagulation of blood which was observed in Group - ii on 20th day was also present in these fishes on 10th day post exposure. Many of the blood vessels appeared more permeable and exhibited fibrinous exudate in perivascular region. These changes became more severe and extensive on 20th day. The degree of coagulative necrosis was also increased. Further extension of coagulative necrosis along with mononuclear cell infiltration was noticed in the liver of animals exposed for 30 days.

Group - iv:

The samples taken on 10th day, showed very extensive vacuolation of hepatic cells with several foci of coagulative necrosis and mononuclear cell accumulation. Perivascular mononuclear cell infiltration and intravascular coagulation of blood were also observed. Hepatic nuclei were either swollen or pyknotic. Samples taken on 20th day revealed further changes like extensive area of necrosis infiltered with mononuclear cells. In 30th day sample these changes have become more severe and extensive.

Group - v:

The fishes under this group started dying from 8 hour post-exposure and all were dead by 16 hours. Liver from these animals revealed acute degenerative changes of hepatic cells with extensive area of necrosis.

Lipid staining:

Liver tissues from Group - ii, Group - iii, and Group - iv, were subjected to lipid staining by Sudan black B method revealed the excessive accumulation of fat in hepatic cells.

The salient changes of liver tissues are shown in Fig. 2 to 8.

Kidney

Group - i:

The kidneys were composed of excretory, haemopoietic and reticuloendothelial tissues. The nephrons consisted of a well vascularised glomeruli which were congested. The glomeruli were surrounded by Bowman's capsule which were lined by squamous epithelial cells. The Bowman's capsule continued through a ciliated neck. Two proximal segments, one with a prominent brush border and other with basal striations which were separated by a ciliated segment were seen. In addition to these tubules there were distal segments which connected second proximal segments to the collecting ducts. The proximal ducts were more eosinophilic in staining and the proximal segment of the tubule were lined by low columnar epithelium with indistinctive borders. Interstitial space was occupied by actively dividing haemopoietic tissue and elements of adrenal tissues. Numerous melanomacrophage centres were also seen. In this group, the architecture of the kidney did not show any major changes except congestion of the glomerular capillaries and occasional degenerative changes in tubules (Fig. 9).

Group - ii:

The samples collected on 10th day revealed no change in glomeruli from that of control. However, swelling of epithelial cells

of proximal segments with casts in the lumen were observed. On 20th day, many of the glomeruli showed more permeability and Bowman's capsule contained proteinaceous fluid and homogenous eosinophilic materials. Some of the glomeruli revealed increased cellularity and sclerotic changes. In addition to necrosis of cells of proximal tubules, swelling of the cells and cast in the lumen were also noticed. By 30th day exposure, in many of the glomeruli the capillaries appeared highly thickened. A few glomeruli revealed mesenchymal proliferation and also increased permeability. Proximal tubules exhibited necrosis and the necrosed cells contained eosinophilic droplets (hyaline droplets).

Group - III:

In this group, the kidneys on 10th day revealed most severe changes in tubules. There were only very mild changes in glomeruli. The tubular epithelial cells were either degenerated or had undergone severe extensive necrosis. The lumen of tubule contained hyaline cast. By 20th day, considerable changes were observed in glomeruli and tubules. The glomerular changes were characterised by accumulation of proteinaceous fluid in Bowman's space, thickening of glomerular capillaries, mesenchymal cell proliferation and adhesion of visceral and parietal layers; and periglomerular fibrosis. In extensive areas, tubular epithelial cells were necrosed and almost all the tubules contained hyaline cast. In 30th day samples also same changes were observed in glomeruli and tubules.

Group - iv:

In this group extensive tubular necrosis, mesenchymal cell proliferation and hypercellularity of glomeruli were seen on 10th day exposure. Extensive thickening of capillaries of glomeruli were observed in many

areas. In 20th day samples, adhesion of glomeruli to parietal layer of Bowman's capsule; and sclerotic changes in glomeruli and Bowman's capsule were evident. The tubular changes which were seen in other groups were also present with much more severity throughout the experimental period. By 30th day, large number of glomeruli revealed sclerotic changes and adhesion to Bowman's capsule in addition to the extensive tubular necrosis.

Group - v:

In this group, the changes in kidney consisted of tubular degenerations. The epithelial cells were swollen and tubular lumen contained some casts. The glomeruli revealed very mild changes.

Polysaccharide staining:

The kidney sections from group - ii, group - iii and group - iv when subjected to staining with PAS method had shown irregular thickening of basal lamina of capillary endothelium.

The pathological changes in kidneys are represented in Fig. 10 to 18.

Brain

Group - i:

The brain and spinal cord was covered with the single meningeal layer and the ventricles were lined with ciliated cuboidal ependymal cells. The brain and spinal cord were divided into grey and white matter, the grey matter contained numerous neurons, the pattern of arrangement or neurons were similar to other vertebrates in general. However there were some neurons which are very large - Mauthnerian group of cells.

The supporting cells, the neuroglia consisted of astrocytes, oligodendrocytes and microglial cells. In this group occasional neurons showed degenerative changes. However these changes were minimal (Fig. 19).

Group - ii:

In this group, the fishes after exposure to toxicant for 10 days showed many changes in the nervous system. These include neuronal lysis, chromatolysis of neurons, vacuolation of neurons and neuronophagia. In the 20th day sample, the cerebral cortex revealed absence of neurons in many places and formation of microglial modules. Many of remaining neurons had their nucleus in the periphery of the cell with vacuolation of cytoplasm. Satellitosis was found around some neurons. By 30th day the above described changes were further aggrevated and liquifaction necrosis of brain substances was evident in some areas.

Group - iii:

In this group, the samples collected on 10th day revealed changes like satellitosis of neurons, proliferation of glial cells, margination of nucleus of neurons and neuronal lysis. By 20th day the brain revealed neuronal vacuolation, liquifactive necrosis, neuronophagia and large number of glial modules. The 30th day sample revealed the above described changes with rounding of many neurons and liquifactive necrosis of many areas. Wallerian degenerations of nerve fibre tracts were also observed. The subependymal tissue was oedematous and the same layer was infiltered with round cells. The blood vessels of choroid plexus appeared hyalinised and leaky.

Group - iv:

This group revealed very severe changes by 10th day itself. Wallerian degeneration of nerve fibres was noticed in the white matter. Several empty spaces indicating neuronal necrosis were seen. Neuronophagia, proliferations of neuroglia, satellitosis, vacuolation of neurons and other neuronal degenerative changes were noticed. Subependymal oedema with vascular changes were also seen. By 20th day and 30th day the changes become more extensive with several regions of liquifactive necrosis. Many of the Purkinje cells of cerebellum and other neurons in many parts of the nervous system were rounded and appeared hyperchromatic.

Group - v:

This group, the capillaries and other blood vessels of brain were severely congested and neuron showed degenerative changes like vacuolation and condensation of nuclei.

The various changes in brain are shown in Fig. 20 to 27.

Heart

Group - i:

The heart from group - i did not reveal any significant changes. The heart was invested with pericardial membrane. It had a sinus venosus, composed mainly of collagenous connective tissue, an atrium which had a thinwall and numerous muscular trabeculae traversing the lumen. The trabeculae were lined with endothelial cells. The ventricle had a very thick wall with a very small lumen. The wall was composed of an outer compact layer of muscle and an innter layer of muscles which formed numerous trabeculae (Fig. 28).

