EFFECT OF HEAVY METAL COPPER ON THE OVARIAN HISTOMORPHOLOGY OF FISH, CYPRINION WATSONI AFTER VARIOUS PERIODS OF EXPOSURE

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بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِيمِ

In The Name Of Allah The Most Beneficent & The Most Merciful

EFFECT OF HEAVY METAL COPPER ON THE OVARIAN HISTOMORPHOLOGY OF FISH, CYPRINION WATSONI AFTER VARIOUS PERIODS OF EXPOSURE

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Philosophy

In

BIOLOGY

(Reproductive Physiology)

By

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CERTIFICATE

This thesis, submitted by ***Mr. Muhammad Azam Zia*** is accepted in its present form by the Department of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Master of Philosophy in Biology (Reproductive Physiology).

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Dated: 15-10.2005

This research work with all respect and honor is dedicated to,

My Sweet and loving Parents and My younger Brother Muhammad Shahzad Akhtar.

CONTENTS

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Muhammad Azam Zia

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ABSTRACT

Copper is an essential micronutrient but its high concentration in water bodies is toxic to aquatic organisms. The present study was carried out to assess the effect of copper on the ovarian histomorpholgy of fish *Cyprinion watsoni*. The fish was exposed to constant dose of copper (0.08ppm) at different periods. Fish were taken out after 7, 14, 21 and 28 days of copper treatments and different parameters were recorded.

Fish length, body weight, ovarian weight, ovarian length and ovarian breadth affected ($P>0.05$) after 7, 14 and 21 days of copper treatment, but after 28 days of copper treatment fish length, body weight, ovarian weight, ovarian length and ovarian breadth significantly decreased ($P<0.05$) in treated group. Condition factor of fish remained unaffected ($P>0.05$) after 7, 14, 21 and 28 days of copper treatment. GSI of fish was not significantly decreased ($P>0.05$) after 7, 14 and 21 days of treatment but it was decreased significantly ($P<0.05$) after 28 days of copper treatment.

In treated group after 7 days, follicle numbers of stage I, II, III, IV and V were not affected ($P>0.05$) but numbers of atretic follicles were significantly increased ($P<0.05$). Follicle numbers of stage I and II were not affected ($P>0.05$) but numbers of follicular stage III, IV, V and atretic follicles were significantly affected ($P<0.05$) after 14 days of copper treatment. Except follicular stage I, number of all follicular stages (II, III, IV, V and atretic follicles) were significantly affected ($P<0.01$) after 21 days of copper treatment. After 28 days of treatment number of all follicular stage (I, II, III.IV, V and atretic follicle) were significantly affected ($P<0.01$).

Follicular diameter of stage III was significantly decreased ($P<0.05$) after 7 days of treatment. But after 14 days of copper treatment follicular diameter of all stages (I, II, III and V) except stage I were significantly decreased ($P<0.05$). Effect of copper was very drastic ($P<0.001$) on follicular diameter of all stages (I, II, III, IV and V) after 21 and 28 days of treatment.

Nuclear diameter of follicular stage I and IV were significantly decreased ($P<0.05$) after 7 days of treatment, while after 14 days of copper treatment nuclear diameter of all stages (I, II, III and IV) except stage V was significantly decreased ($P<0.001$). Nuclear diameter of all follicular stages was significantly decreased ($P<0.01$) after 21 and 28 days of copper treatment. Nucleoli number of follicular stages (II, III and IV) was significantly decreased ($P<0.01$) after 7 days of treatment. After 14 days of treatment nucleoli number of Follicular stages III, IV and V were significantly decreased ($P<0.001$). After 21 days of copper treatment nucleoli numbers of all follicular stages were significantly decreased ($P<0.05$). After 28 days of copper treatment nucleoli numbers of stages (I, II III and IV) except Stage V were significantly decreased ($P<0.001$).

As far as ovarian histology is concerned disintegration of developing granulosa, disintegration of nuclear membrane, increased atresia of yolked and non yolked follicles (stage III and stage IV follicles) was observed after 7, 14, 21 and 28 days of treatment, In stage IV and V very interesting and unique abnormalities, clumping of yolk globules, absence of non staining yolk vacuoles and is replaced by thick condensed striations of yolk material, separation of follicular layer from inner yolk material, decline in thickness of zona radiata and conversion of striations of zona radiata into black striations were noticed after 28 days of treatment.

INTRODUCTION

INTRODUCTION

Water is the most abundant of all compounds on earth, and is essential for the continued existence of life on this planet. It covers approximately 71% of the planet and is present in all living matter, at times comprising as much as 90% of the body's tissues. Of all available water on earth, only 0.25% can be classified as surface water. Over the years there has been an increased interest in the effects of human habitation and industrialization on water resources. With the earth's population increasing steadily every year, more pressures are being placed on this precious resource (Davis and Day, 1998).

Water, which in its three states has played and still plays an important role on the earth, is an element upon which all creatures that breed in it rely heavily for support. Water has static physical properties such as its specific heat, density and transparency, chemical properties such as its solubility and stability and dynamic characteristics, such as the lantic facies of still waters and lotic facies of flowing waters (Jacques, 1999)

Inland waters have a particular fascination for us. The expenses of lake water surrounded by the hills and unique habitat of corridors, through which stream and rivers flow towards the sea, evoke a sense of timeless and feeling of contentment. Although lakes (0.33%) and rivers (0.04%) contain only a very small proportion of earth's fresh water, they have played an important part in the development of our civilization. They provide necessary supply of drinking water, so that settlement develops along their banks. The fish is harvested for food but now the aquatic environment is showing signs, which are unwelcome by the aquatic organisms including fish. These signs are mostly the result of discharging of chemicals and wastes (Lolyd, 1992).

Freshwater ecosystems exhibit a high natural variability in their physical and chemical properties due to local differences in geology and climate. They are therefore more susceptible to anthropogenic influences than the more consistent and stable marine environments (Rainbow & Dallinger, 1993). Consequently, both the

quality and quantity of water are affected by an increase in anthropogenic activity. Any pollution, either physical or chemical, cause changes to the quality of the receiving waters. These changes may include increased dissolved nutrients which may result in eutrophication, changes in stream temperatures and bottom characteristics which lead to habitat destruction and alteration of species diversity, and the addition of toxic substances which can have either acute or chronic effects on aquatic organisms (Sanders, 1997).

Fish occupy a significant position in the socio-economic fabric of south Asian countries by providing the populations not only the nutritious food but also income and employment opportunities of the 21,723 fish species known to science, over 40 % live in the fresh waters and majority of them live in tropics between latitude 2 3 °5 N and 23 °5 S. Nowhere in the world is a geographic region so blessed as Indian subcontinent (India, Nepal, Burma, Sri Lanka and Bangladesh) in respect of the diversity of fish wild life that dwells the inland waters (Talwar and Jhingran, 1992).

Cyprinion watsoni belongs to cyprinide group found in the streams in hilly areas of Northern Pakistan and extends up to Afghanistan, Iran, some parts of Syria and eastern corner of Arabian Peninsula (Jaya Ram, 1981).

This is a small (maximum size 12 cm) cyprinid fish, which is commonly found in the hilly stream of Islamabad (33.3 N, 73.0 E). Where the surface water temperature ranges between 14°C in the coldest month and 29°C in the warmest months. The reproductive cycle of this species comprises of a spawning season between March and May (Spring to early Summer) followed by post spawning period extending between June and August. This is followed by a quiescent period lasting from September to November. Gonadal recrudescence begins in December (preparatory period) when the gonads show first signs of proliferative activity. The fish enter a pre-spawning phase during January to March when gametogenic progress becomes pronounced and the fish ultimately reach the final stage of preparedness to start spawning in March/April and May/June (Shaikh and Jalali, 1986).

The ovaries of many adults' teleosts are paired, although fusion of ovarian tissues occurs in some species (Franchi, 1962). Many teleosts ovaries are of the cytovarian type (valming, 1972) and consist of a cortex derived from epithelial elements; no medullary tissue is present (Barr, 1965). The ovary has a central cavity lined by germinal (Ovarian folds) transverse the ovarian tissue and projects to this cavity; these lamellae are lined by germinal epithelium and contain "cell nests" of oogonia. Ovarian follicles developed along the lamellae, and oocytes are ovulated into the ovarian cavity. Externally, many teleosts ovaries are covered by a muscular sac (Valming, 1972).

In many single brooded species with a short breeding season, synchronous development of a crop of vitellogenic follicles occur, and these follicles are accompanied by synchronously developing crops (one or more) of small pre-vitellogenic follicles (Valming, 1972). Most studies of teleosts ovaries deal with species sowing synchronous development of a crop of vitellogenic follicles from a synchronously developing crop of previtellogenic follicles. This recruitment occurs once a year, few times a year or continuously. In these species, pre-vitellogenic follicles are produced from germinal tissue and serve as a pool from which vitellogenic follicles are derived. Atresia of growing follicles is common; thus the larger the size of follicles, the less their number. In some species, Clarias batrachus the smaller follicles may be of two size classes (Lehri, 1968).

Toxicity is the study of poisons. It includes identification of poisons, their chemical properties and their biological effects as well as the treatment of the disease condition that they cause. Toxicologists deal with many different chemicals, feed additives, environmental contaminants, heavy metals and natural toxins of plants and animal origin which may adversely affect the health of animal (Gary, 1996).

Within the last three decades studies in fish biology and ecology have made rapid advances. In recent years, a growing social demand has lead to an increase in studies on pollution in its various forms and on its impact on aquatic ecosystem. Interrelations between fish and its environment are so complex that answers are seldom unequivocal and it becomes necessary to organize regular contacts between

specialists in various fields. Apart from its commercial or heritage value, "fish" is also at the heart of issue because it is very sensitive to environmental factors and because of its high biodiversity level. Fish is a valuable bio-indicator when describing habitats and monitoring changes within them (Purnet *et al*, 1988).

Water pollution has focused the attention of both the scientific community and the public on environmental problems. Not only does water pollution affect the health and welfare of people and organisms, but it also damages vegetation (Sehgal and Saxena, 1986). Amongst the pollutants contaminating water bodies, metals play an important role (Witeska *et al.*, 1995). According to Mason (1991), the toxic pollutants known to man, metals arising from industrial processes and some agricultural applications (Lead, Copper, Nickel and Zinc). The random discharge of various wastes, pollute most ecosystems and affect survival and physiological activities of organisms in these systems (James *et al.*, 1998). In natural waters, metal ions occur as free aqueous ions, complexes with organic/inorganic ligands or sorbed onto the surfaces of particles (Brezonik *et al.*, 1991), and most metals are taken up in the ionic form (Kotze *et al.*, 1999). Free metal ions cause more serious damaging effects on aquatic organisms than their more complex forms (James *et al.*, 1998). The duration of exposure of a specific concentration of toxin can influence whether it will kill an aquatic organism (Skidmore, 1964).

The health of fish may be affected, either directly through uptake from the water, or indirectly through their diet of vegetation, invertebrates or smaller fish (Kime *et al.*, 1996). Metals released into aquatic ecosystems, are responsible for several fish physiology irregularities (Sehgal and Saxena, 1986). They can also disturb the ionoregulatory mechanism in aquatic organisms (Hansen *et al.*, 1996). All of these effects of heavy metals usually affect fish negatively leading to stress and eventually, in most cases, death.

Heavy metals are continuously released into the aquatic environment from natural resources such as volcanic activity or weathering of rocks. Moreover, industrial processes and some agricultural uses (e.g. $CuSO_4$ is used to control aquatic vegetation) have greatly increased the mobilization of many metals in fresh water (Dunnick and Fowler, 1988). Therefore, in recent years concern has increased

over heavy metal pollution. Heavy metals include both essential elements (Mn^{++}, Zn^{++}, Cu^{++} etc) and metals with no known biological functions such as Cd^{++}, Hg^{++}, Ag^{+} and Sn^{++}. These metals are potentially harmful to most of the organisms at some level of exposure and absorption. Some aquatic species can regulate the body level of essential metals such as copper and zinc at constant levels but the regulation is mainly achieved by the rate of metal excretion being increased to match the rate of the metal uptake. However the body concentrations of nonessential metals such as cadmium and mercury are not regulated and it is accumulated in proportion to dissolved cadmium concentrations (Rainbow and White, 1989; Amiard *et al,* 1987; Cuvin-Aralar, 1994). It is also known that final body concentrations of a metal are dependent on accumulation strategy of the species for that metal (Rainbow and White, 1990). Aquatic organisms have been widely used to assess environmental pollution because of their ecological and economic importance and their morphological, physiological and ecological diversity in aquatic habitats (Williams and Dusenbery, 1990). Many studies have been carried out on the effect of environmental pollutants on various aquatic organisms, and on biological assessment of water quality using certain indicator organisms (Sheedy *et al,* 1991). Most of toxicity tests have been concerned with measure of acute lethality. The results are expressed as a concentration or dose of toxicant at which a specific percentage (e.g. LC_{50}) of the test organism are killed over a standard period of time (e.g. 24, 48, 72 or 96 hours) (Mason, 1991; Green *et al,* 1988; Migliore and Giudici, 1990; Handy, 1994).

Heavy metals induce changes in histology, metabolism, biochemistry, and physiology; inhibit synthesis of proteins and nucleic acids (Choz, 1983; Mur and Ramamurti, 1987; Diuga and Penni, 1989; wicklund *et al,* 1992; Wilson and Taylor, 1993). Heavy metals are known to induce genetic alterations and teratogenesis (Weis and Weis, 1977; Birge *et al,* 1983; Dawson *et al,* 1988; Alsabti, 1994; DeYoung *et al,* 1996; Poongothai *et al,* 1996). It is known from field and laboratory studies that fish reproduction is one of the most sensitive target sites to heavy metals and other aquatic contaminants (Bilard *et al,* 1981; Donaldson and Scherer, 1983; Donaldson, 1990; Tracy *et al,* 1992).

Reproductive toxicity is defined as adverse effect on male and female reproductive system that results from exposure to chemical substances. Reproductive toxicity may be expressed as alteration in sexual behavior, decrease in fertility or loss of fetus during pregnancy. A reproductive toxicant may interfere with sexual functioning or reproductive ability if exposed individuals from puberty throughout adulthood. (Vose *etal*. 2000).

Such toxicants may affect virtually all phases of reproduction, including embryonic development, egg hatchability, growth and survival of alevins and juveniles. The effect of heavy metals and other chemicals on fish reproduction can be segregated according to a specific action on the ontogenetic stage (Eaton *et al., 1987).* Copper is an essential trace element to plants, animals and even humans (DWAF, 1996). It is in fact a required element for all living organisms, and although the absolute concentration of copper is usually low in nature, it occurs in adequate quantities for growth in all aquatic environments (Steemann Nielsen *et al.*, 1970). In animals, copper is important for bone formation, maintenance of myelin within the nervous system, synthesis of haemoglobin, a component of key metallo enzymes, plus it forms an important part of cytochrome oxidase and assorted other enzymes involved in redox reactions in cells (Sorensen, 1991; Dallas and Day, 1993). It is essential for cellular metabolism, where its concentration is well regulated (Pelgrom *et al.*, 1995a) but becomes toxic at elevated levels. The liver is an important storage organ of copper in mammals and most fish (Pelgrom *et al.*, 1995b). Copper compounds are used for prophylactic purposes to control fish diseases and parasites (Moore *et al.*, 1984). Copper compounds are also used to control algae, kill slugs and snails in irrigation water systems and municipal water treatment systems. The United States Environmental Protection Agency (U.S.E.P.A.) classifies copper sulphate as a pesticide. Although copper is important, it is toxic when concentrations exceed that of natural concentrations (<0.05mol/L) (Stouthart *et al.*, 1996). At concentrations even found in natural waters, the ionic form of copper is very poisonous towards photosynthesis and growth of unicellular algae (Steemann Nielsen *et al.*, 1970). Copper is one of the world's most widely used metals (DWAF, 1996), with the electrical industry probably making use of it

the most. It reaches aquatic systems through anthropogenic sources such as industrial, mining, plating operations, usage of copper salts to control aquatic vegetation or influxes of copper containing fertilizers (Nussey, 1998). Copper concentrations in locations receiving anthropogenic inputs such as mine tailing discharges can vary anywhere from natural background to 100 µg/L (Hem 1989; Lopez and Lee 1977) and have in some cases been reported in the 200,000 µg/L range in mining areas (Robins et al. 1997). Mining, leather and leather products, fabricated metal products, and electric equipment are a few of the industries with copper-bearing discharges that contribute to anthropogenic inputs of copper to surface waters (Patterson et al. 1998). Copper, a common toxin in water, has an unclear mode of action on aquatic organisms, but toxicity is largely attributable to Cu2+ (EIFAC, 1978), that forms complexes with other ions (Nussey, 1998). Changes in the amount of free Cu2+ in solution will affect the amount of copper that is bioavailability and hence its toxicity (Welsh *et al.*, 1993). A reduction in water dissolved oxygen, hardness, temperature, pH, and chelating agents can increase the toxicity Cu2+ (Nussey, 1998). Organic and inorganic substances can easily complex the cupric form of copper, which is the most common speciation of this metal, and it's then adsorbed on to particulate matter. Therefore, the free ion is rarely found except in pure acidic soft water (EIFAC, 1978). The chemical speciation of copper strongly depends on the pH of water (Stouthart *et al.*, 1996). Copper, in water, precipitates at high pH (alkaline) and is thus not toxic, whilst at low pH (acidic) it is mobile, soluble and toxic (Nussey, 1998). The main difference in copper toxicity between mammals and fish concerns environmental uptake, occurring almost exclusively through the gills in fish. This organ is the principal site of toxic insult and important in the start of compensatory responses (Pelgrom *et al.*, 1995b).