Group - ii:

In group - ii the heart muscles did not reveal any significant difference from the control group till 10th day exposure. But by 20th day there were foci of necrosis and loss of striation of muscle fibres. As the days of exposure increased number of foci of necrosis increased with infiltration of mononuclear cells.

Group - iii:

In fishes which were exposed to 5 ppm zinc on 10th day, the muscle fibres lost their striated appearance and sarcoplasm appeared granular. By 20th day, vacuolation of sarcoplasm and focal necrosis were evident. Accumulation of mononuclear cells were also seen. 30th day samples also had focal necrosis, loss of striations and mononuclear cells infiltration. Deposition of fibrin was seen in between the myofibrils.

Group - iv:

In this group, loss of striation of myofibrils, focal leucocytic infiltration and necrosis were evident by 10th day. On 20th day and 30th day sample the same changes with more severity were observed.

Group - v:

The heart was not examined.

The salient changes of heart muscles are shown in Fig. 29 and 30.

Gill

Group - i:

The control gill had structure very similar to normal gill. The gill arch was covered by typical epidermal tissue which at the ori[illegible]

of primary lamellae was much thicker and endowed with mucus cells. Below this epidermis there was an array of lymphoid tissue consisting of lymphocytes and large cells containing eosinophilic granules. The primary lamellae had so many lateral projections, the secondary lamellae which were covered with epithelial cells - one layer thick which was supported and protected by the pillar cells. The pillar cells forms the lining of the blood sinuses or lamellar sinus which connect the afferent and efferent lamellar arteries. The structure of control group did not reveal any major anatomical abnormalities except occassional areas of sloughing of epithelial cells.

Group - ii, Group - iii, Group - iv:

Gill tissues from these groups on 10th, 20th and 30th day post-exposure did not reveal any significant changes from that of control group.

Group - v:

In this group severe epithelial necrosis was seen in secondary lamellae and many lamellae were oedematous and exhibited desquamation of epithelium.

The control and group - v gills are shown in Fig. 31 to 33.

Skeletal muscle

The muscle of group - i showed normal architecture of muscle fibres. But in all other groups occassional Zenker's type of necrosis was observed.

Spleen

The haemopoietic tissue of control and experimental group did not show much significant changes in Haematoxylin and Eosin stained sections.

Table 1. Histopathological changes due to sublethal toxicity

Vital organs/ tissues	Period of exposure	Group - ii (2.5 ppm Zinc)	Group - iii (5 ppm zinc)	Group - iv (7.5 ppm zinc)
Liver	10 days	Vacuolation, pyknotic nuclei	Vacuolation, swollen/ pyknotic nuclei, coagulative necrosis with mononuclear cell accumulation. Intra vascular coagulation	Extensive vacuolation, swollen/ pyknotic nuclei, foci of coagulative necrosis with mononuclear cell accumulation.
	20 days	-do- Intravascular coagulation, focal necrosis	-do-	-do- Extensive area of necrosis
	30 days	-do- Severe changes	-do- Severe changes	-do- Severe changes
Kidney	10 days	Glomeruli : no significant change Tubule : Degenerative changes	Glomeruli : very mild changes Tubule : necrosis and cast	Glomeruli : capillary thickening Tubule : necrosis, hyper cellularity
	20 days	Glomeruli : exudation of proteinaceous fluid, increased cellularity, increased capillary thickening Tubule : necrosis, swellir g, cast	Glomeruli : exudation of proteinaceous fluid, increased capillary thickening Tubule : necrosis, swelling, cast	More glomeruli showing sclerotic changes and adhesion
	30 days	-do- Severe changes	-do- Severe changes	-do- Severe changes

Cont.....

Table 1 .....Contd

Brain	10 days	neuronal lysis chromatolysis, vacuolation of neurons and neuronophagia.	Neuronophagia, satellitosis, gliosis, margination of nucleus of neuron, neuronal lysis	Wallerian degeneration, satellitosis, neuronophagia, gliosis and other neuronal degenerative changes.
	20 days	Satellitosis, gliosis, marginat-ion of nucleus of neuron.	Liquifactive necrosis	-do- rounding of neurons
	30 days	Severe than above with liquifaction necrosis	-do- rounding of many neurons, wallerian degeneration, subep-endymal oedema, hyalinised and leaky blood vessel of chroid plexus.	-do more severe.
Heart	10 days	No significant change	Muscle fibres:- loss of striations	Muscle fibres:- loss of striations, necrosis.
	20 days	foci of necrosis, loss of striations of muscle fibres	-do- Vacuolation, focal necrosis	-do- severe
	30 days	-do- necrosed area infiltered by mo-nonuclear cells	-do- mononuclear cells accumulation. De-position of fibrin in between myofibrils	-do- more severe

Cont.....

Table 1 .....Contd

Gill	10 days	No significant change	No significant change	No significant change
	20 days	-do-	-do-	-do-
	30 days	-do-	-do-	-do-
Skeletal muscle	10 days	Occassional Zenker's type of necrosis	Occassional Zenker's type of necrosis	Occassional Zenker's type of necrosis
	20 days	-do-	-do-	-do-
	30 days	-do-	-do-	-do-
Spleen	10 days	No significant changes	No significant changes	No significant changes
	20 days	-do-	-do-	-do-
	30 days	-do-	-do-	-do-

Table 2. Histopathological changes due to lethal toxicity (30 ppm zinc)

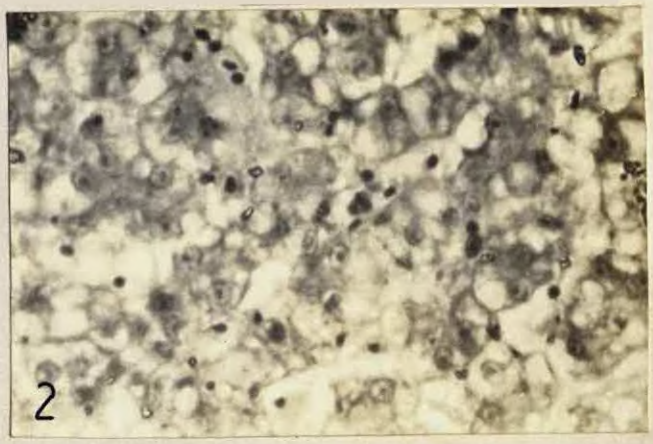
Vital organs/ tissues	Extent of damage
Liver	Acute degenerative changes of hepatic cells with extensive area of necrosis.
Kidney	Glomeuli:- Very mild cahgnes Tubule: Swollen epithelial cells, cast in the tubule
Brain	Blood vessels and capillaries severely congested, degenerative changes in neurons,
Heart	Not examined
Gill	Epithelial necrosis, desquamation and oedema of secondary lamellae
Skeletal muscle	Occassional Zenker's type of necrosis
Spleen	Not examined

Fig. 1. Liver hepatocytes from control fish. H&E X320.

Fig. 2. Liver of treated fish showing extensive vacuolation of hepatic cells. H&E X320.

Fig. 3. Liver of treated fish showing intravascular coagulation of blood. H&E X320.