Effects of copper on fish

In polluted water copper is often present with other heavy metals. Toxic effect of copper on fish is also influenced by the chemical nature of water, such as calcium hardness (Tabata, 1969; Albaster and Lloyd, 1982), temperature and pH (Laws, 1981). Copper can affect life of fish when copper sulphate is used as algicide

and molluscicide in fish ponds (Lloyd, 1992). Shah *et al. (1995)* have shown the effect of copper sulphate on feeding activity of fish has also been demonstrated. Copper's toxic effects in fish include; changes in biochemistry, anatomy, physiology, histology and behaviour. The lowest treatment of copper (0.03 mg/L) caused little change in fish behavior, which may be the avoidance behavior of animals to pollutants in the receiving water. A significant response to 0.06 mg Cu/L was noted: the swimming activity and breathing rate of the fish increased. In the highest treatment (0.12 mg Cu/L) fish became lethargic and lost equilibrium. The lowest treatment of copper (0.03 mg/L) did not cause any significant change in fish behavior and the highest treatments (0.12 mg Cu/L) caused lethargic conditions and loss of equilibrium in exposed animals (Shah, 2002).

Observations on rainbow trout also exposed to zinc pointed to vertebral damage, but no histological evidence was presented (Bengtsson, 1974b). According to Lewis and Lewis (1971) damage to the gill and head area of fish, could probably cause mucous to accumulate on the gill area. This could then lead to respiratory problems, which in turn affects the fish even more negatively resulting in stress and eventually death. A decrease in heart rate (bradycardia), ventilation increases and anaemia may occur, whilst the locomotors activity increases, although glycogen content of liver and muscle is reduced (Heath, 1987). Copper exposures cause reduced growth, often with impacts to specific growth rates most evident during initial exposure times (Marr *et al.*, 1996). It also interferes with bronchial ion transport and affects various blood parameters such as plasma ion concentrations, hematological parameters, and enzyme activities in blood and liver (Stagg and Shuttleworth, 1982). It may also cause immunosuppression, vertebral deformities and neurological disorders (Stouthart *et al.*, 1996).

The toxicological aspects of the metallic contamination of aquatic environment in relation to its icthyofauna have been well documented. It has been shown that the sublethal concentrations of heavy metals that do not affect the survival and growth of fish over a given period are capable of impairing reproduction (Brungs, 1969; Sephar, 1976). However very little information is

available regarding the effects of metallic pollutants on the gonads of fish (Gardner and Yevich, 1970; Tafanelli and Summerfelt, 1975).

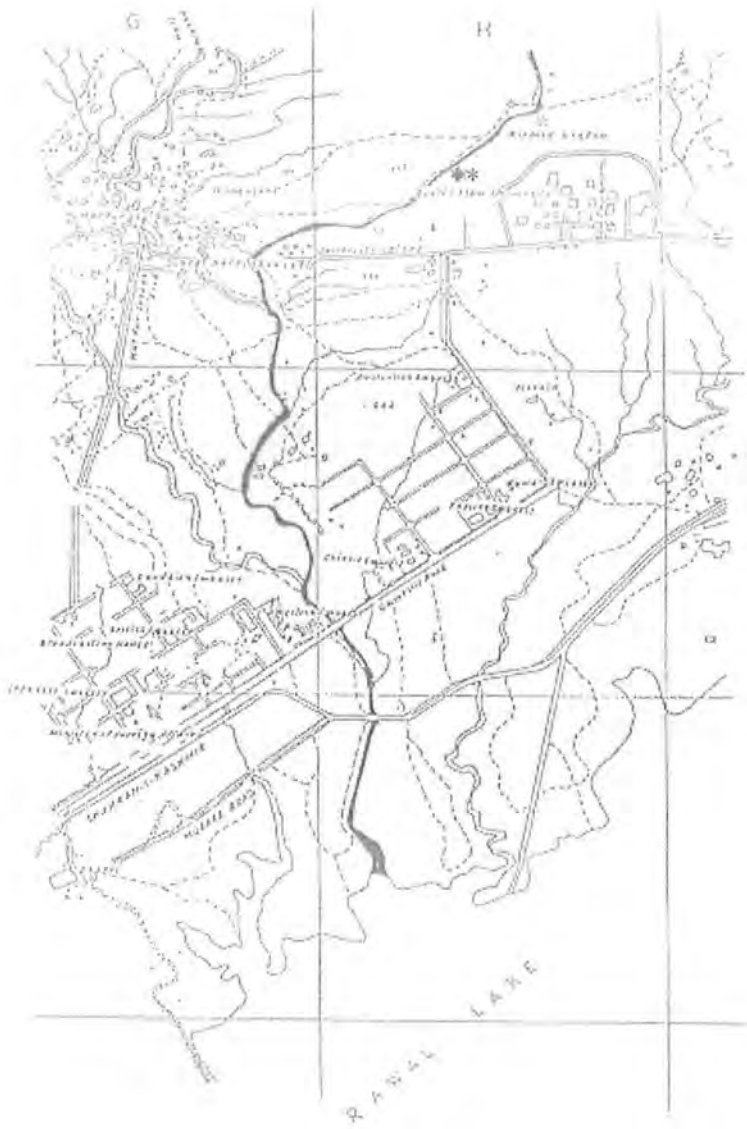
Copper has been shown to affect swimming performance growth, and reproductive success in a variety of teleosts (Stagg and Shuttleworth, 1982). Handy *et al.* (1999) noted that copper affected routine swimming activity of rainbow trout. It is possible that locomotors activity of contaminated fish was reduced as a type of metabolic 'sparing effect' to enable copper detoxification without associated feeding efficiency and growth rate parameter reductions.

Copper adversely affects fry survival, fry growth and reproduction of blunt nose minnows, *Pimephales notatus,* according to Horning and Neiheisel (1979). They found that the size of adult fish was affected negatively at higher concentrations, with lower concentrations resulting in increased size. The copper was also found to affect sexual maturation in males with effects such as immaturity and a reduction in spermatozoa in the testes, with mature males full of motile sperm. Eggs present in females were either poorly developed, or in the process of being resorbed in exposure ranges of 0.018 to 0.119 mg/L. Copper (Cu) interfered with spermatogenesis temporarily. Copper induced significant atresia in the ovary. Copper was more effective on relatively older oocyte (Kumar and Pant, 1984).

Mount (1968) discovered that copper also delayed sexual development and growth in the fathead minnow *P. promelas.* Marr *et al.* (1996) observed significant accumulation of copper in rainbow trout fry, and this accumulation was both dose- and time-dependent.

Main objective of the present study was to investigate the potential effects of exposure to heavy metal copper on several reproductive parameters with the ovary considered as the most probable target organ of fish (*Cyprinion watsoni*), keeping in view the follicular development at various stages, follicle numbers, atresia, gonadosomatic index (GSI), and condition factor (K).

MATERIALS AND METHODS



Part map of Islamabad showing Ramly stream (solid line) and sampling site **

MATERIAL AND METHOD

Collection of Fish specimens:

Live specimens of *Cyprinion watsoni* were collected with cast nets from Ramly stream in the start of July 2004. The size of fish used for present study ranged from 6-9 cm. The fish were transported to the experimental fish laboratory of Department of Biological Sciences, Quaid-i-Azam University Islamabad and kept in stocking glass aquaria total capacity 90 litres containing 70 litres of water. They were allowed to acclimate to the ambient environmental conditions for at least two weeks prior to the start of experiment.

The fish were fed daily on tropical fish food and were maintained in a photoperiod of 12 light and 12 dark using fluorescent tube light and automatic timer clock placed 10 inches above the water surface. The water was changed after alternate day. Experiment was performed at room temperature.

Preparation of copper solution, route of administration and dose:

The desired concentrations of copper were achieved using copper sulphate ($CuSO_4$. $5H_2O$). The copper sulphate for research purpose was taken from store of Department of Biological sciences, Quaid-i-Azam University Islamabad. 0.08ppm (parts per million) concentration of copper was selected. Copper solution was administrated through water of aquaria by dissolving calculated amount of the solution in the water of aquaria .Two aquaria were administrated with 0.08ppm copper and other two aquaria were controlled.

Experimental Design:

Before the start of experiment, placing them in Petri dish containing ice flakes stunned the fish. The fish lengths were then measured from the tip of the snout to the tail with the help of vernier caliper. The fish were than weighed on Metter, s balance to the nearest mg were grouped in different experimental aquaria. Before treatment, the fish having length 6-9 cm were divided into four groups (n=24 each). Two groups were maintained in separate glass aquaria as a control group (n=48) and two groups were maintained in separate glass aquaria as treated group (n=48). The desired

concentrations of copper were achieved using copper sulphate ($CuSO_4$. $5H_2O$). The aquaria were cleaned and the test concentration restored after every alternate day. Length of experiment was 28 days.

Dissection:

After 7 days of copper treatment, fish were stunned by ice flakes, body weight were measured to nearest grams, body length in centimeters. Ovaries of fish were also dissected out, weighed to nearest mg and their length and breadth in (cm) was measured. Left ovary was frozen immediately at -20° C, while the right ovary was immersed in fixative sera. Same process was repeated after 14, 21 and 28 days of treatment respectively.

In the present study following parameters were studies.

- Gonadosomatic index (GSI)
- Condition factor (K)
- Number of developing follicles.
- Follicular diameter of developing oocyte.
- Nuclear diameter of developing oocyte.
- Number of nucleoli in the developing oocyte.
- Number of atretic follicles

Histology and Cytometry:

Two stains were used in staining slides Hematoxylin and Eosin, the procedure to prepare these stains as follows:

Ehrlich's Alum Hematoxylin:

Hematoxylin	6g	dye
Ethanol 95%	300 ml	solvent
Potassium Alum	50 g (excess)	mordant
Distilled water	300 ml	solvent
Glycerol	300 ml	stabilizer
Glacial Acetic Acid	30 ml	acidifier

Procedure:

Hematoxylin was dissolved in ethanol and mixed with acetic acid. Alum was dissolved in water and mixed with glycerol in an oversized container. Hematoxylin solution was added to alum solution. Container was plugged loosely with cotton wool. Then ripened it by leaving in a warm, sunlit place for several weeks. When sufficiently ripened, stored it in a cool, dark place. The solution is stable for years. The solution may be chemically ripened by adding 0.5g sodium iodate, but chemically ripened solutions are inferior in longevity.

Eosin Preparation:

Ethanol 70%	100 ml
Eosin	1g

Take 100 ml of ethanol (90%) and add 1g Eosin dye in it and mix thoroughly and than use to stain the slides. The process followed during histology is mentioned below. The ovaries fixed in fixative sera for 4-5 hours before further processing. The composition of sera is:

Absolute Alcohol = 60 ml
Formaldehyde =30 ml
Glacial acetic Acid =10 ml

After fixation ovaries were dehydrated in ascending grades of alcohol in a following manner.

80 %	over night
90 %	for 2 hours
100 %	for 5 hours

The ovaries were then transferred to cedar wood oil and left in it until they became transparent. The ovaries were embedded in paraplast by the following method.

Benzol I	=	10 minutes (At room temperature)
Benzol II	=	10 minutes (At room temperature)
Benzol +Paraplast	=	20 minutes at 60 °C

Paraplast I	=	12 hours at 60 °C
Paraplast II	=	12 hours at 60 °C
Paraplast III	=	12 hours at 60 °C

After this process ovaries were ready to make blocks. The sections were cut out of paraffin block at the thickness of 6µm by using Reichert Microtome. Sections were affixed to pre cleaned albumenized glass slides and stretched at 60 °C on Fisher slide warmer and then transfered to paraffin oven for 12 hours for complete deparafinizing. The slides were then transferred to xylene for half an hour to remove any remaining wax. The slides were then dehydrated in the descending grades of alcohol, washed in tap water and stained in haematoxylin and dehydrated in the ascending grades and counter stained with eosin. The slides were hydrated, dehydrated in the following way:

Xylene I	=	15 minutes
Xylene II	=	15 minutes
100 % alcohol	=	2-5 minutes
90 % Alcohol	=	2-5 minutes
70 % Alcohol	=	2-5 minutes
50 % Alcohol	=	2-5 minutes
30 % Alcohol	=	2-5 minutes
Tap water	=	2-3 dips
Hematoxylin	=	2-3 dips
Tap water	=	10 minutes
30 % Alcohol	=	2-5 minutes
50 %Alcohol	=	2-5 minute
70% Alcohol	=	2-5 minutes
90 % Alcohol	=	2-5 minutes
Eosin	=	2-3 dips
90% Alcohol	=	1 dip
100 %Alcohol=	=	3-5 minutes
Xylene	=	10 minutes

Now the slides were mounted with Canada balsam. Microscopic examination of slides was carried out under a Nikon optiphot research microscope equipped with an automatic microphotographic system.

Number of follicles in selected sections of each group was counted and categorized. Staging of developing oocyte was done. Measurement of oocyte diameter and nuclear diameter was made by precalibrated ocular microscope in order to obtain their mean size and mean nuclear diameter. The number of nucleoli in each category of follicles was also counted to observe the effect of copper at this level.

Histological details and morphometric data, in combination with macroscopic features of the ovaries were used to determine any change caused by the toxicant in treated groups with reference to control group.

Gonadosomatic Index:

Record of body weight and ovarian weight were used to determine gonadosomatic index (GSI), which was calculated according to the formula

$$GSI = \frac{\text{Weight of the gonad (g)}}{\text{Weight of the fish (g)}} \times 100$$

Condition Factor (K):

Condition factor was calculated according to formula.

$$\text{Condition Factor} = \frac{\text{Body weight in (g)}}{\text{Length (cm)}^3}$$

RESULTS

RESULTS

Behaviour and Mortality:

During the exposure period, behavioural changes were recorded in fish. After addition of copper sulphate solution the first observation was uneasiness of fish and they moved towards the bottom of the aquarium, moving very fast in circular motion. After one week, increased mucus production was observed as indicated by slimy surface of water in the aquarium. Occasional jumping and hitting the wall of aquarium also observed during experiment. Mortality was also recorded at 27^{th} day of the experiment. No change in the behaviour of control fish was observed.

Gross morphology of ovaries:

The ovary in *Cyprinion watsoni* is paired, elongated organ situated in the dorsal region of the coelom, ventrolateral to the swim bladder. Each ovary is suspended in the body cavity along its dorsal side and surrounded by a peritoneal membrane. The size and colour of the ovaries varies according to the development. The ovaries of inactive fish are thin, transparent and of grey colour, while those of active individual occupy most of the abdominal cavity are large, light yellow in the beginning and dark yellow at the peak of the breeding season.

Effect of copper on body weight:

Group I; Day 7:

Body weight of control fish was 8.07±0.77 g. Body weight of fish exposed to copper was 8.87±0.26 g. Body weight of treated fish showed no significant difference ($P>0.05$) as compared to control (Table.1 and Figure.1)

Group II; Day 14:

Mean ± SE values of body weight in control and treated fish were given in Table.1 and Figure.1. There was no significant difference ($P>0.05$) in body weight of control (9.72±0.52 g) and treated fish (9.36±0.48 g).

Group III; Day 21:

Body weight of control (9.38±1.29 g) and treated fish (8.50±1.24 g) showed no significant difference ($P>0.05$) after 21 days of treatment. (Table.1, Figure.1).

Group IV; Day 28:

After 28 days body weight of treated fish (6.71±1.28 g) showed significant decrease ($P<0.05$) as compared to body weight of control fish (11.23±0.35 g). Mean ± SE values of body weight of control and treated fish are shown in Table.1 and Figure.1.

Effect of copper on standard length:

Group I; Day 7:

When the standard body length of both control and treated fish were compared, there was no significant difference ($P>0.05$). Standard body length of control and treated fish were 7.83±0.45 cm and 8.11±0.24 cm (Table.1, Fig.1) respectively.

Group II; Day 14:

Exposure of copper to fish showed no significant difference ($P>0.05$) in standard body length of control fish, compared to that of treated fish. Standard body length of control and treated fish was 7.76±0.30 cm and 7.40±0.27 cm respectively. (Table.1.Figure.1).

Group II; Day 21:

There was no significant difference ($P>0.05$) in standard body length of control fish, which was 7.97±0.51 cm and body length of fish exposed to copper, which was 7.90±0.41 cm. (Table.1, Figure.1)

Group II; Day 28:

Standard body length of control fish was 8.72±0.11 cm and standard body length of fish exposed to copper was 7.01±0.46 cm. Standard body length of treated fish showed significant difference ($P<0.05$) as compared to control. (Table.1, Fig.1).

Effect of copper on ovarian weight:

Group I; Day 7:

Mean ±SE values of right and left ovaries as well as total ovarian weight are given in Table 1 and Figure.2. When weight of right ovary (176.7±29.44 mg), left

ovary (167.32±32.53 mg) and total ovarian weight (344.02±61.81mg) of control fish were compared with weight of right (168.85±39.79 mg), left (157.35±38.65 mg) and total ovarian weight (326.175±78.43 mg) of treated fish, there was no significant difference (P>0.05).

Group II; Day 14:

There was no significant difference (P>0.05) in the weight of right ovary (137.2±14.32 mg), left ovary (136.27±13.58 mg) and total ovarian weight of control fish (273.47±27.85 mg), when compared with the weight of right ovary (136.72±13.18 mg), left ovary (135.6±13.1) and total ovarian weight (272.32±26.32 mg) of fish exposed to copper. Mean ±SE values are given in Table.1 and Figure .2

Group III; Day 21:

Mean ±SE values of ovarian (right, left and total) are given in Table.1 and Figure.2. After treatment weight of right ovary (87.16±8.08 mg), left ovary (86.03±6.12 mg) and total ovarian weight (173.2±14.06 mg) of control fish showed no significant difference (P>0.05), when compared with weight of right ovary (73.87±10.28 mg), left ovary (73.27±13.27 mg) and total ovarian weight (147.5±23.44 mg) of fish exposed to copper.

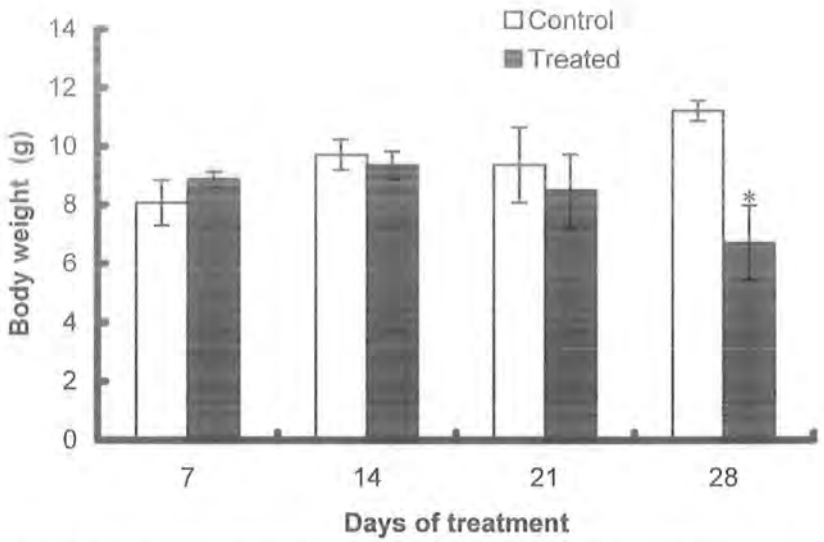
Group IV; Day 28:

Total ovarian weight of control and treated fish was 498.67±87.90 and 133.2±27.43 mg respectively (Table.1, Fig.2). There was significant difference (P<0.01) in the total ovarian weight of control compared to treated fish. Weight of right and left ovary (250.3±41.77 mg and 248.37±46.15 mg respectively) of control fish showed significant difference (P<0.01) as compared to weight of right ovary (65.25 ± 14.26 mg) and weight of left ovary (67.95±13.22 mg) of treated fish.

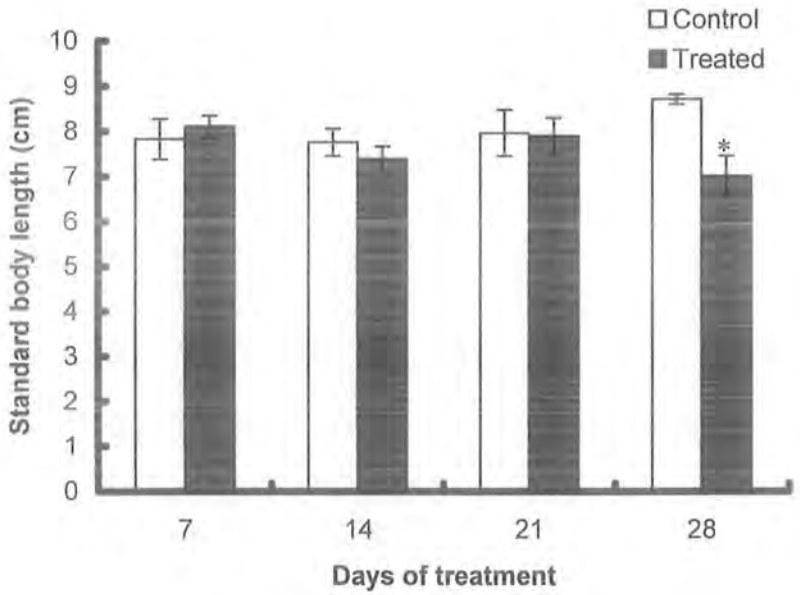
Table.1. Effect of copper (0.08 ppm) on body weight, standard body length and ovarian weight of fish *Cyprinion watsoni*.

Groups	Body weight (g)	Std. Body Length (cm)	Right Ovary Weight (mg)	Left Ovary Weight (mg)	Total Ovarian Weight (mg)
Control. Day 7 (n =5)	8.07±0.77	7.83±0.45	176.7±29.44	167.32±32.53	344.02±61.81
Treated. Day 7 (n =4)	8.87±0.26	8.11±0.24	168.85±39.79	157.35±38.65	326.175±78.43
Control. Day 14 (n =4)	9.72±0.52	7.76±0.30	137.2±14.32	136.27±13.58	273.475±27.85
Treated. Day14 (n =5)	9.36±0.48	7.40±0.27	136.72±13.18	135.6±13.1	272.32±26.32
Control. Day21 (n =4)	9.38±1.29	7.97±0.51	87.16±8.08	86.033±6.12	173.2±14.068
Treated. Day 21 (n =3)	8.50±1.24	7.90±0.41	73.87±10.28	73.27±13.27	147.5±23.44
Control. Day 28 (n =4)	11.23±0.35	8.72±0.11	250.3±41.77	248.37±46.15	498.67±87.90
Treated. Day 28 (n =5)	6.71±1.28*	7.01±0.46*	65.25±14.26**	67.95±13.22**	133.2±27.43**

Mean (±SE); Student's t test *P<0.05; **P<0.01; ***P<0.001 vs Control

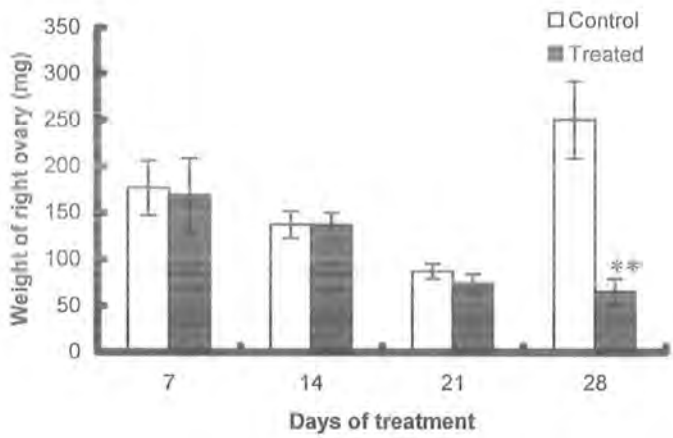


a. Effect of copper (0.08ppm) on body weight (g) of fish.

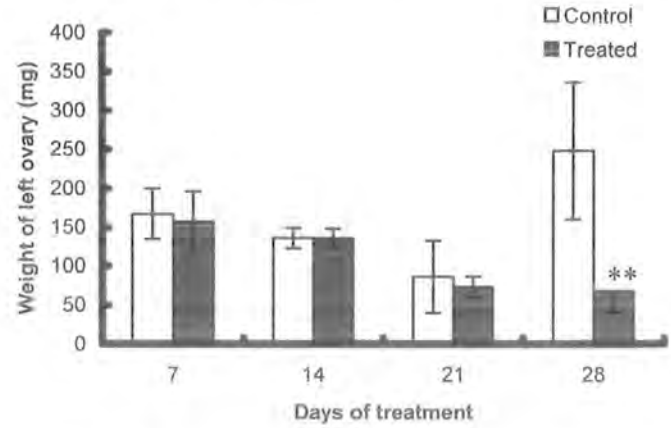


b. Effect of copper (0.08ppm) on standard body length (g) of fish.

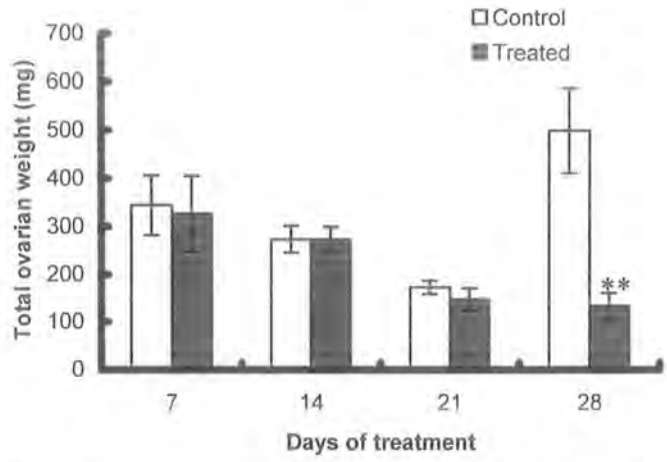
Fig.1
Effect of copper (0.08ppm) on body weight (a) and standard length (b) of fish.
Mean ± SE, Student's t test, significantly different from control. $P<0.05$ (*)



a. Effect of copper (0.08ppm) on weight of right ovary (mg) of fish.



b. Effect of copper (0.08ppm) on weight of left ovary (mg) of fish.



c. Effect of copper (0.08ppm) on weight of total ovarian weight (mg) of fish.

Fig.2:
Effect of copper (0.08ppm) on right ovarian weight (a), left ovarian weight (b) and total ovarian weight (c) of fish. Mean ± SE, Student's t test, significantly different from control. $P<0.05$ (*), $P<0.01$(**)

Effect of copper on ovarian length and breadth:

Group I; Day 7:

Length and breadth of right and left ovaries are given in Table2, Figure.3 and 4.There was no significant difference (P>0.05) in the length and breadth of right and left ovaries of fish exposed to copper, compared to control fish. Lengths of right and left ovaries of control fish were 1.82±0.13 cm and 1.81±0.13 cm respectively. While Length of right and left ovaries of treated fish were 1.6±0.082 cm and 1.59±0.07 cm respectively. Breadths of right and left ovaries of control fish were 0.40±0.013 cm and 0.40±0.011 cm respectively. While breadth of right and left ovaries of treated fish were 0.36±0.017 cm and 0.36±0.02 cm respectively.

Group II; Day 14:

After day 14 of treatment, length of right and left ovaries of control fish was 1.91±0.18 cm and 1.92±0.18 cm respectively. While Length of right and left ovaries of treated fish was 1.82±0.13 cm and 1.91±0.12 cm respectively. Breadth of right and left ovaries of control fish was 0.39±0.0095 cm and 0.40±0.0091 cm respectively. While breadth of right and left ovaries of treated fish was 0.35±0.026 cm and 0.33±0.033 cm respectively (Table2, Fig.3, 4).There was no significant difference (P>0.05) in the length and breadth of ovaries of treated fish, compared to control fish.

Group III; Day 21:

There was no pronounced effect of copper on length and breadth of ovaries after 21 days of treatment. Length of right and left ovaries (1.99±0.066 cm and 1.97±0.075 respectively) of control fish showed no significant difference (P>0.05), compared to length of right and left ovaries (1.64±0.11 cm and 1.67±0.12 cm respectively) of treated fish. There was no significant difference (P>0.05) in breadth of right and left ovaries (0.41±0.008 cm and 0.42±0.006 cm respectively) of control fish and breadth of right and left ovaries (0.40±0.043 cm and 0.41±0.039 cm) of treated fish. (Table2, Fig.3, 4).

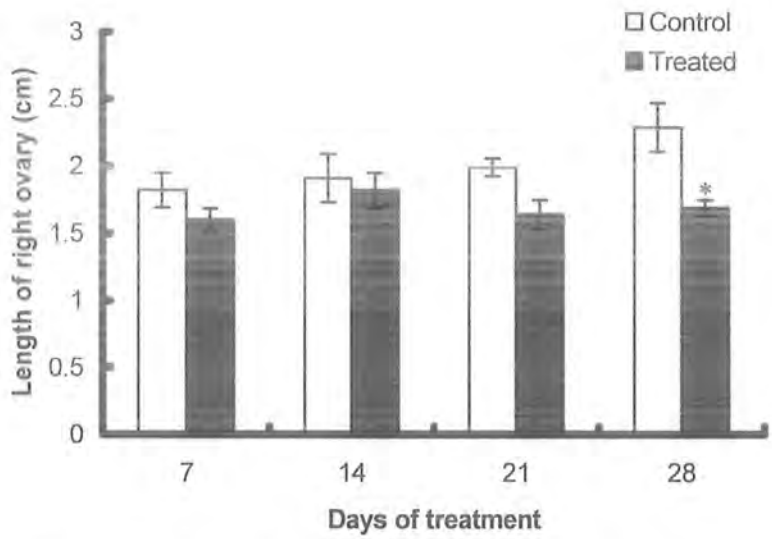
Group IV; Day 28:

After 28 day, effect of exposure to heavy metal copper on length and breadth of fish was significant ($P<0.05$). Length (1.69±0.06 cm) of right ovary of treated fish was significantly decreased ($P<0.05$), compared to length (2.29±0.18 cm) of right ovary of control fish Similarly copper showed drastic effect on the length of left ovary. Length (1.67±0.06 cm) of left ovary of treated fish significantly decreased ($P<0.05$), as compared to length (2.26±0.201 cm) of left ovary of control fish. Breadth (0.58±0.036 cm) of right ovary of control fish showed significant difference ($P<0.01$) as compared to breadth (0.38±0.02 cm) of right ovary of treated fish. There was significant decrease ($P<0.05$) in the breadth (0.38±0.023 cm) of left ovary of fish exposed to copper and breadth (0.58±0.503 cm) of left ovary of control fish. (Table2, Fig.3, 4).

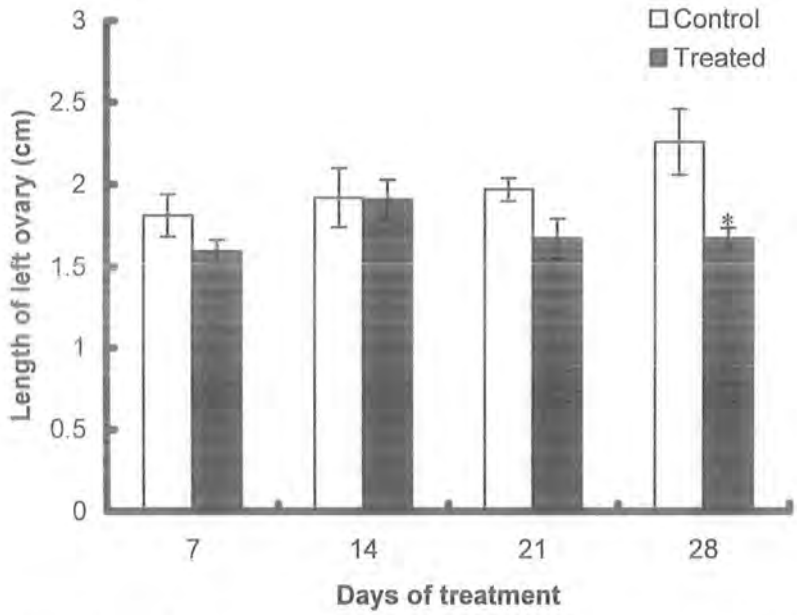
Table.2 Effect of copper (0.08 ppm) on ovarian length and breadth of *Cyprinion watsoni*.