Fig. 4. Liver of treated fish exhibiting intravascular coagulation and perivascular fibrinous exudate (arrow), note also the extensive vacuolation of hepatic cells. H&E X320.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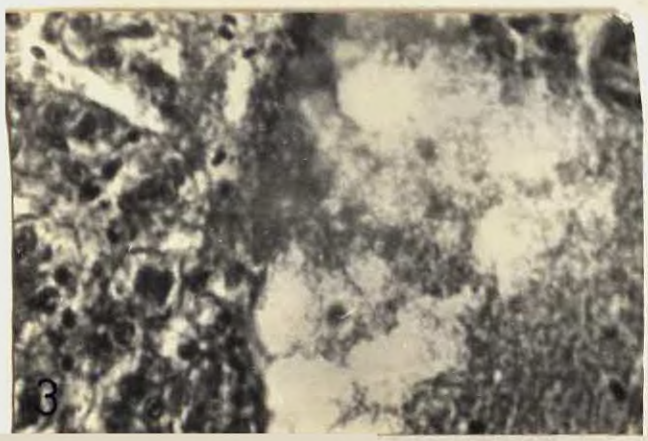
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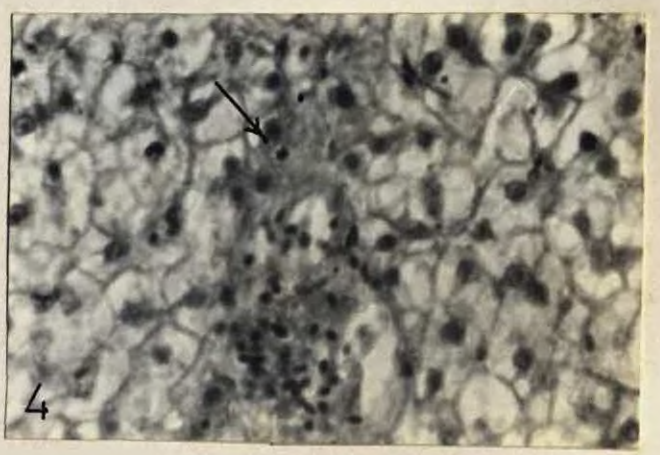
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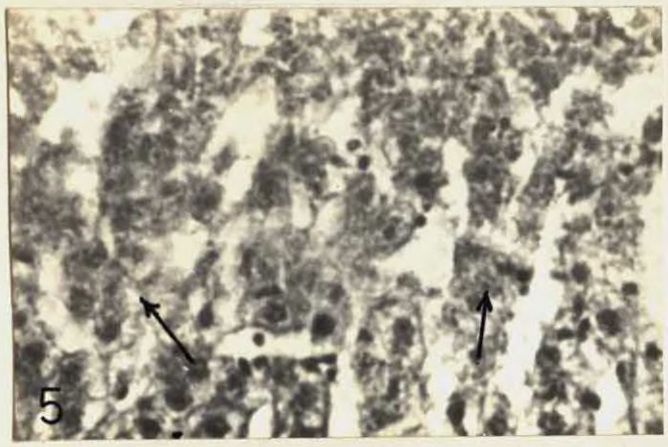
Fig. 5. Liver of treated fish showing of the necrotic regions (arrows). H&E X320

Fig. 6 and Fig. 7 Liver of treated fish showing accumulation of mononuclear cells in perivascular region of hepatic parenchyma (arrows). H&E X320

Fig. 8. Frozen section of liver from treated animals showing lipid accumulation. Sudanblack B. X800



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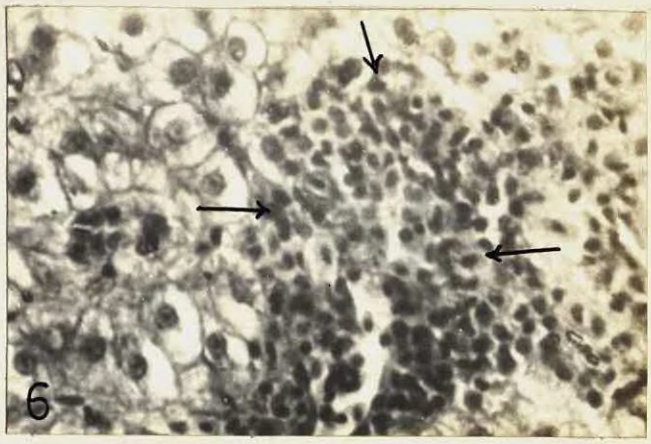

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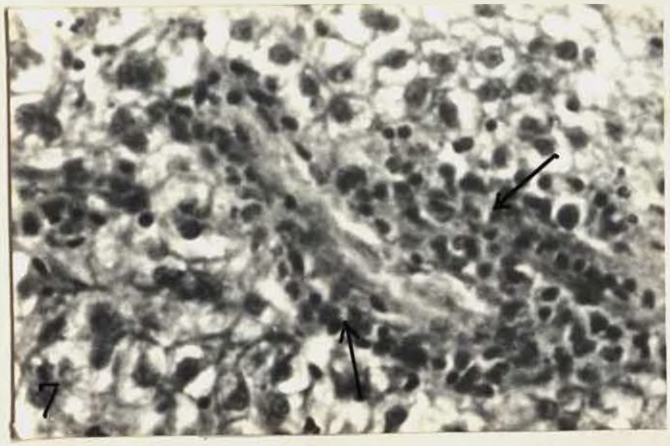

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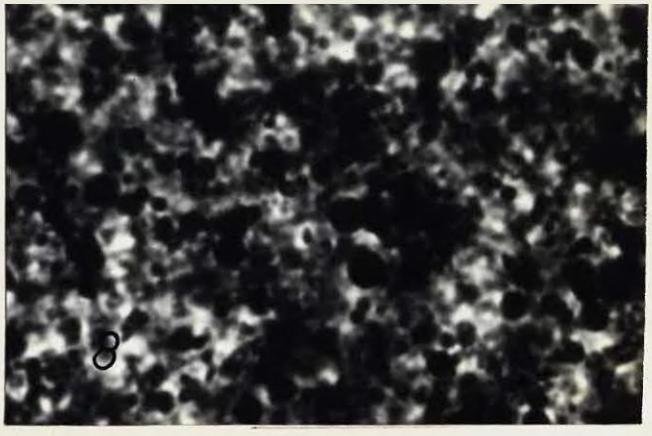

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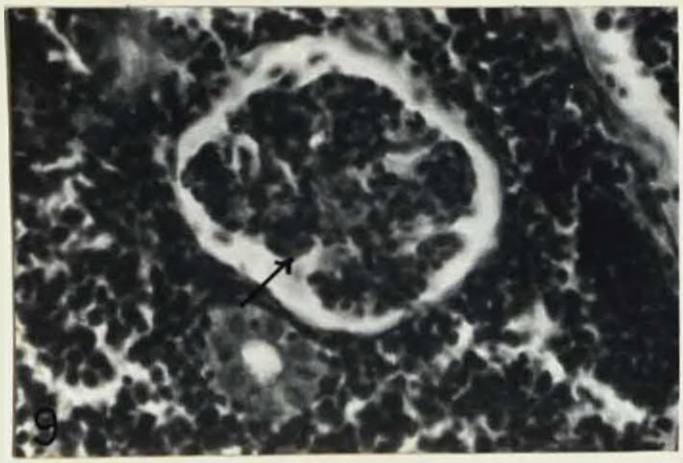
Fig. 9 Section of kidney from control fish showing congested glomerulus (arrow), haemopoietic tissue and tubules. H&E X320

Fig. 10 Kidney from treated fish showing increased permeability of glomerulus and accumulation of exudate in Bowman's capsule (arrows). H&E X400