Groups	Length (cm)		Breadth (cm)	
	Right Ovary	Left Ovary	Right Ovary	Left Ovary
Control. Day 7 (n =5)	1.82±0.13	1.81±.0.13	0.40±0.013	0.40±0.011
Treated. Day 7 (n =4)	1.6±0.082	1.597±0.07	0.36±0.017	0.36±0.02
Control. Day14 (n =4)	1.91±0.18	1.92±0.18	0.39±0.0095	0.4±0.0091
Treated. Day14 (n =5)	1.82±0.13	1.91±0.12	0.35±0.026	0.33±0.033
Control. Day 21 (n =4)	1.99±0.066	1.97±0.075	0.41±0.008	0.42±0.006
Treated. Day 21 (n =3)	1.64±0.11	1.67±0.12	0.40±0.043	0.41±0.039
Control. Day 28 (n =4)	2.29±0.18	2.26±0.201	0.58±0.036	0.58±0.503
Treated .Day 28 (n =5)	1.69±0.06*	1.67±0.06*	O.38±0.02**	0.38±0.023*

Mean (±SE); Student's t test *P<0.05; **P<0.01; ***P<0.001 vs Control

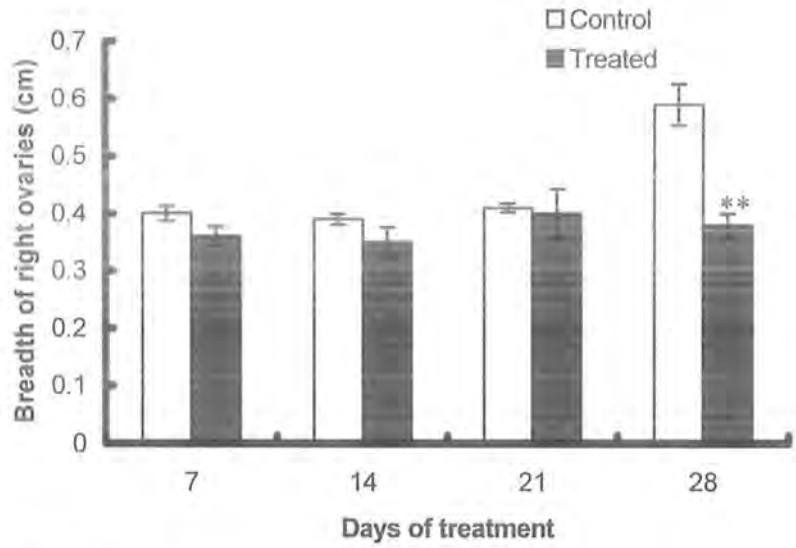


a. Effect of copper (0.08ppm) on length (cm)of right ovary of fish.

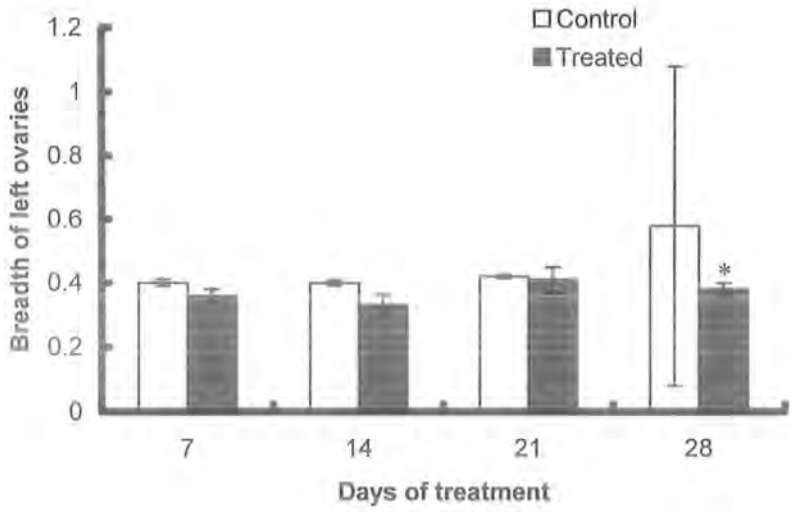


b. Effect of copper (0.08ppm) on length (cm)of left ovary of fish.

Fig.3
Effect of copper (0.08ppm) on length of right (a) and left ovaries (b) (cm) of fish.
Mean ± SE, Student's t test, significantly different from control. $P<0.05$ (*)



a. Effect of copper on breadth (cm) of right ovary of fish.



b. Effect of copper on breadth (cm) of left ovary of fish.

Fig.4
Effect of copper (0.08ppm) on breadth of right (a) and left ovaries (b) (cm) of fish.
Mean ± SE, Student's t test, significantly different from control. $P<0.05$ (*), $P<0.01$(**).

Effect of copper on gonadosomatic index (GSI):

Group I; Day 7:

Mean ± SE values of GSI are given in Table 3 and Figure.5. Gonadosomatic index of control fish (4.18±0.45) as compared to gonadosomatic index (5.97±1.31) of fish exposed to copper showed no significant difference (P>0.05).

Group II; Day 14:

There was no significant difference (P>0.05) on gonadosomatic index of control fish (2.80±0.21) and gonadosomatic index (2.89±0.19) of fish exposed to copper after 14 day of treatment. All average values are given in Table 3 and Figure.5.

Group III; Day 21:

Exposure of copper to fish showed no significant difference (P>0.05) on gonadosomatic index of control fish compared to that of treated fish after 21 day of treatment. Gonadosomatic index of control fish was 1.95±0.38. Gonadosomatic Index of fish exposed to copper was 1.72±0.066. (Table.3, Fig.5).

Group IV; Day 28:

When the GSI of both control and treated fish were compared, there was significant (P<0.05) difference. GSI of control and treated fish was 4.40±0.73 and 1.98±0.22 respectively (Table.3, Fig.5).

Effect of copper on condition factor (K):

Group I; Day 7:

Mean ± SE values of condition factor are given in Table 3 and Figure.5. Condition factor (0.017±0.0013) of control fish compared to condition factor (0.016±0.0016) of fish exposed to copper showed no significant difference (P>0.05).

Group II; Day 14:

There was no significant difference (P>0.05) in condition factor of control fish (0.020±0.0015) and condition factor of treated fish (0.023±0.0018). Mean ± SE values of condition factor are given in Table 3 and Figure.5.

Group III; Day 21:

Condition factor 0.0169±0.0003of treated fish showed no significant difference (P>0.05) compared to condition factor 0.018±0.0011 of control fish. (Table 3, Fig.5) on day 21.

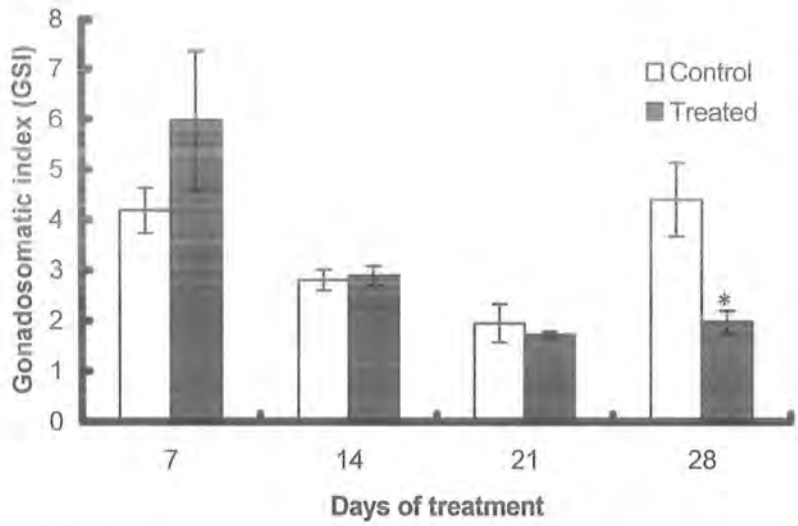
Group IV; Day 28:

After 28 days condition factor of treated fish (0.019±0.0011) showed no significant decrease (P>0.05) as compared to condition factor of control fish (0.0169±0.0006). Mean ± SE values of condition factor are given in Table 3 and Figure.5.

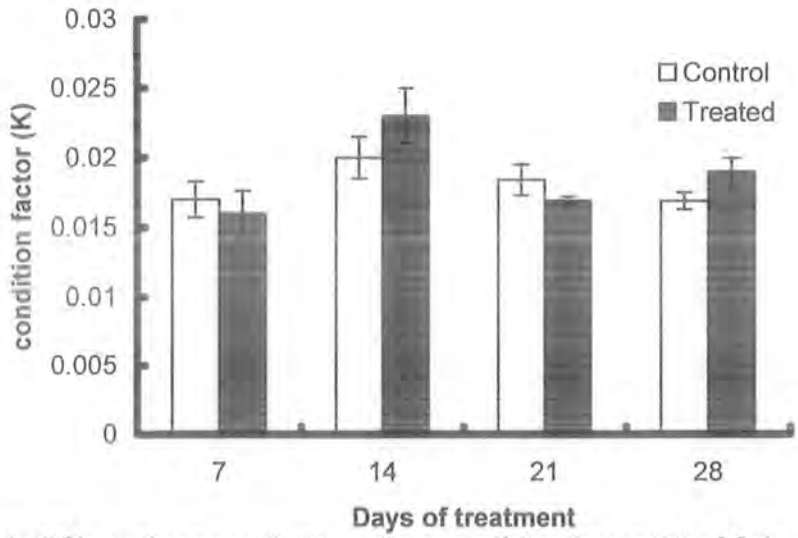
Table.3.Effect of copper on Gonadosomatic Index (GSI) and Condition Factor (k) of fish *Cyprinion watsoni*.

Groups	Gonadosomatic Index (GSI)	Condition Factor (K)
Control. Day 7 (n =5)	4.18±0.45	0.017±0.0013
Treated. Day 7 (n =4)	5.97±1.31	0.016±0.0016
Control. Day 14 (n =4)	2.80±0.21	0.020±0.0015
Treated. Day14 (n =5)	2.89±0.19	0.023±0.0018
Control. Day 21 (n =4)	1.95±0.38	0.018±0.0011
Treated. Day 21 (n =3)	1.721±0.066	0.0169±0.0003
Control. Day 28 (n =4)	4.401±0.73	0.0169±0.0006
Treated .Day 28 (n =5)	1.98±0.22*	0.019±0.0011

Mean (±SE); Student's t test *P<0.05; **P<0.01; ***P<0.001 vs Control



a. Effect of copper (0.08ppm) on gonadosomatic index (GSI) of fish.



b. Effect of copper (0.08ppm) on condition factor (K)of fish.

Fig.5
Effect of copper (0.08ppm) on gonadosomatic (a) index and condition factor (b) of fish. Mean ± SE, Student's t test, significantly different from control. $P<0.05$ (*)

Histological studies:

The wall of ovary consists of tunica albuginea, a thick layer made up of connective tissue containing numerous blood vessels. The tunica albuginea projects into the ovarian cavity to form relatively few ovigerous folds containing germ cells or oogonia and other follicles in various developmental stages. These are embedded in loose connective tissue, the stroma. Five developmental stages were observed which are described as.

Stage I; primary oocyte:

They have darkly stained basophilic and homogenous cytoplasm. Follicular diameter ranges from 21-65 μm. The range of nuclear diameter 14-26 μm with number of 2-4 nucleoli (Fig.14 a).

Stage II; Perinucleolar follicle:

These follicles are larger than (diameter 60-126 μm) stage I, have strongly basophilic and homogenous cytoplasm. The nucleus (diameter 39-63μm) posses a number of nucleoli 7-22.The nucleoli lie towards the inner periphery of the nuclear membrane and the chromatin material is evenly distributed throughout the nucleus smoothly. Single layered granulosa appears but not fully organized around the developing follicle (Fig. 14 a)

Stage III; Yolk precursor:

These follicles are even larger than the Perinucleolar follicles; the diameter ranges between 128-195 μm while the nuclear diameter range of stage III follicles between 55-94μm. The cytoplasm is basophilic and has a slightly granular appearance at the periphery. Moreover, yolk vesicles are present only in the peripheral region of the cytoplasm. Chromatin material is evenly distributed and nucleoli (22-55) are arranged in peripheral region of nucleus towards the inner border of nuclear membrane, which is irregular in appearance (Fig.14 b).

Stage IV; Oocyte with vacuolated cytoplasm:

The oocyte at this stage has further increased in size (diameter176-315μm) compared to stage III follicle, while their nuclear diameter range is 50-140 μm. The nucleus at this stage has characteristic arrangement of nucleoli and has irregular nuclear membrane. The numbers of nucleoli are 24-55.The Oocyte at this stage are

surrounded by a non-cellular layer, the zona radiata (2 μm). At this stage zona radiata shows light striations but not prominent. Around the outer periphery of zona radiata, there is single layer of flattened granulosa cells, which have oval nucleoli. Layers of very thin elongated thecal cells surround the granulosa layer. The cytoplasm shows the accumulation of yolk which is in the form of brightly stained yolk globules and lightly stained yolk vesicles .The yolk globules occupy the area just on the outer periphery of the nucleus, while the yolk vesicles are observed outer to this, below the zona radiata (Fig.14 c).

Stage V; Opaque oocyte with yolk granules:

Follicles at this stage are deeply eosinophilic and are characterizes by the presence of eosinophilic yolk granules scattered throughout the cytoplasm .The periphery is invaded with large number of yolk vesicles. At this stage follicular diameter is maximum i.e.312-763μm. Several nucleoli are present toward the peripheral region throughout the evenly distributed chromatin. The nuclear membrane is not clearly visible. Germinal vesicle moved towards the periphery .The theca, granulosa and zona radiata increased in thickness. The striations on zona radiata are deeply eosinophilic and very conspicuous (Fig.14 d).

Atretic follicles/Past spawning ovary:

The regressed ovary contains postovulatory follicles, immature oocyte and a number of those follicles, which had not undergone hydration at the time of spawning and have become Atretic. Two types of Atretic follicles were observed i.e. atresia of vitellogenic oocytes and atresia of previtellogenic oocytes. (Fig. 15)

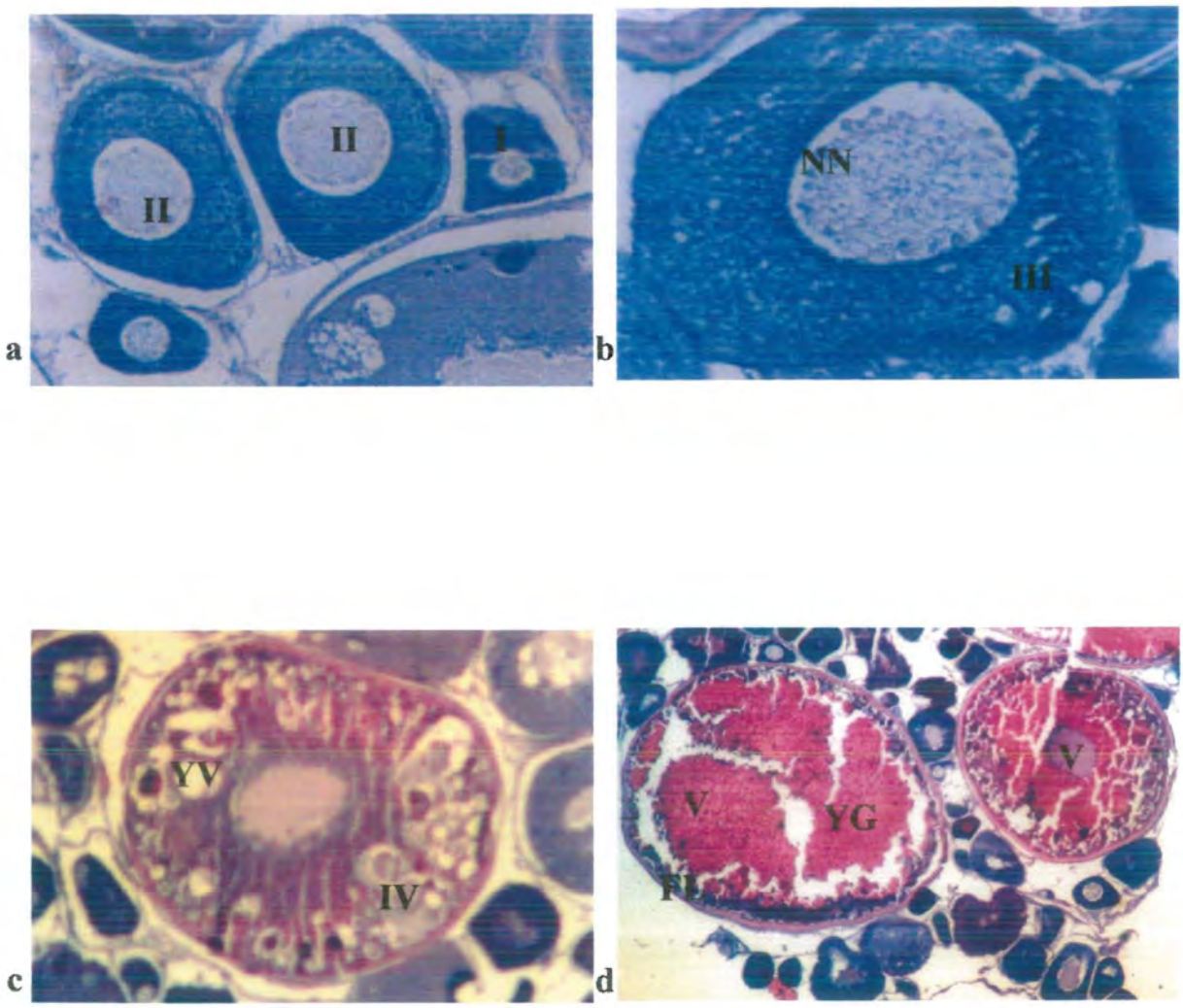


Fig 14: Photomicrographs of developmental stages of oocytes in *Cyprinion watsoni*, showing a (stage I and II), b (stage III), c (stage IV) and d (stage V) and nucleoli number (NN), follicular layer (FL), yolk vacuoles (YV) and yolk globules (YG).

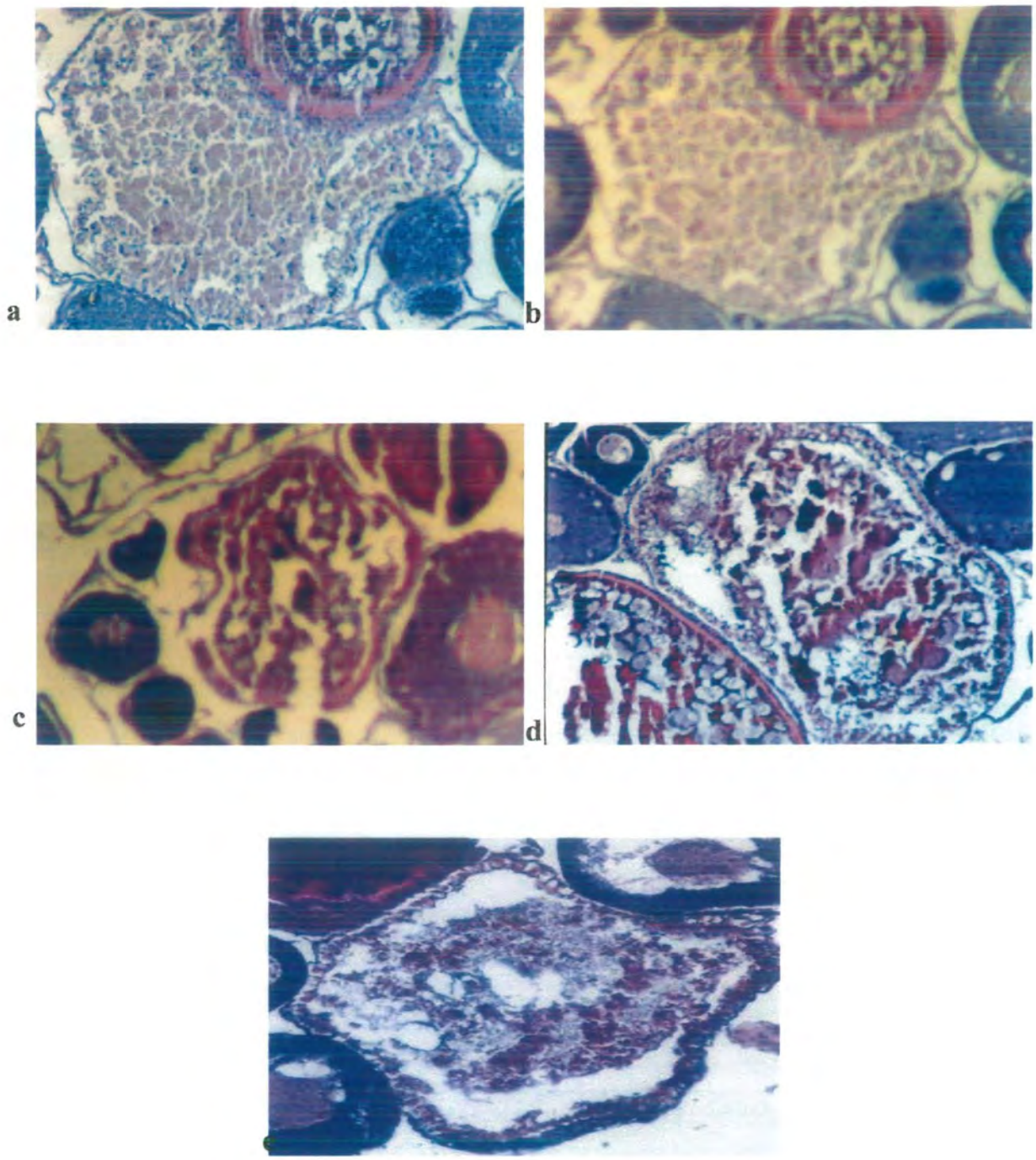


Fig 15: Photomicrographs of Patterns of atresia in *Cyprinion watsoni* (a-e).

Effect of copper on number of developing and atretic follicles:

Number of developing follicles per cross section of follicular stages I, II, III, IV, V and atretic follicles were counted in control and treated groups. The results are presented in Table.4

Group I; Day7:

Numbers of follicles per cross section were counted according to follicular developmental stages. Table 4 and figure 6 shows the number of follicles against each follicular stage after 7 days of treatment. There was no significant difference ($P>0.05$) on number of follicles of all stages. Number of follicles of follicular stages I, II, III, IV and V of control fish were 11.18± 0.66, 35.93 ± 4.35, 24.06± 1.10, 9.53± 0.6 and 3.53±0.23 respectively. While number of follicles of follicular stages I, II, III, IV and V of treated fish were 12.2±0.64, 32.92±1.35, 25.93±1.16, 8.46±3.33 and 3.06±0.24 respectively. Number of atretic follicles of treated fish (4.3±0.38) showed significant difference ($P<0.05$) compared to number of atretic follicles of control fish (3.2±0.34).

Group II; Day14:

Mean ± SE values of no of developing and atretic follicles per cross section are given in Table 4 and figure 6.There was no significant different ($P >0.05$) in number of follicles of follicular stages I and II of control fish (9.4±0.53 and 37±1.11 respectively) and number of follicles of follicular stages I and II (9.2 ±0.65 and 35.95± 1.05 respectively) of fish exposed to copper. But number of follicles of follicular stages III and V of control fish (20.1±1.07 and 3.45±0.22 respectively) showed significant difference ($P<0.001$) compared to number of follicles of follicular stages III and V of treated fish (12.35±0.83 and 2.3±0.17 respectively). Number of follicles of follicular stage IV of treated fish (7.75±0.75) significantly decreased ($P<0.05$) compared to number of follicles of follicular stage IV of control fish (10.05±0.54). In treated fish number of atretic follicles (13.15±3.73) significantly increased ($P<0.001$) compared to number of atretic follicles of control fish (2.4±0.35).

Group III; Day 21:

After 21 days of treatment number of developing and atretic follicles per cross section were counted. There was no significant difference ($P>0.05$) in number of follicles of follicular stage I of control (10.2±0.63) and that of treated fish (8.86±0.61). But there was drastic effect on the number of follicles of follicular stages II, III, IV and V of treated fish. Number of follicles of follicular stages II, III and IV of control fish (45±1.35, 16.93±1.07 and 6.73±0.79 respectively) showed significant difference ($P<0.001$) compared to number of follicles of follicular stages II, III and IV of treated fish (36.4±1.09, 9.4± 0.66 and 2.66±0.70 respectively). Number of follicles of follicular stage V of treated fish (2.66±0.18) showed significant decrease ($P<0.01$) compared to number of follicles of follicular stage V of control fish (3±0.21). After 21 days of treatment number of atretic follicles (8.8±0.54) in fish exposed to copper was significantly increased ($P<0.001$) compared to that of control fish (3.66±0.3). All mean ± SE values are given in Table 4 and figure 7.

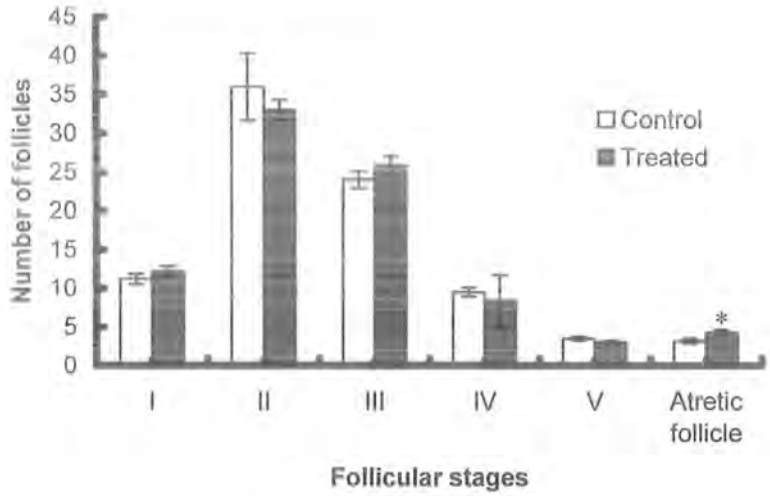
Group IV; Day 28:

Effect of exposure to heavy metal copper was very drastic on number of follicles (per cross section) of follicular stages I, II, III, IV and V of treated fish as compared to that of control fish after 28 days of treatment. Number of follicles of follicular stages II, III, IV and V of control fish (50.2±1.67, 14.2±0.60, 6.73±0.49 and 3.8±0.32 respectively) showed significant difference ($P<0.001$) compared to number of follicles of follicular stages II, III, IV and V of treated fish (35.46±1.05, 5.93±0.38, 3.06±0.24 and 2.2±0.22 respectively). Number of atretic follicles of treated fish (8.6±0.63) showed significant increase ($P<0.01$) compared to number of atretic follicles of control fish (3.26±1.03). Copper also showed drastic effect on number of follicles of follicular stage I of treated fish as compared to control. There was significant difference ($P<0.01$) in number of follicles of follicular stage I of control fish (9.86±0.85) and number of follicles of follicular stage I of treated fish (6.86±0.46). All mean ± SE values are given in Table 4 and figure 7.

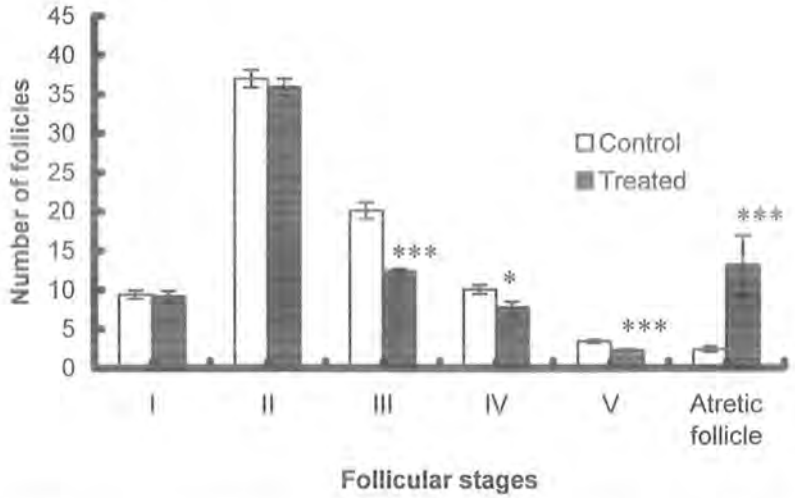
Table.4. Effect of copper (0.08 ppm) on number of developing and atretic follicles of fish *Cyprinion watsoni.*

Groups	Follicular stages					
	Stage I	Stage II	Stage III	Stage IV	Stage V	Atretic follicle
Control Day. 7 (n=5)	11.18±0.66	35.93±4.35	24.06±1.10	9.53±0.60	3.53±0.23	3.2±0.34
Treated. Day.7 (n=4)	12.2±0.64	32.92±1.35	25.93±1.16	8.46±3.33	3.06±0.24	4.3±0.38*
Control. Day. 14 (n=4)	9.4±0.53	37.00±1.11	20.1±1.07	10.05±0.54	3.45±0.22	2.4±0.35
Treated. Day 14 (n=5)	9.2±0.65	35.95±1.05	12.35±0.3***	7.75±0.75*	2.3±0.17***	13.15±3.73***
Control Day.21 (n=4)	10.2±0.63	45.00±1.35	16.93±1.07	6.73±0.79	3.00±0.21	3.66±0.38
Treated. Day.21 (n=3)	8.86±0.61	36.4±1.09***	9.4±0.66***	2.66±0.70***	2.66±0.18**	8.8±0.54***
Control. Day.28 (n=4)	9.86±0.85	50.2±1.67	14.2±0.60	6.73±0.49	3.8±0.32	3.26±1.03
Treated Day.28 (n=4)	6.86±0.46**	35.46±1.05***	5.93±0.38***	3.06±0.24***	2.2±0.22***	8.6±0.63***

Mean (±SE); Student's t test *P<0.05; **P<0.01; ***P<0.001 vs Control

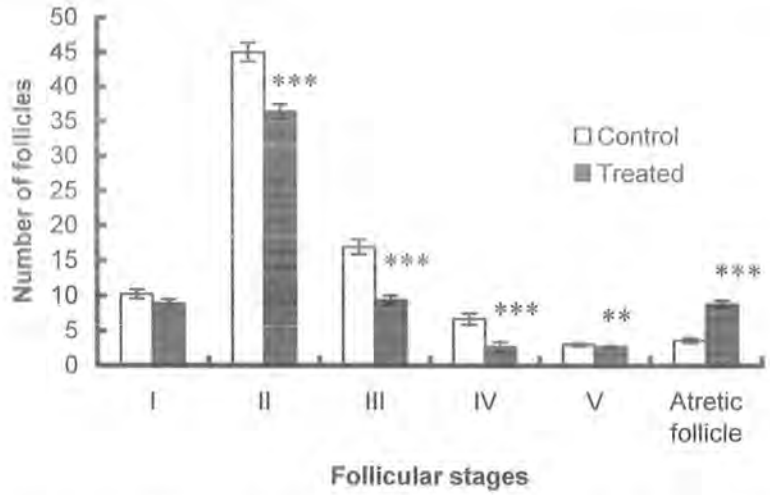


a. Effect of copper (0.08ppm) on number of follicles after 7 days of treatment.

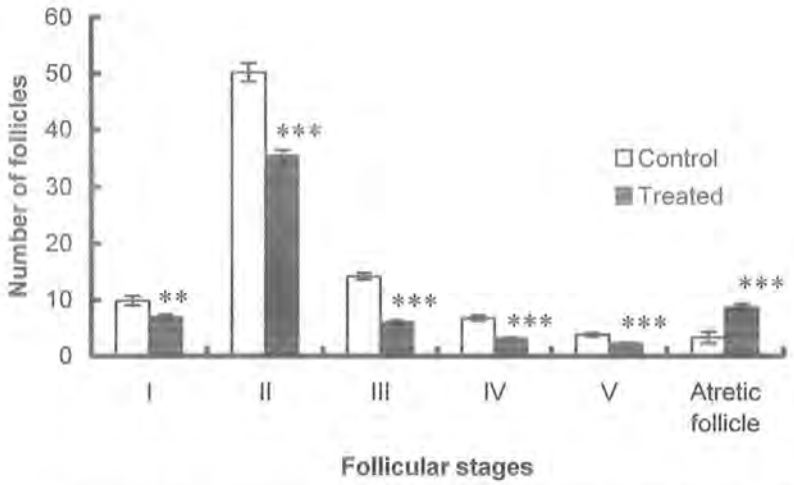


b. Effect of copper (0.08ppm) on number of follicles after 14 days of treatment.

Fig.6
Effect of copper (0.08ppm) on number of follicles of fish.
Mean±SE, Student's t test, significantly different from control, $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***).



a. Effect of copper (0.08ppm) on number of follicles after 21 days of treatment.



b. Effect of copper (0.08ppm) on number of follicles after 28 days of treatment.

Fig.7
Effect of copper (0.08ppm) on number follicles of fish.
Mean±SE, Student's t test, significantly different from control, $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***).