Fig. 11 High power view of Fig. 10. H&E X800

Fig. 12 Section of kidney showing extensive thickening of capillaries (arrows). H&E X400.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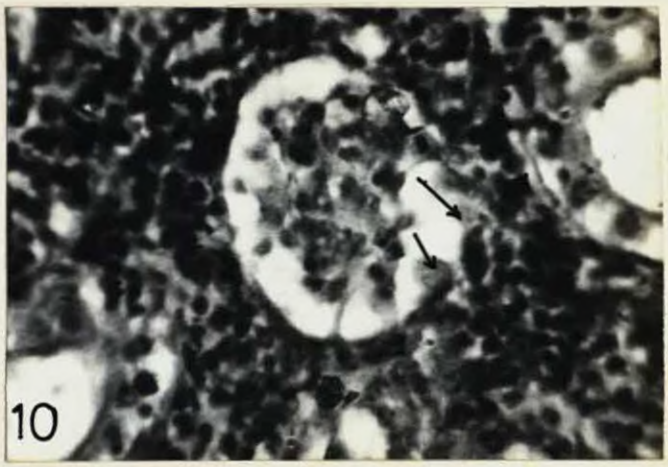
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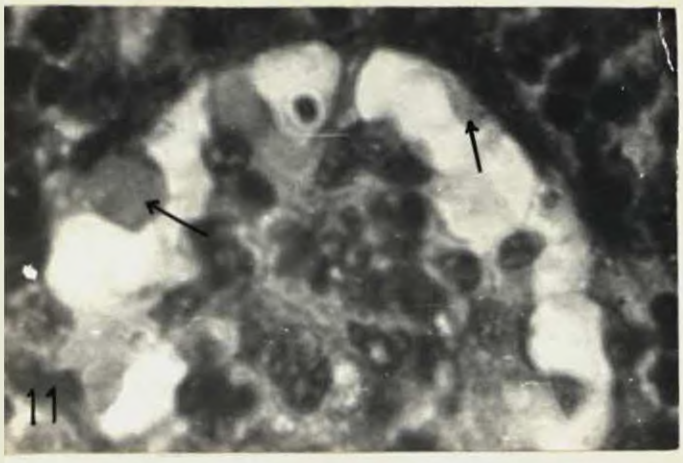
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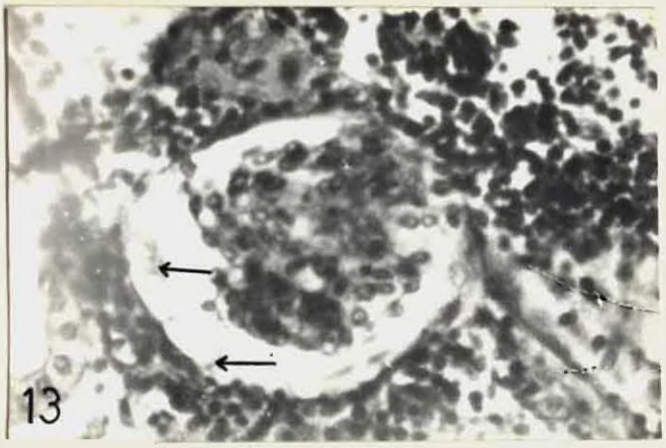
Fig. 13. Glomerulus showing increased cellularity, note also accumulation of exudate in Bowman's capsule (arrows). H&E X400

Fig. 14. Glomerulus showing sclerotic changes and adhesion (arrows) H&E X400

Fig. 15. Glomeruli showing the sclerotic changes, note also the thickening of Bowman's capsule. H&E X320

Fig. 16. Section of kidney showing necrosis of tubules. (arrows). H&E X320.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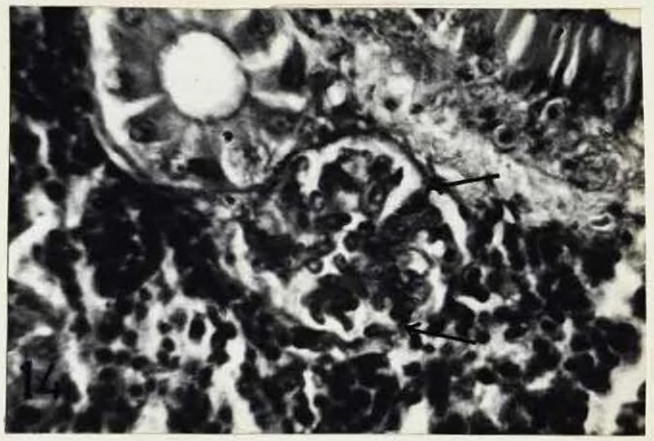
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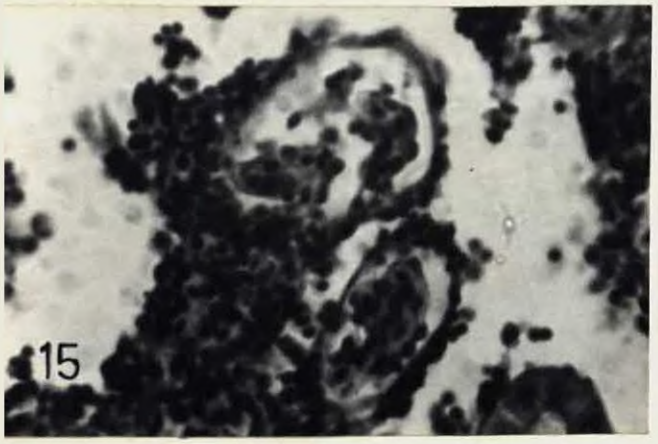
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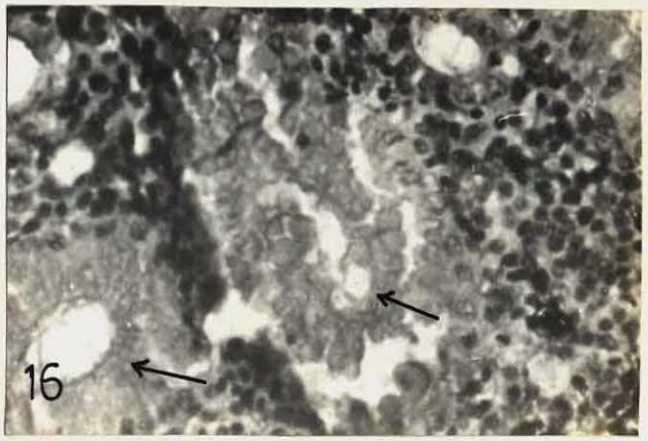
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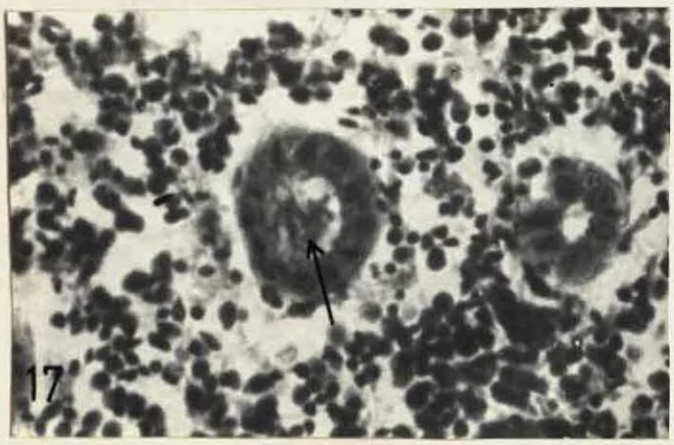
Fig. 17 Section of kidney depicting appearance of cast in tubule (arrow). H&E X320.