Effect Of copper on follicular diameter:

Group I; Day7:

Follicular diameters of stage I of control fish (50.54±1.47 μm) compared to follicular diameter of stage I of treated fish (48.12±1.42μm) showed no significant difference (P>0.05). Similarly there was no significant difference (P>0.05) in the follicular diameter of stage II of control fish (106.09±2.65 μm) compared to follicular diameter of stage II of fish exposed to copper (105.34±2.84μm). But follicular diameter of stage III of control fish (166.25±2.98μm) showed significant difference (P<0.05) compared to follicular diameter of treated fish (156.64±2.64μm). Copper was not able to produce any drastic effect on follicular diameter of stages IV and V of treated fish. Follicular diameter of stage IV and V of control fish (220.47±4.54 μm and 531.97±33.77μm respectively) compared to follicular diameter of stage IV and stage V of treated fish (206.53±7.73μm and 499.17±27.34μm respectively) showed no significant difference (P>0.05). Mean ± SE values are given in Table.5 and Figure.8.

Group II; Day14:

Effect of exposure to heavy metal copper was very drastic on follicular diameter of follicular stages I, II, III of treated fish as compared to that of control fish. The follicular diameters of stages I, II and III of treated fish (40.8±1.10μm, 92.24±2.57μm and 147.74 ±2.13μm respectively), showed significant decrease (P<0.001) compared to follicular diameter of stages I, II and III of control fish (49.98±0.13μm, 111.31±2.46μm and 166.15 ±2.57μm respectively). There was no significant effect (P>0.05) on follicular diameter of stage IV of control fish (301.59±68.43μm) and follicular diameter of stage IV of treated fish (211.86±3.19μm). Follicular diameter of stage V of control fish (481.75±28.88μm) showed significant difference (P<0.05) as compared to follicular diameter of stage V of fish exposed to copper (381.3±11.64μm). Mean ± SE values are given in Table.5 and Figure.8.

Group III; Day 21:

The follicular diameters of stages I, II, III, IV and V of fish exposed to copper were 37.56±1.25μm, 86.17±2.05μm, 142.37 ±2.51μm, 179.62±2.96μm and

358.75±7.42μm respectively, which showed significant decrease (P<0.001) compared to follicular diameters of stages I, II, III, IV and V Of control fish (45.09±1.04μm, 113±2.77μm, 155.25 ±2.67μm, 219.03±4.7 μm and 456.12±5.61μm respectively). All mean ± SE values of follicular diameters are given in Table.5 and Figure.9.

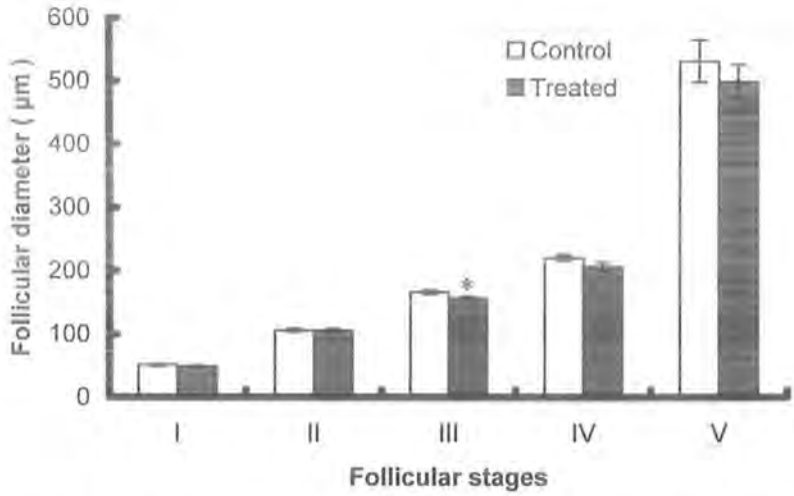
Group IV; Day 28:

Drastic changes were observed in follicular diameters of all follicular stages after 28 days of experiment. The follicular diameters of stages I, II, III, IV and V of fish exposed to copper were 34.7±1.33μm, 74.13±2.63μm, 141.23±2.61μm, 191.10±2.55μm and 509.93±36.52μm respectively, which showed significant decrease (P<0.001) compared to follicular diameter of stages I, II, III, IV and V Of control fish .The follicular diameters of stages I, II, III, IV and V of control fish were 45.92±1.46μm, 105.92±1.82 μm, 157.33 ±2.60 μm, 229.53±6.03μm and 687.77±26.33μm respectively. (Table.5, Fig.9).

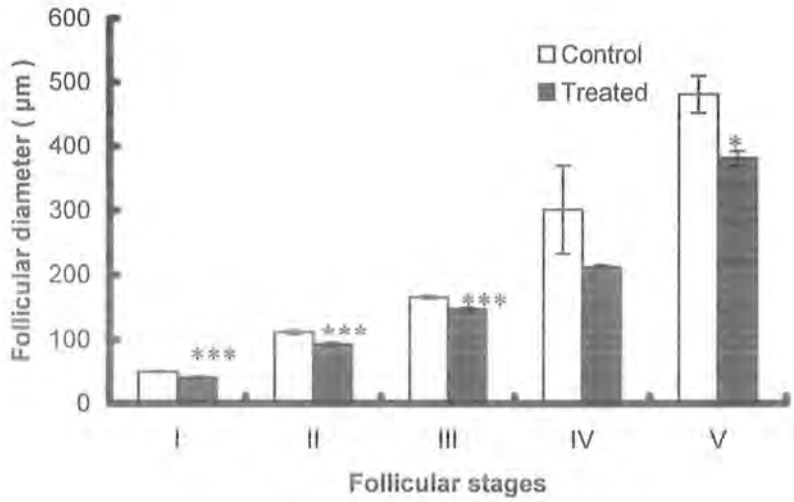
Table.5. Effect of copper (0.08 ppm) on follicular diameter (μm) of fish *Cyprinion watsoni.*

Groups	Follicular Stages				
	Stage I	Stage II	Stage III	Stage IV	Stage V
Control. Day 7 (n =5)	50.54±1.47	106.09±2.65	166.25±2.98	220.47±4.54	531.97±33.77
Treated. Day 7 (n =4)	48.12±1.42	105.34±2.84	156.64±2.64*	206.53±7.73	499.17±27.34
Control. Day 14 (n =4)	49.98±0.131	111.31±2.46	166.15±2.57	301.59±68.43	481.75±28.88
Treated. Day14 (n =5)	40.80±1.10***	92.24±2.57***	147.74±2.13***	211.86±3.19	381.3±11.64*
Control. Day21 (n =4)	45.09±1.04	113.00±2.77	155.25±2.67	219.03±4.7	456.12±5.61
Treated. Day 21 (n =3)	37.56±1.25***	86.17±2.05***	142.37±2.51***	179.62±2.96***	358.75±7.42***
Control. Day 28 (n =4)	45.92±1.46	105.92±1.82	157.33±2.60	229.53±6.03	687.77±26.23
Treated . Day 28 (n =5)	34.70±1.33***	74.13±2.63***	141.23±2.61***	191.10±2.55***	509.93±36.52***

Mean (±SE); Student's t test *P<0.05; **P<0.01; ***P<0.001 vs Control

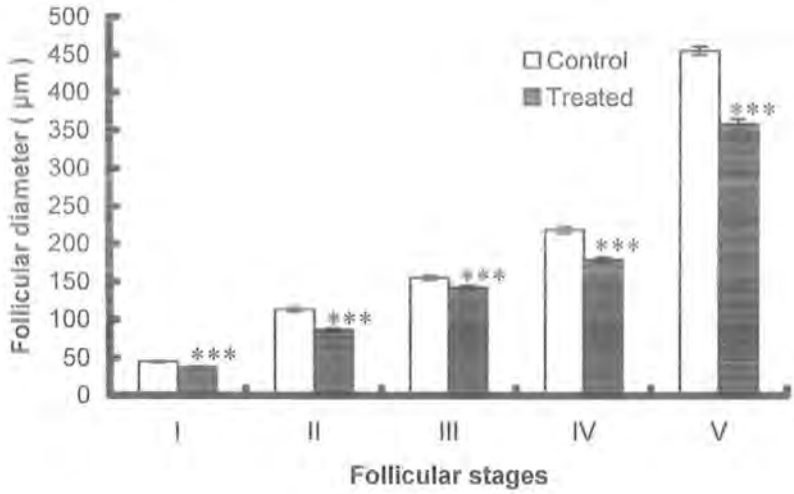


a. Effect of copper (0.08ppm) on follicular diameter (µm) after 7 days of treatment.

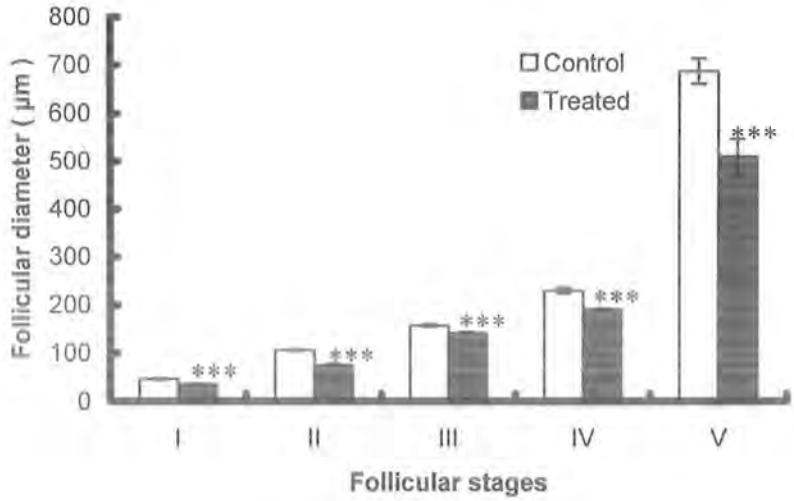


b. Effect of copper (0.08ppm) on follicular diameter (µm) after 14 days of treatment.

Fig.8
Effect of copper (0.08ppm) on follicular diameter of fish.
Mean ± SE, Student's t test, significantly different from control, $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***).



a. Effect of copper (0.08ppm) on follicular diameter (µm) after 21 days of treatment.



b. Effect of copper (0.08ppm) on follicular diameter (µm) after 28 days of treatment.

Fig.9
Effect of copper (0.08ppm) on follicular diameter of fish.
Mean ± SE, Student's t test, significantly different from control, $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***).

Effect Of copper on nuclear diameter:

Group I; Day7:

Mean values of nuclear diameter are tabulated in Tabe.6 and Figure.10. Nuclear diameter of follicular stage I of control fish (24.74±0.91μm) showed significant difference ($P<0.05$) as compared to nuclear diameter of follicular stage I of treated fish (21.22±0.98μm). There was no significant change ($P>0.05$) in nuclear diameter of follicular stages II and III of control fish (53.16±1.52μm and 81.93±2.74 μm respectively) and nuclear diameter of follicular stages II and III of treated fish (50.75±1.31μm and 74.19±2.86μm respectively). Nuclear diameter of follicular stage IV (79.8±2.476μm) of fish exposed to copper showed significant decrease ($P<0.001$) compared to nuclear diameter of follicular stage IV (93.3±2.30μm) of control fish. There was no significant change ($P>0.05$) in nuclear diameter of follicular stage V of control fish (156.4±13μm) and nuclear diameter (143.5±13.26μm) of follicular stage V of fish exposed to copper

Group II; Day14:

A pronounced change was observed in nuclear diameter of oocytes after 14 days of experiment. The nuclear diameters of follicular stages I, II, III and IV of fish exposed to copper were 19.55±0.61μm, 49.57±1.15μm, 70.81 ±1.46μm and 82.45 ±1.55 μm respectively, which showed significant decrease ($P<0.001$) compared to nuclear diameters of follicular stages I, II, III and IV of control fish (24.06±1.04 μm, 55.95±1.33 μm, 83.39±2.22 μm and 93.72±2.78μm respectively). Nuclear diameter (129.15±8.46μm) of follicular stage V of control fish showed no significant difference ($P>0.05$) compared to nuclear diameter (102.5±4.58 μm) of follicular stage V of fish exposed to copper. Mean ± SE values are given in Table.6 and Figure10.

Group III; Day 21:

The nuclear diameters of follicular stages I, II, III and IV of fish exposed to copper were 18.82±0.59 μm, 44.35±0.97μm, 70.30±1.79 μm and 74.73 ±1.84 μm respectively, which showed significant decrease ($P<0.001$) compared to nuclear diameters of follicular stages I, II, III and IV of control fish .The nuclear diameters of follicular stages I, II, III and IV of control fish were 22.01±0.52μm, 58.56±1.58μm, 78.54±1.62μm and 87.22±2.71μm respectively. Nuclear diameter (91.22±3.76 μm) of

follicular stage V of fish exposed to copper showed significant decrease ($P<0.01$) compared to nuclear diameter (125.05±6.19μm) of follicular stage V of control fish. Mean ± SE values are given in Table.6 and Figure.11.

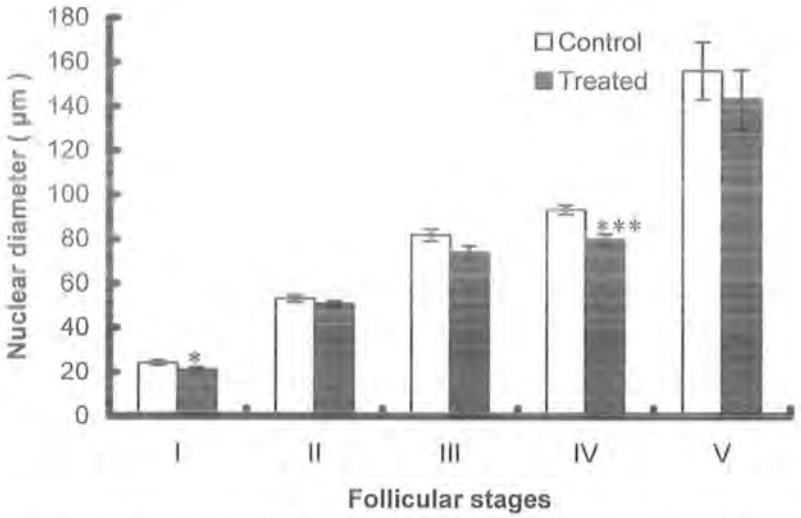
Group IV; Day 28:

Effect of exposure to heavy metal copper was very drastic on nuclear diameter of follicular stages I, II, III, IV and V of treated fish as compared to that of control fish. There was significant decrease ($P<0.001$), when nuclear diameters of follicular stages I, II, III, IV of treated fish (18±0.57μm, 39.33±1.35μm, 67.65±1.70μm, 77.55±1.40μm and 135.04±6.48μm respectively) were compared with nuclear diameters of follicular stages I, II, III, IV and V of control fish (21.54±0.56μm, 51.74±1.15μm, 79.62±1.63 μm, 94.74±2.66 μm and 190.13±6.08μm respectively). All mean± SE values are given in Table.6 and Figure.11.

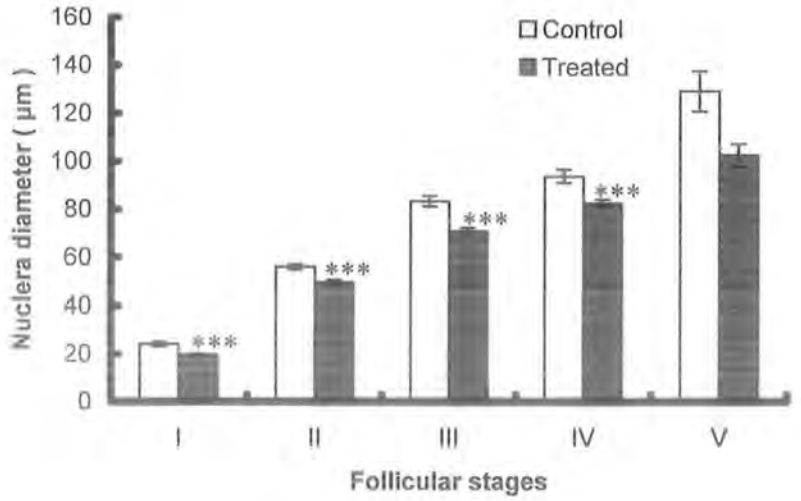
Table.6.Effect of copper (0.08 ppm) on nuclear diameter (μm) of fish *Cyprinion watsoni*.

Groups	Follicular Stages				
	Stage I	Stage II	Stage III	Stage IV	Stage V
Control, Day 7. (n =5)	24.74±0.91	53.16±1.52	81.93±2.74	93.30±2.30	156.4±13
Treated. Day 7. (n =4)	21.22±0.98*	50.75±1.31	74.19±2.86	79.86±2.47***	143.5±13.26
Control. Day 14. (n =4)	24.06±1.04	55.95±1.33	83.39±2.22	93.72±2.78	129.15±8.46
Treated. Day14 (n =5)	19.55±0.61***	49.57±1.15***	70.81±1.46***	82.45±1.55***	102.50±4.58
Control. Day21 (n =4)	22.01±0.52	58.56±1.58	78.54±1.62	87.22±2.71	125.05±6.19
Treated. Day 21. (n =3)	18.82±0.59***	44.35±0.97***	70.30±1.79**	74.73±1.84***	91.22±3.76**
Control. Day 28. (n =4)	21.54±0.56	51.74±1.15	79.62±1.63	94.74±2.66	190.13±6.08
Treated. Day 28. (n =5)	18.00±0.57***	39.33±1.35***	67.65±1.70***	77.55±1.40***	135.04±6.48***

Mean (±SE); Student's t test *P<0.05; **P<0.01; ***P<0.001 vs Control

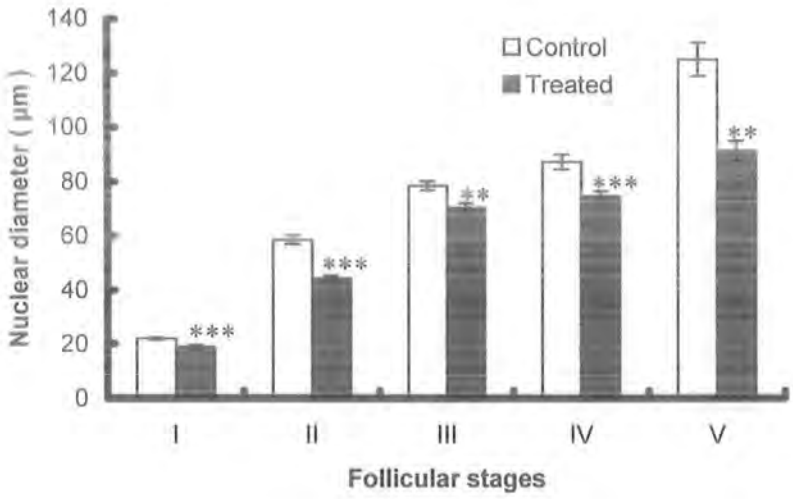


a. Effect of copper (0.08ppm) on nuclear diameter (µm) after 7 days of treatment.

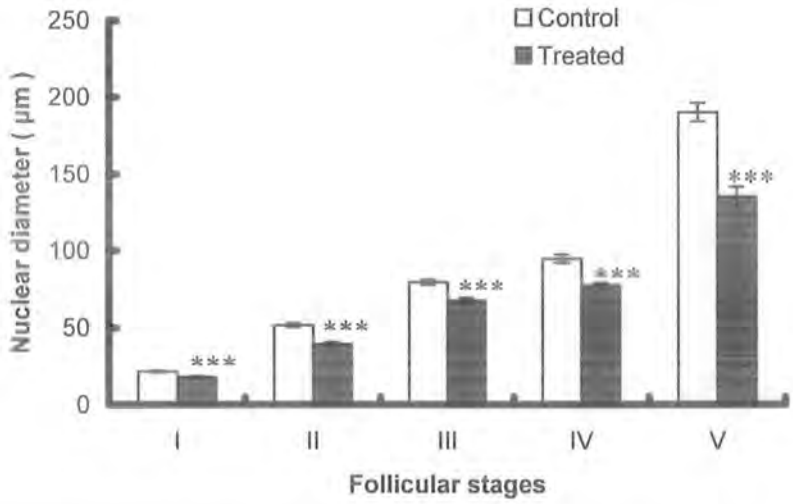


b. Effect of copper (0.08ppm) on nuclear diameter (µm) after 14 days of treatment.

Fig.10
Effect of copper (0.08ppm) on nuclear diameter of fish.
Mean ± SE, Student's t test, significantly different from control, P<0.05 (*), P<0.01 (***), P<0.001 (***).



a. Effect of copper (0.08ppm) on nuclear diameter (µm) after 21 days of treatment.



b. Effect of copper (0.08ppm) on nuclear diameter (µm) after 28 days of treatment.

Fig.11
Effect of copper on nuclear diameter of fish.
Mean ± SE, Student's t test, significantly different from control, $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***).

Effect of copper on number of nucleoli:

Group I; Day 7:

Number of nucleoli of follicular stages I and V of control fish were 2.96±0.16 and 27.4±0.74 respectively, which showed no significant difference (P>0.05) compared to number of nucleoli of follicular stages I and V (2.86±0.12 and 25.4±1.20 respectively) of fish exposed to copper. While number of nucleoli of follicular stages II and IV of control fish were 13.96±0.42 and 28.2±0.91 respectively, which showed significant difference (P<0.01) compared to number of nucleoli of follicular stages II and IV (10.8±1.31 and 24.16±0.73) of fish exposed to copper. Similarly number of nucleoli of follicular stage III of control fish was 33.03±0.82,which showed significant difference (P<0.001) compared to number of nucleoli of follicular stage III (22.6±0.43) of fish exposed to copper. All mean ± SE values are given in Table.7 and Figure.12.

Group II; Day 14:

There was no significant change (P>0.05) in nucleoli number of follicular stages I and II of control fish (2.82±0.12 and 12.75±0.41) and nucleoli number of follicular stages I and II of treated fish (2.55±0.10 and11.25±0.40 respectively). While nucleoli number of follicular stages III, IV and V of control fish (29.55±0.83, 34.27±0.81 and 28.5±0.5 respectively) showed significant difference (P<0.001) compared to nucleoli number of follicular stages III, IV and IV (24.02±0.44; 27.75±0.44 and 24.4±0.6 respectively) of fish exposed to copper. All mean± SE values are given in Table.7 and Figure.12.

Group III; Day 21:

Average values of nucleoli number of follicles of control and treated fish are given in Table.7 and Figure.13. Number of nucleoli of follicular stage I of control fish was 2.73±0.11 showed significant difference (P<0.05) compared to number of nucleoli of follicular stage I of treated fish (2.33±0.11). While number of nucleoli of follicular stages II, III, IV and V of control fish (13.6±0.44, 30.4±0.54, 32.83±0.47 and 29.4±0.74 respectively) showed significant difference (P<0.001) compared to number of nucleoli of follicular stages II, III, IV and V (8.76±0.30, 25.73±0.68, 28.2±0.60 and 23.2±0.37 respectively) of treated fish.

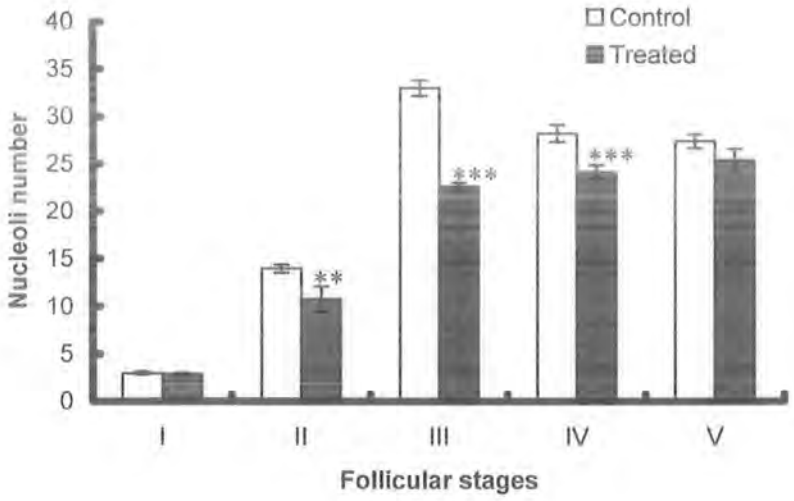
Group IV; Day 28:

No significant difference ($P>0.05$) was in the number of nucleoli of follicular stage V of control fish (32.4±1.40) and number of nucleoli (29.3±0.86) of follicular stage V of treated fish. Number of nucleoli of follicular stages I, II, III and IV of control fish were 2.92±0.13, 11.75±0.38, 31.07±0.48 and 32.62±0.49 respectively, which showed significant difference ($P<0.001$) compared to number of nucleoli of follicular stages I, II, III and IV of treated fish (2.07±0.11, 8.65±0.29, 25.8±0.50 and 26.55±0.64 respectively). All mean± SE values are given in Table.7 and Figure.13.

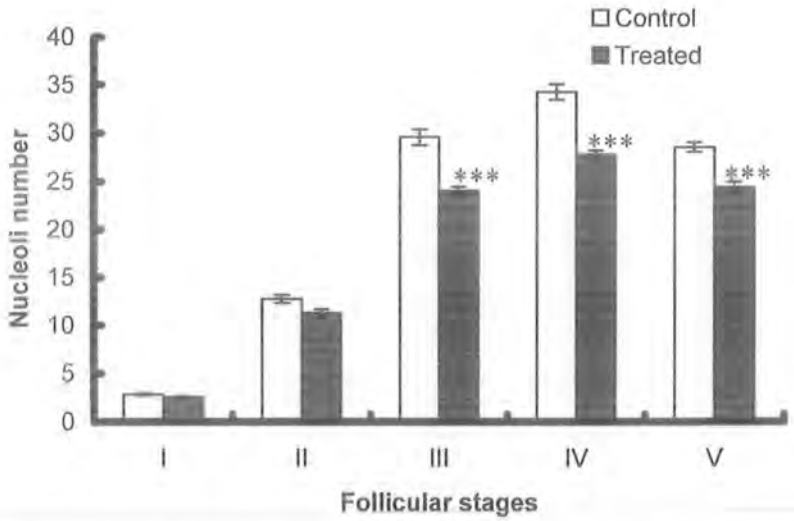
Table.7.Effect of copper (0.08 ppm) on number of nucleoli in fish *Cyprinion watsoni*.

Groups	Follicular Stages				
	Stage I	Stage II	Stage III	Stage IV	Stage V
Control. Day 7 (n =5)	2.96±0.162	13.96±0.42	33.03±0.82	28.2±0.91	27.4±0.74
Treated. Day 7 (n =4)	2.86±0.12	10.8±1.31**	22.6±0.43***	24.166±0.73**	25.4±1.20
Control. Day 14 (n =4)	2.82±0.12	12.75±0.41	29.55±0.83	34.27±0.81	28.5±0.5
Treated. Day14 (n =5)	2.55±0.10	11.25±0.40	24.02±0.44***	27.75±0.44***	24.4±0.6***
Control. Day21 (n =4)	2.73±0.11	13.6±0.44	30.4±0.54	32.83±0.47	29.4±0.74
Treated. Day 21 (n =3)	2.33±0.11*	8.76±0.30***	25.73±0.68***	28.2±0.60***	23.2±0.37***
Control. Day 28 (n =4)	2.92±0.13	11.75±0.38	31.07±0.48	32.62±0.49	32.4±1.40
Treated. Day 28 (n =5)	2.07±0.11***	8.65±0.29***	25.8±0.50***	26.55±0.64***	29.3±0.86

Mean (±SE); Student's t test *P<0.05; **P<0.01; ***P<0.001 vs Control

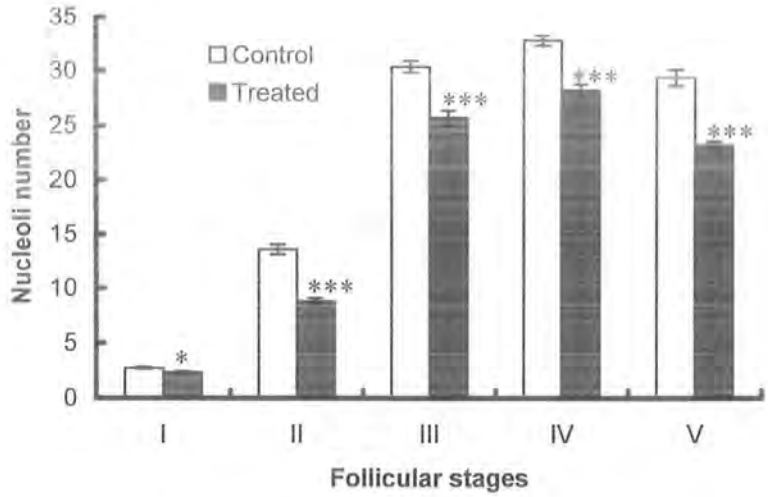


a. Effect of copper (0.08ppm) on nucleoli number after 7 days of treatment.

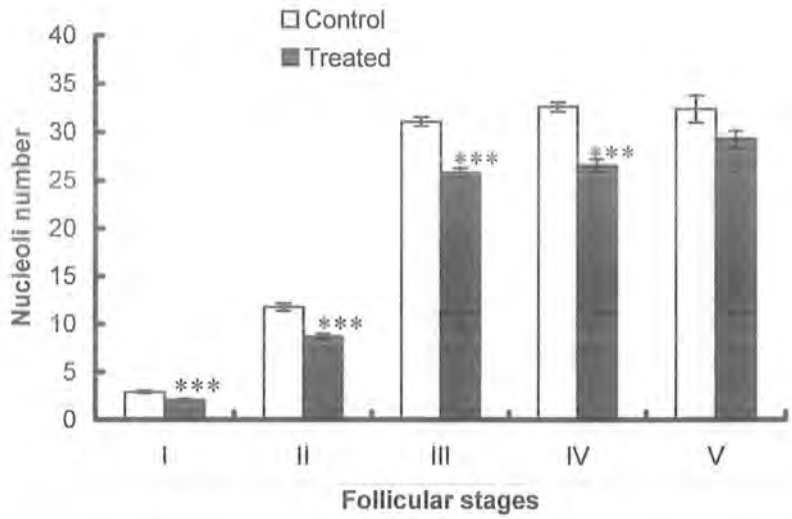


b. Effect of copper (0.08ppm) on nucleoli number after 14 days of treatment.

Fig.12
Effect of copper (0.08ppm) on nucleoli number of fish.
Mean ± SE, Student's t test, significantly different from control, $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***).



a. Effect of copper (0.08ppm) on nucleoli number after 21 days of treatment.



b. Effect of copper (0.08ppm) on nucleoli number after 28 days of treatment.

Fig.13
Effect of copper (0.08ppm) on nucleoli number of fish.
Mean ± SE, Student's t test, significantly different from control, P<0.05 (*), P<0.01 (**), P<0.001 (***).

Effect of copper on histology of ovary of fish *Cyprinion watsoni*:

Gross histology of ovary indicated a great number of atretic follicles in the treated groups (exposed to 0.08 ppm copper) after 7, 14, 21, 28 days of treatment respectively and atresia was observed in stage III, IV and stage V follicle. Atretic follicles in the control ovaries were lesser in number compared to treated group. Effect of exposure to heavy metal copper was very drastic on zona radiata, granulosa and nuclear membrane of stage III, IV and stage V follicle respectively after 7,14, 21, 28 days of treatment respectively. Effect of exposure to copper was very pronounced on yolk vacuoles and yolk globules of stage IV and stage V follicles respectively after 7, 14, 21 and 28 days of treatment respectively.

Group I; Day7:

The gross histology of ovary of control fish showed that follicles of different stages were present, which include immature stage I, II and stage III yolk precursor follicles (Fig, 16a). Vacuolated Stage IV follicles and stage V vitellogenic follicles were also present in ovarian tissue of control ovary. Stage II follicles were present with normal and homogeneous cytoplasm and nucleus with nucleoli arranged toward the periphery or attached with the nuclear membrane. Stage III (Yolk precursor) follicle, showed basophilic homogenous cytoplasm with peripheral appearance of yolk vacuoles (Fig 16a). In the cross section of ovary of fish exposed to copper (0.008 ppm) all follicular stages (I, II, III, IV and V) were present. Gross histology showed stage I and stage II follicles remained unaffected in appearance but increased atresia of yolked follicles compared to control (Fig.16b). Stage III (yolk precursor) follicles displayed drastic changes including disintegration (wrinkling) of the developing granulosa layer and irregular nuclear membrane (Fig, 16c). Stage IV vacuolated and stage V vitellogenic follicles remained unaffected respectively after 7 days of copper treatment.

Group II; Day14:

Gross histology of ovarian tissue of control fish showed a great number of stage II and III follicles but less number of stage I, IV and V follicles were observed in the ovarian tissue. Stage II follicles were present near the ovarian wall, showing darkly stained cytoplasm, evenly distributed chromatin and nucleoli with well-defined

nuclear membrane (Fig, 17a). There was no pronounced effect of copper on number of stage I and stage II follicles after 14 days of copper treatment but stage III, IV and stage V follicles were greatly reduced in number in treated groups compared to control. In this group stage III (yolk precursor) i.e. previtellogenic and stage IV (Vaculoated) follicles were affected in appearance, these showed disintegrating of the developing granulosa layer and nuclear membrane became irregular (Fig, 18b). Another very prominent effect was increased atresia of stage III and IV yolked follicles (Fig, 18a). Stage V vitellogenic follicle remained unaffected after 14 days of copper treatment.