Fig. 18. Section of necrosed tubule showing presence of hyaline droplets (arrows). H&E X320.

Fig. 19 Cerebral cortex of brain showing the organizations of nervous tissues. H&E. X320.

Fig. 20 Rounding of Purkinje cell in cerebellum (arrow). H&E. X320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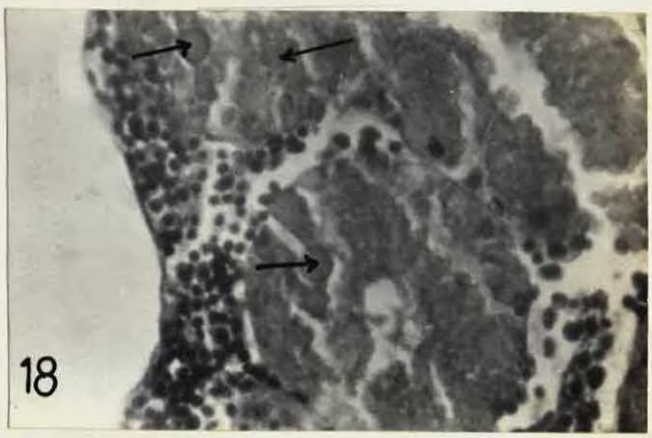
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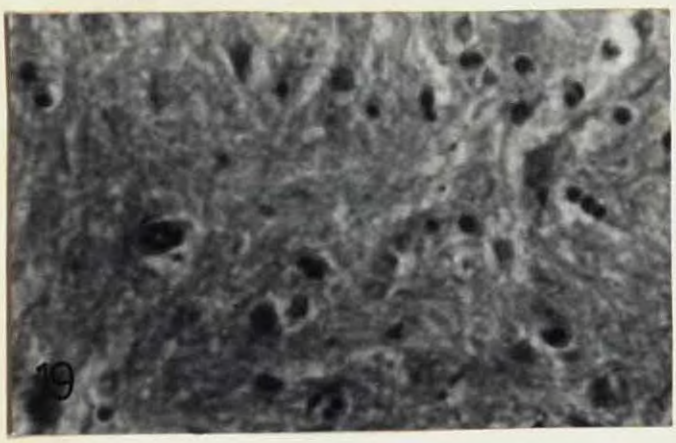
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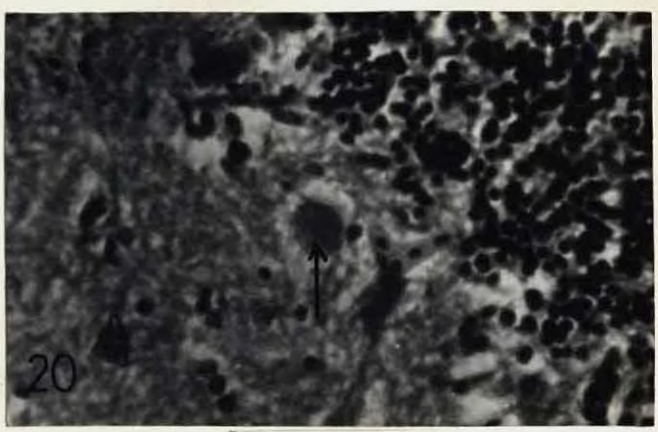
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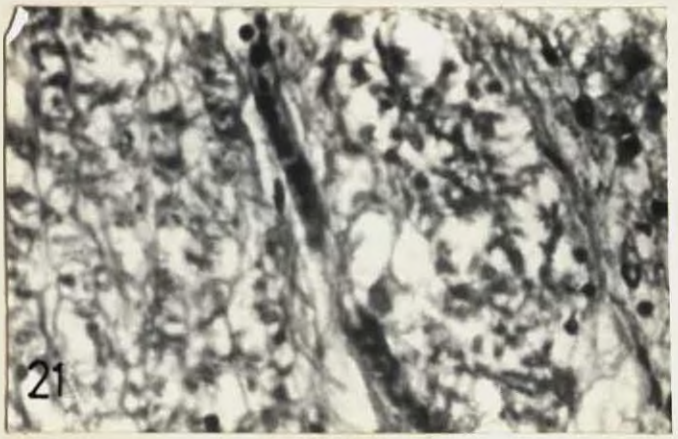
Fig. 21. Section showing area of malacia in part of white matter. H&E X320.

Fig. 22. Section depicting satellitosis of neurous (arrows) and proliferation of glial cells. H&E X320

Fig. 23. Section of brain showing satellitosis and starting of neuronophagia (arrow) and glial cell accumulation. H&E. X320

Fig. 24. Section of brain in which a neuron undergoing neuronophagia (arrow). H&E X320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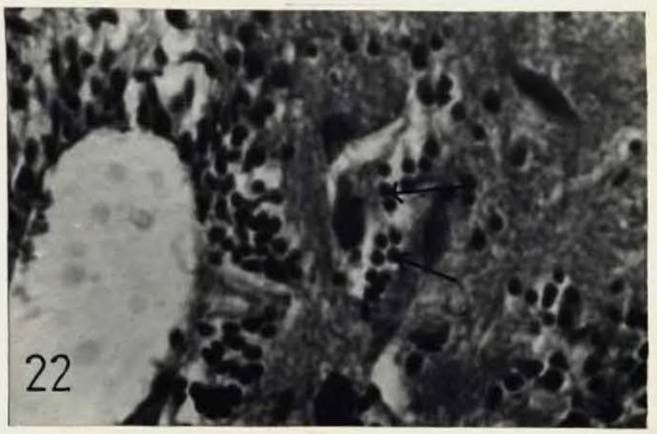
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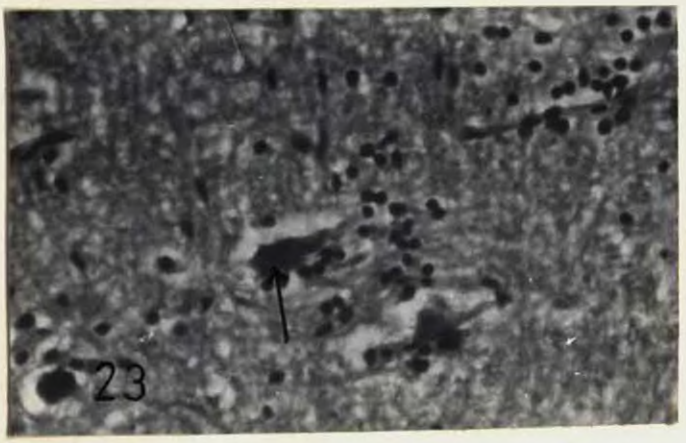
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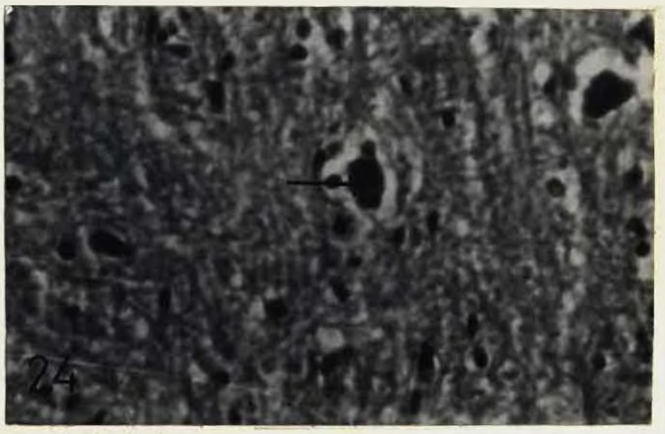
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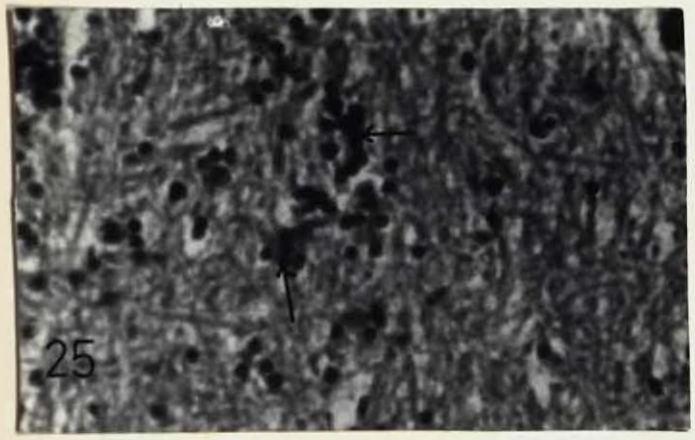
Fig. 25. Section of brain in which several neurous undergoing neuronophagia (arrows). H&E X320

Fig. 26. Brain section showing area of liquifactive necrosis invaded by neuroglial cells (arrows). H&E X320

Fig. 27. Brain section showing subependymal accumulation of round cells, also note hyalinisation of wall of blood vessel (arrow). H&E X320

Fig. 28. Muscle trabeculae of heart covered with endothelial cells from control fish. H&E X320.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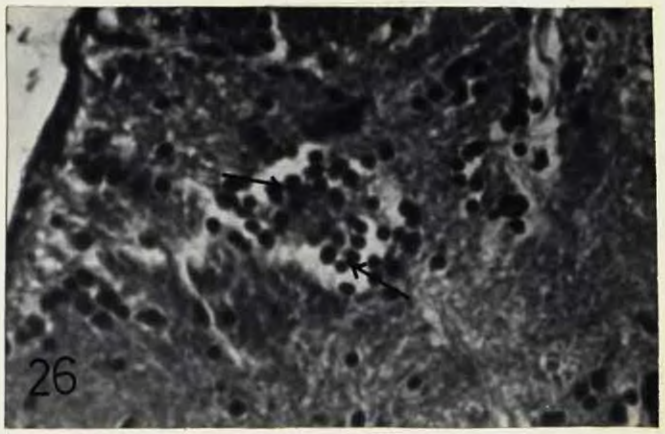
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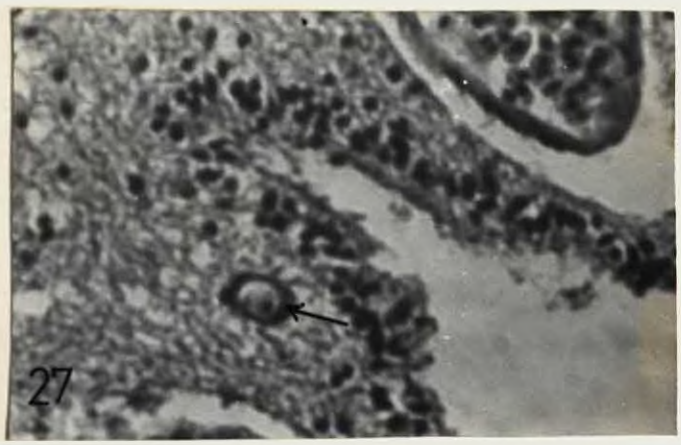
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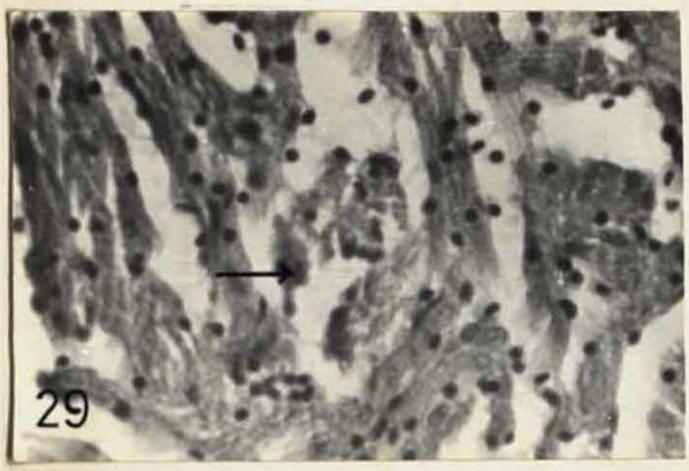
Fig. 29. Loss of striation and focal necrosis (arrow) of heart musculature from treated fish. H&E X320

Fig. 30. Focal accumulation of inflammatory cell in heart musculature (arrows). H&E X320

Fig. 31. Section of gill lamellae from control fish. H&E X320

Fig. 32. Gill, after exposure to lethal concentration; note the extensive necrotic changes of gill lamellae (arrows). H&E X80

Fig. 33. Section of necrosed gill lamellae (arrows). H&E X320.



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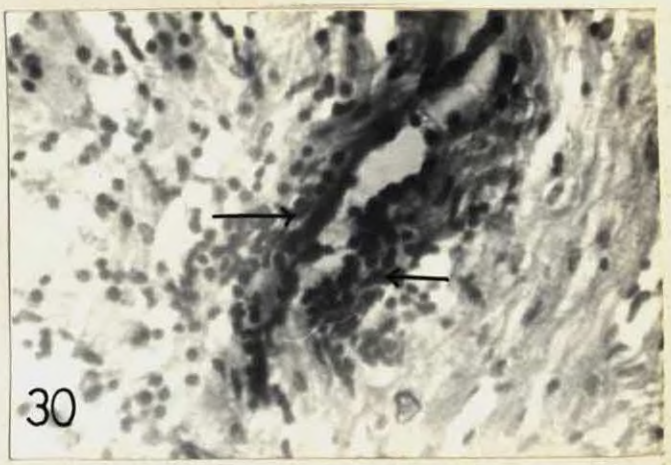

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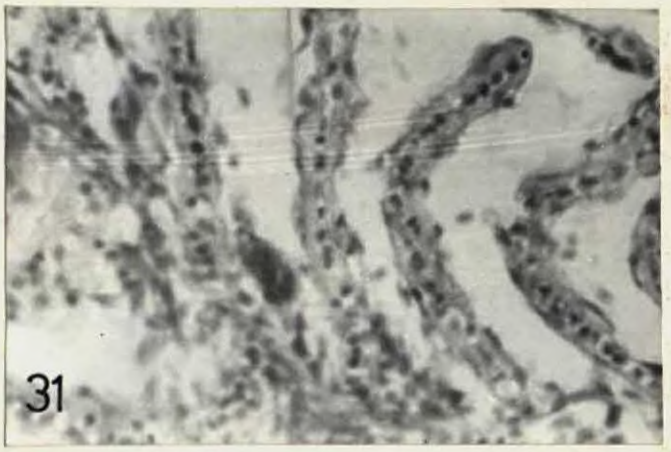

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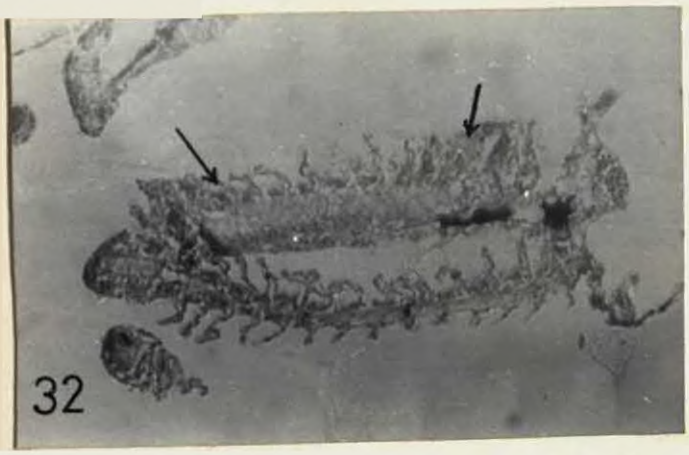

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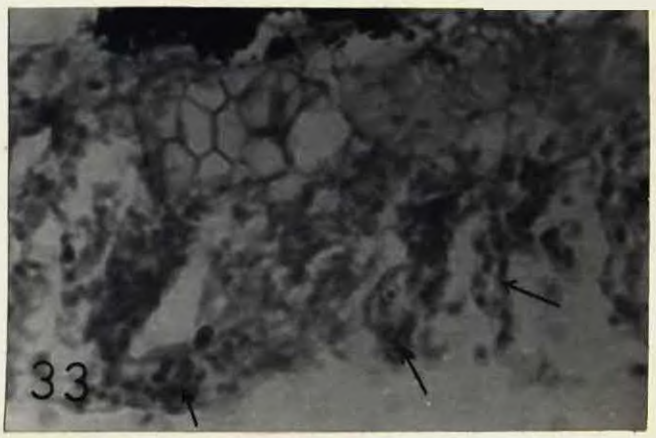

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DISCUSSION

The study involved estimation of approximate median lethal concentration (LC_{50}) value of zinc for milkfish (Chanos chanos) and histopathological alterations of tissues in different concentrations of zinc. It was felt necessary to estimate approximately LC_{50} for the test species in order to arrive at sublethal concentrations of zinc. Present study revealed the LC_{50} value was around 15 ppm for 72 hours at salinity 20 ppt, pH 7.2 and temperature 24 - 28°C. The exact median lethal concentration (LC_{50}) could not be arrived due to the non-availability of required size group of fishes during the period of study. Taylor (1981) suggested that 96 hour LC_{50} of zinc for marine fish was between 4 to 100 mg/l. Skidmore (1964) reviewed the variation of LC_{50} values, mainly for different species of freshwater fishes. However, Metlev et al,. (1971) was of the opinion that 15 ppm of zinc was toxic for all fishes within eight hours.

Histopathological studies:

Histopathological studies of group - i did not show any major alterations. The structure of liver was very similar to that described for normal fish liver by Varichak (1938), Ferguson (1974), Ellis et al., (1976), Hinton and Pool (1976) and Ellis et al., (1978). In group - ii, group - iii, group - iv and group - v the liver samples revealed vacuolation of hepatic cells from 10th day onwards. This vacuolation was present in all the treated groups throughout the experimental period and increased in severity in proportion to the time of exposure and dose. Crandall and

and Goodnight (1963) also reported the same type of changes in hepatic parenchyma by prolonged exposure of zinc in fishes. Kumar and Pant (1981), Sultan and Khan (1981), Bhattacharya et al.,(1985). Cruz and Tamse (1986), Razani et al.,(1986a), Waster and Canton (1986, 1987), Ansari and Kumar (1987), Jambulingam (1988) have also reported hepatic vacuolation in toxic condition associated with zinc, mercury, copper, cadmium etc. This study also supports the same observations. In the present study, the vacuolation was proved to be due to fat accumulation.

Liver suffers from lipid accumulation in a number of conditions like deficiency of ∝-Tocopherol, lipotropic factors, excessive fat in the diet and toxic damage to the liver which interfere with transport and metabolism of fat or protein synthesis in liver. Many of the toxins like antimony, arsenic, cadmium, carbon tetrachloride etc., were reported to have caused fatty liver in higher vertebrates (Runnells et al.,1965; Meiss et al., 1982; Jones and Hunt, 1983).

The heavy metals including zinc were reported to have effect on respiratory metabolism and protein synthesis of hepatic cells in fishes (Hiltibran, 1971; Shukla and Pande, 1986). However the exact mechanism of zinc interference with hepatic cellular function was not studied in this work. Hence from the present study, it is very difficult to arrive at the mechanism of zinc toxicity in the liver of milkfish.

Pyknotic nuclei, karyorrhexis and other changes indicating necrosis of liver cells were seen in all experimental groups except control. This changes became more apparent and severe as the concentration and period of exposure increased. It is believed that the injurious effect of

the toxin might have produced the same result. Severe necrotic changes were observed in the liver of fish exposed to lethal concentration of zinc by Kumar and Pant (1981). Necrosis of hepatocytes was a common finding in many of the studies involving heavy metal pollution and other toxic condition in fishes (Bhattacharya et al., 1985; Cruz and Tamse, 1986; Jambulingam, 1988).

Intravascular coagulation and perivascular accumulation of fibrinous material observed in this study, have also been reported by some workers like Di Michele and Taylor (1978). Fish generally has very high count of thrombocytes in blood; hence fish blood clot very rapidly (Ellis et al.,1978). A number of workers have reported vascular damage as well as poor development of blood vessels in the liver in zinc toxicity and other toxic conditions (Crandall and Goodnight, 1963; Ellis et al,1976). Increased permiability of vasculature and intravascular clotting were observed in the present study also. The changes probably may be due to the vascular damage which might have initiated the clotting and exudation of fibrin.

Generally, necrosis is accompanied by inflammatory reaction and accumulation of leucocyte in the periphery of those affected areas (Jones and Hunt, 1983). The leucocytic infiltration which was observed in present study may be due to an inflammatory response against the necrotic tissue.

In the control group, the structure of kidney was very similar to the euryhaline fish which was described by Ellis et al.,(1978). Occassionally some tubules showed degenerative changes and many glomeruli appeared congested. Since the changes were mild in nature, they were considered not very significant.

In group - ii, group - iii, group - iv considerable changes were observed in glomeruli and tubules. Glomerular changes consisted of increased permeability leading to accumulation of proteinaceous fluid in Bowman's capsule, thickening of capillaries, mesenchymal cell proliferation and sclerotic changes in glomeruli. Initially the changes in glomeruli were characterised by increased permeability indicating vascular damage to the glomerular capillaries. These glomerular changes were morphologically very similar to changes described for glomerulonephritis, in other vertebrates (Cassey et al.,1979; Slauson and Lewis,1979; George and Seshadri, 1983; Jones and Hant, 1983; George and Somvanshi, 1984). Glomerulonephritis was classified as membranous, acute proliferative, membrano-proliferative and chronic sclerosing glomerulonephritis. The mechanisms of glomerular injury in glomerulonephritis is generally unknown. However, it is believed that injury results from immunologically mediated inflammatory reaction at glomeruli. They include deposition of circulating antigen antibody complexes, autoimmune reactions and compliment activation (Heyman et al.,1959; Lewis et al., 1963, 1965; Cassey et al., 1979; Slauson and Lewis, 1979; and Jones and Hunt, 1983). Glomerulonephritis is a frequent condition observed in fishes during histological examination. However no proper study with reference to its etiology and pathogenesis has been done (Roberts, 1978). The changes like thickening of glomerular capillaries, hyalinisation of capillaries, dilation of glomerular capillaries, shrinkage of glomeruli and dilation of Bowman's capsule were also observed in experimental toxic studies on zinc, copper, cadmium, $KMnO_4$, β - hexachlorocyclohexane etc., in fishes including milkfish (Kumar and Pant, 1981; Saxena, 1981;

Cruz and Tames, 1986; Wester and Canton, 1986). The exact mechanisms involved in glomerular injury in the toxic conditions of fishes need further elucidation.

Tubules in group - ii, group - iii and group - iv had undergone degenerative and necrotic changes depending on concentration of toxin and period of exposure. These changes were more severe in proximal tubules and consisted of swelling, appearence of hyaline droplets and complete necrosis of epithelial cells. The tubular lumen contained hyaline casts. The tubular changes described above usually occur in toxic and anoxic conditions. A large number of chemical poisons like cadmium, copper, mercury, arsenic, bismuth, chromium, potassium, dichromate, etc., were reported to have produced same conditions in higher animals (Runnells et al., 1965, Jones and Hunt 1983). The hyaline droplets, which appeared in many tubular epithelial cells were reported to have occurred in kidneys where protein leakage through glomeruli occurred (Jones and Hunt 1983). In this case also glomeruli were damaged and Bowman's capsule contained the proteinaceous fluid. Tubular degeneration and necrotic conditions were common findings in many experimental studies involving chemical and insecticide toxins in different groups of fishes including Chanos chanos (Gardner and Yevich, 1970; Koyama et al.,1977; Kumar and Pant, 1981; Cruz and Tamse, 1986; Forlin et al., 1986). Findings in this study also support the same observations.

In group - v, kidney changes were of the nature of degeneration. Glomeruli did not reveal any significant change. These mild changes may be due to rapid death of the animals.

The brain of control group showed a few areas of neuronal degenerations. These changes were very mild. group - ii, group - iii, group - iv and group - v exhibited very severe changes. They consisted of neuronal changes like vacuolation, pyknosis and margination of nucleus, chromatolysis and in some cases hyperchromatosis of neurons were also noticed. In many cases satellitosis, neuronophagia, glial cells proliferation leading to glial nodule formation were observed. Brain substance revealed areas of liquefactive necrosis and Wallerian degeneration of nerve fibre tracts, Oedema of the choroid plexus with vascular changes were also observed.

Very limited information is available regarding the histological changes in brain of fishes in response to heavy metal toxicity. Many organic compounds had induced necrosis, haemorrhages and other deleterious effect on nervous tissue of fishes (Di Michele and Taylor, 1978; Van Leeuven et al., 1986; Tripathi et al.,1987). Lesions of olfactory organs were observed in copper toxicity (Gardner and La Roache, 1973). Some studies involving sensory organs and behavioral changes in relation to heavy metal toxicity in fishes indicated neuronal damage (Gardner, 1975). Ultra structure studies on brain cells from fish larvae exposed to toxic conditions exhibited extensive damage of endoplasmic reticulum, mitochondria and nuclear membrane (Cameron and Smith, 1980; Somasundaram et al.,1984).

There were reports from the studies of higher vertebrates that brain get affected by chemical toxins like mercury, lead etc., (Runnells et al.,1965 Jones and Hunt, 1983). The findings of this study also agree with the above observations. In many experimental fishes choroidplexus were oedematous. The capillaries and blood vessels were thickened and hyalinised. These blood

vessels appeared leaky. Such conditions were not reported form fishes.

Hepatopathy is known to produce brain lesions in higher vertebrates. The mechanism is believed to have operated through excessice production of ammonia (Hooper, 1972; Hooper et al., 1974). In this study also all treated animals had hepatopathy. Whether this has caused brain lesions could not be ascertained at present, because no study was undertaken about the metabolism and detoxifying functions of liver in milkfish.

The heart revealed the normal structure in group - i as described by Roberts (1978). Heart in the treatment groups revealed myofibrillar degeneration and necrosis with mononuclear cell accumulation. The histo-pathological studies on cardiac muscle in fishes is very scanty. A study by Crandall and Goodnight (1963) on zinc toxicity indicated accumulation of inflammatory cells in cardiac musculature. In mammals, focal myocardial necrosis is a common finding in toxicity studies (Jones and Hunt, 1983). The picture of this study also agrees with the above findings.

In group - i, gills revealed more or less normal architecture. Group - ii, group - iii, and group - iv did not reveal any alterations compared to group - i. This finding is in agreement with the view of Crandall and Goodnight (1963) and Skidmore (1964). However, Tuurala and Soivio (1982) did not agree with the view of the above cited authors. In group - v, extensive necrosis and oedema of lamellar epithelial layer was noticed. Same changes were observed by exposing the fishes to heavy metals including zinc and other toxic conditions (Skidmore and Tovell, 1972; Kumar and Pant, 1981; Sultan and Khan, 1981; Cruz and Tamse, 1986).

In the case of skeletal muscle, Zenker's necrosis was seen occassionally in muscle fibres of all the treated groups. Crandall and Goodnight (1963) reported the underdeveloped and vacuolated skeletal muscles in fishes after prolonged exposure to zinc. However, in fishes not much information is available regarding this aspect. In the case of spleen, eventhough many workers had reported several changes like alteration in size of melanomacrophage centre, necrosis, etc., no such changes were noticed in any of the experimental groups.

SUMMARY

1. Milkfish (Chanos chanos) were collected from Kerala Agriculture University Farm and Matsyafed Farm at Vypeen island were used for determining approximate LC_{50} value and histopathological studies for both lethal and sublethal concentration of zinc.

2. Median lethal concentration determined approximately was found to be 15 ppm zinc for 72 hours.

3. Histopathological studies were carried out on liver, kidney, brain, heart, gill, skeletal muscles and spleen.

4. The liver in all treatment groups (Lethal and various sublethal concentrations) revealed extensive vacuolation, necrosis, mononuclear cell accumulation, intravascular coagulation and perivascular fibrinous exudation.

5. The kidneys exhibited remarkable changes in glomeruli and in tubules. The glomerular changes were of the nature of increased capillary permiability, basement membrane thickening of capillaries, shrinkage of glomeruli, increased cellularity and sclerosis of capillary walls. The tubules generally showed degenerative changes leading to necrosis and accumulation of hyaline droplets in epithelial cells. The tubular lumen contained proteinaceous cast.

6. The brain in acute toxic condition revealed congestion of blood vessels, vacuolation of neurons and necrosis of neuron. In

sublethal levels, the changes were very significant. The neuronal degenerative changes like vacuolation, pyknosis of nucleus margination of nucleus, rounding of cells and chromatolysis were present in all sublethal and lethal groups. There were areas of liquifactive necrosis, satellitosis and neuronophagia. These changes varied in severity depending on the concentration of zinc in water and duration of treatment.

7. Cardiac muscles showed changes like loss of striations, granularity of cytoplasm and foci of necrosis. These changes were seen in the initial stages of experiment. But as the duration increased, the foci of necrosis were numerous and were infiltrated with mononuclear cells.

8. The gills of the group which were treated with sublethal amount of zinc did not reveal much significant alteration compared to the control. However, the gills of fishes in group which was treated with lethal amount of zinc revealed extensive oedema of the lamellae, necrosis and disquamation of epithelial layers of secondary lamellae.

9. The skeletal muscle of the body wall in group treated with lethal and sublethal amount of zinc showed foci of hyalinised muscle fibres indicating Zenker's type of necrosis.

10. Spleen from the treated groups did not reveal any significant changes.

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