Group III; Day21:

Ovary of control fish showed a variety of follicles. Small immature stage I follicles were present throughout the ovarian region in between the previtellogenic and vitellogenic follicles. Stage II follicles were also present in the ovarian section, showing darkly stained cytoplasm, and nucleoli with well-defined nuclear membrane. Granulosa layer was developed in stage IV follicles and yolk vacuoles were present near the periphery (Fig, 19a).

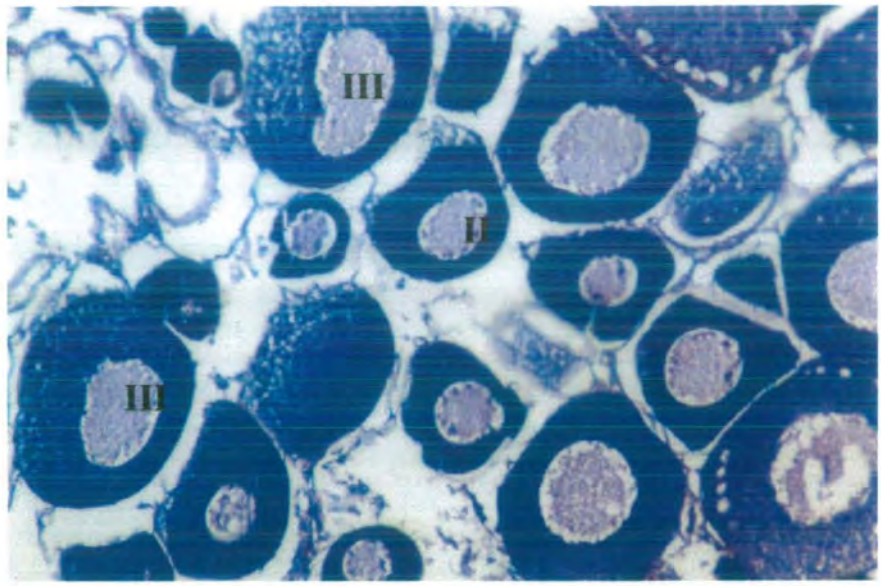
After 21 days of copper treatment, number of stage I follicles remained unaffected but number of stage II, III, IV and stage V follicles reduced. Number of atretic follicles significantly increased in treated group compared to control. Copper was unable to effect stage I and stage II follicles in appearance but drastic changes were observed in stage III (yolk precursor) and IV (vacuolated) follicles. In these follicles disturbed granulosa (Fig, 19b), disintegrating cytoplasm, irregular nuclear membrane and atresia can be observed. (Fig 19c). Stage V vitellogenic follicles remained unaffected in appearance after 21 days of copper treatment.

Group IV; Day28:

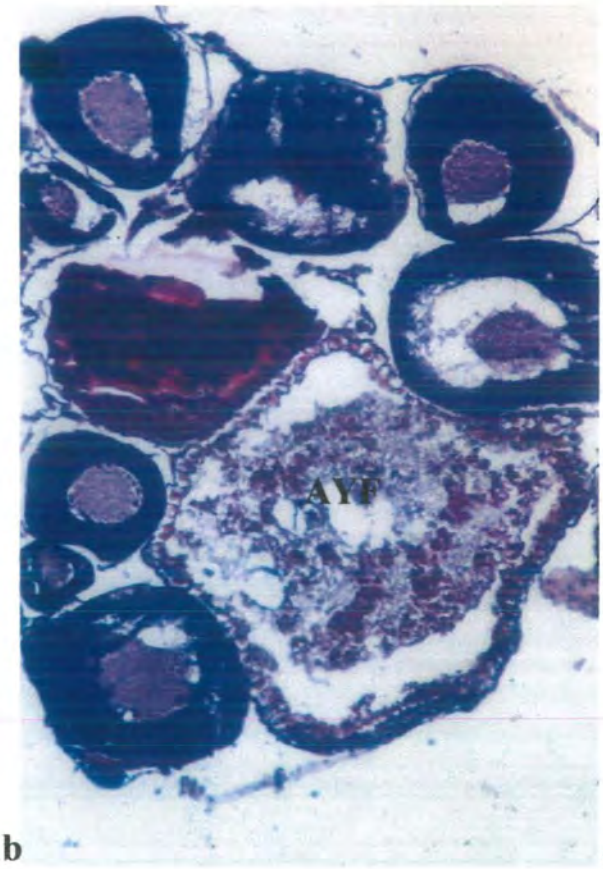
Ovarian tissue of fish showed great number of stage II follicles, but other follicles (I, III, IV and V) were less in number. Gross histology of ovary of control fish showed follicles of different stages, which include stage I to stage V follicles. Some stage I follicles were present near the ovarian wall and other were present throughout the ovarian tissue. Stage II follicles were present with normal and homogeneous cytoplasm with nucleus, having nucleoli arranged towards the

periphery or attached with the nuclear membrane. Stage III (Yolk precursor) follicle showed basophilic homogenous cytoplasm with peripheral appearance of yolk vesicles. Granulosa layer was developed in stage IV follicle and yolk vacuoles were present near the periphery. Nuclear region contain homogenous nucleoplasm with nucleoli arranged in the periphery of nucleus (Fig, 20a). Stage V follicles i.e. vitellogenic follicles displayed bright red stained yolk globules in between nucleus and non-staining yolk vacuoles. Yolk vacuoles were present near the zona radiata layer (Fig, 23a). At this stage these mature vitellogenic follicles developed granulosa, zona radiata externa, and zona radiata interna and well developed thecal cells formed outer to granulosa layer (Fig, 22a). Striations were also prominent in zona radiata of vitellogenic follicles (Fig, 22a). Yolk vacuoles were tightly attached to follicular layer (Fig, 23a).

Copper was unable to affect theca cells after 28 days of copper treatment but follicular layer (theca, granulosa and zona radiata) in stage IV and V follicles separated from inner yolk in a gradual manner (Fig, 22d). There was also complete disappearance of non-staining yolk vacuoles and replaced by thick condensed striations of yolk material. (Fig, 23b), in addition striations of zona radiata were converted into blackish striation in treated groups (Fig, 22b). Stage IV follicles showed increased yolked follicular atresia, vacuolation in peripheral yolk and disintegration of nuclear membrane (Fig, 20b,c). In addition other drastic changes were also observed in stage V follicles, these changes were decrease in thickness of zona radiata and granulosa layer, atresia of yolked follicles, degeneration of nucleoli and clumping of yolk globules (Fig, 21b).



a



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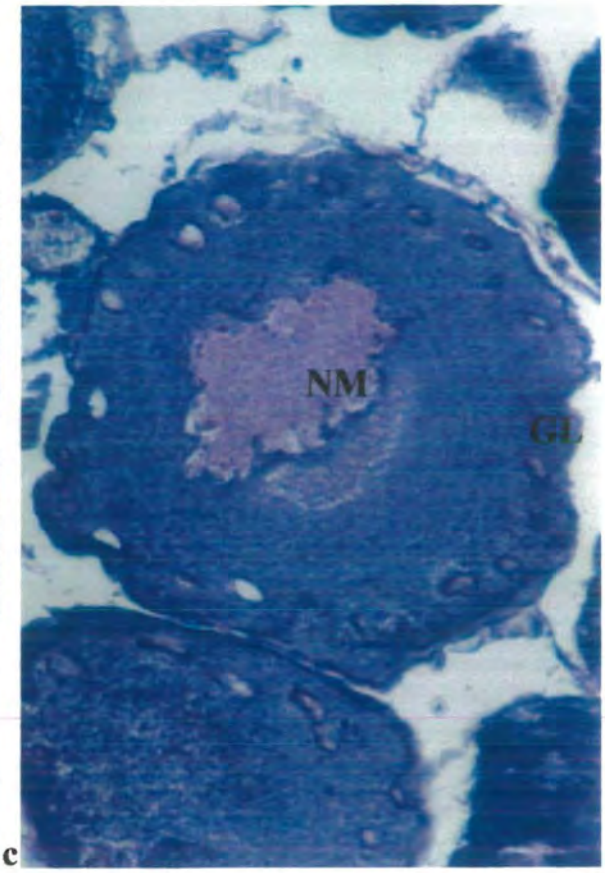


c

Fig 16: Photomicrographs of section of control and treated ovary of *Cyprinion watsoni*. **a.** Control, showing a number of stage II & III oocytes with well-defined nuclear membrane (NM) X 163.5. **b.** (exposed to copper, 0.08 ppm for 7 days), showing atresia of yolk follicles (AYF) X 164.97. **c.** Follicles of same (treated) ovary, showing disintegration of developing granulosa layer (GL) and nuclear membrane (NM) X 329.95.

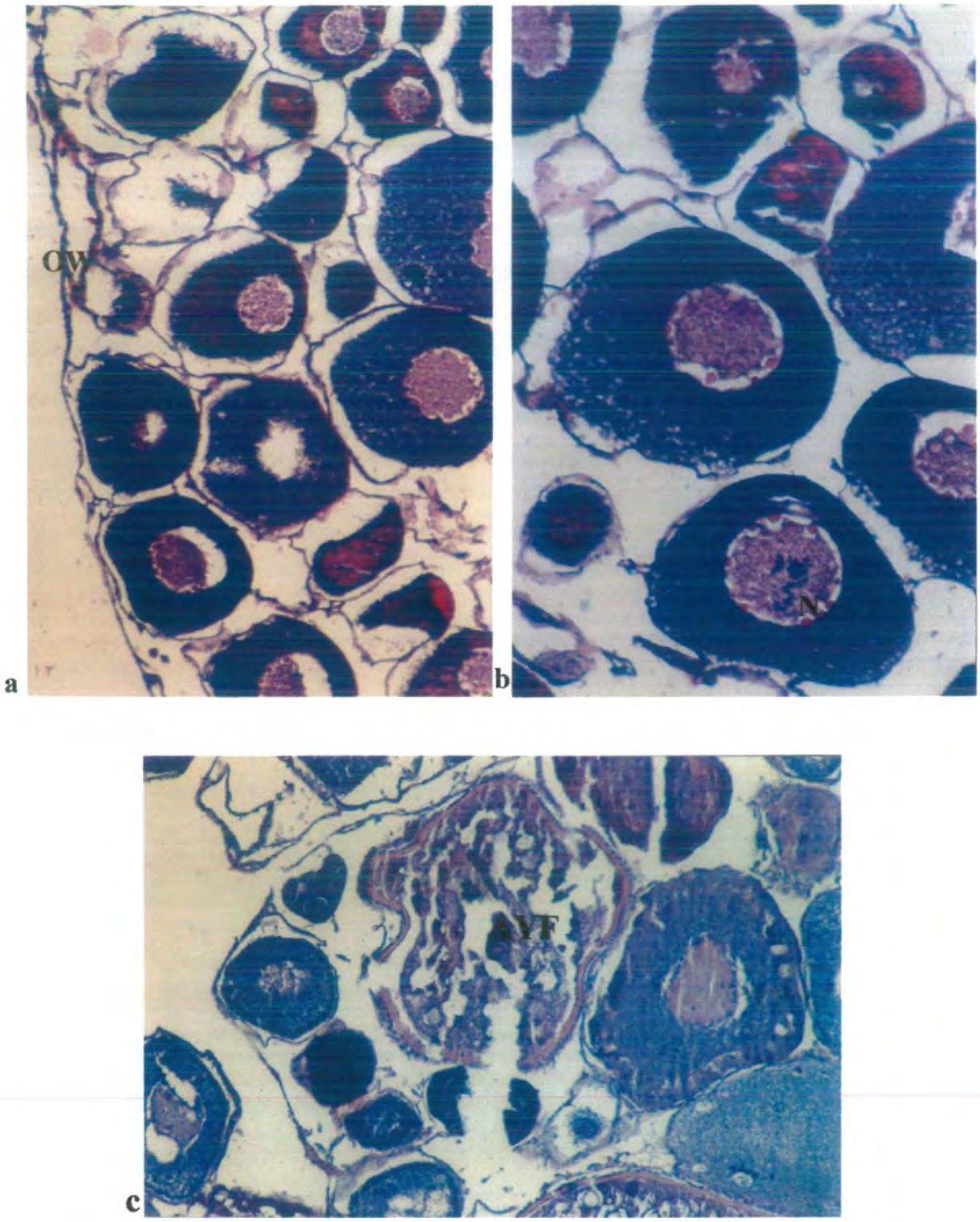
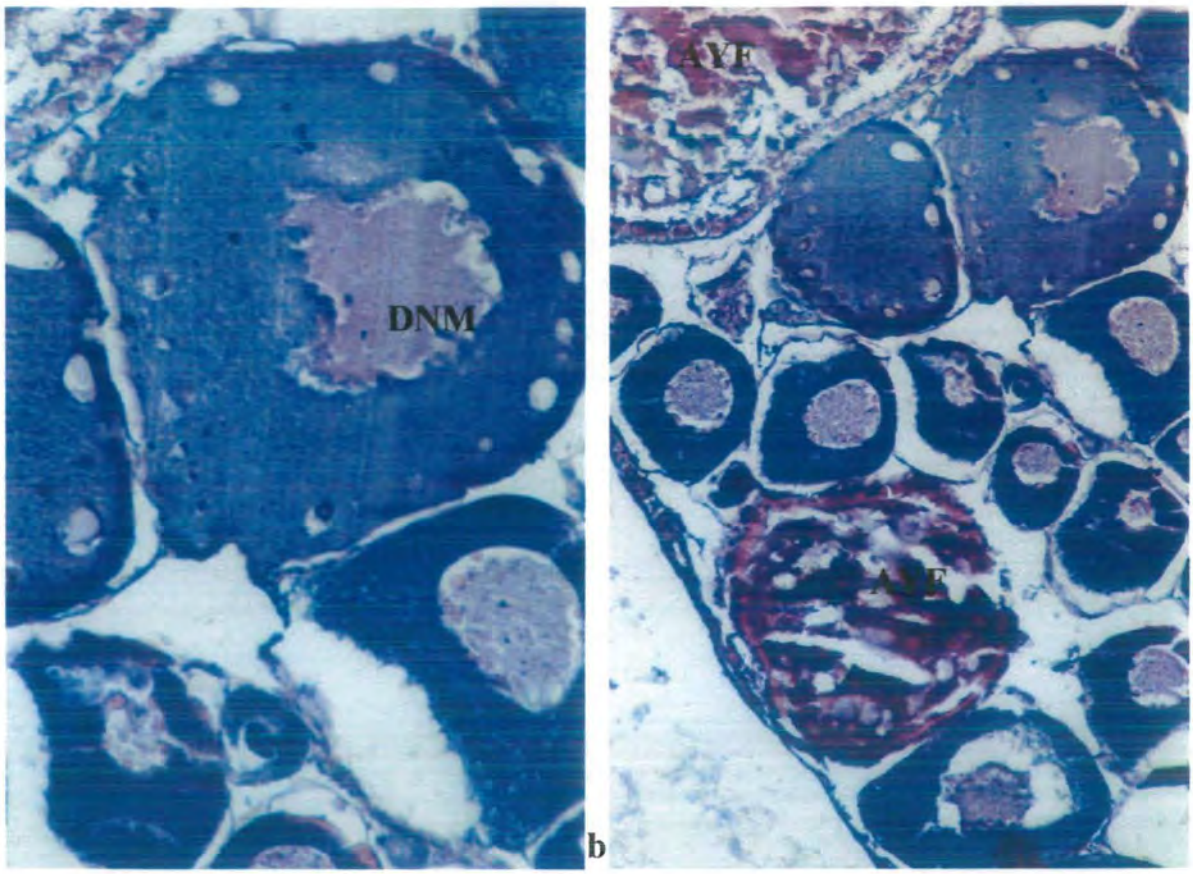


Fig 17: Photomicrographs of section of control and treated ovary of *Cyprinion watsoni.* **a.** Control, showing ovarian wall (OW) with variety of follicles X 65.99. **b.** Control, showing stage II oocytes with well-defined nuclear membrane and nucleoli (N) X 329.95. **c.** (exposed to copper, 0.08 ppm for 14 days), showing increased atresia of yolked follicles (AYF) X 163.5.



a b

Fig 18: Photomicrographs of section of treated ovary of *Cyprinion watsoni.* **a.** (exposed to copper 0.08ppm for 14 days), follicle showing disintegration of nuclear membrane (DNM) 327.11 X. **b.** Follicles of same ovary, showing atresia of yolk follicles (AYF), disintegration of developing granulosa layer 163.55 X

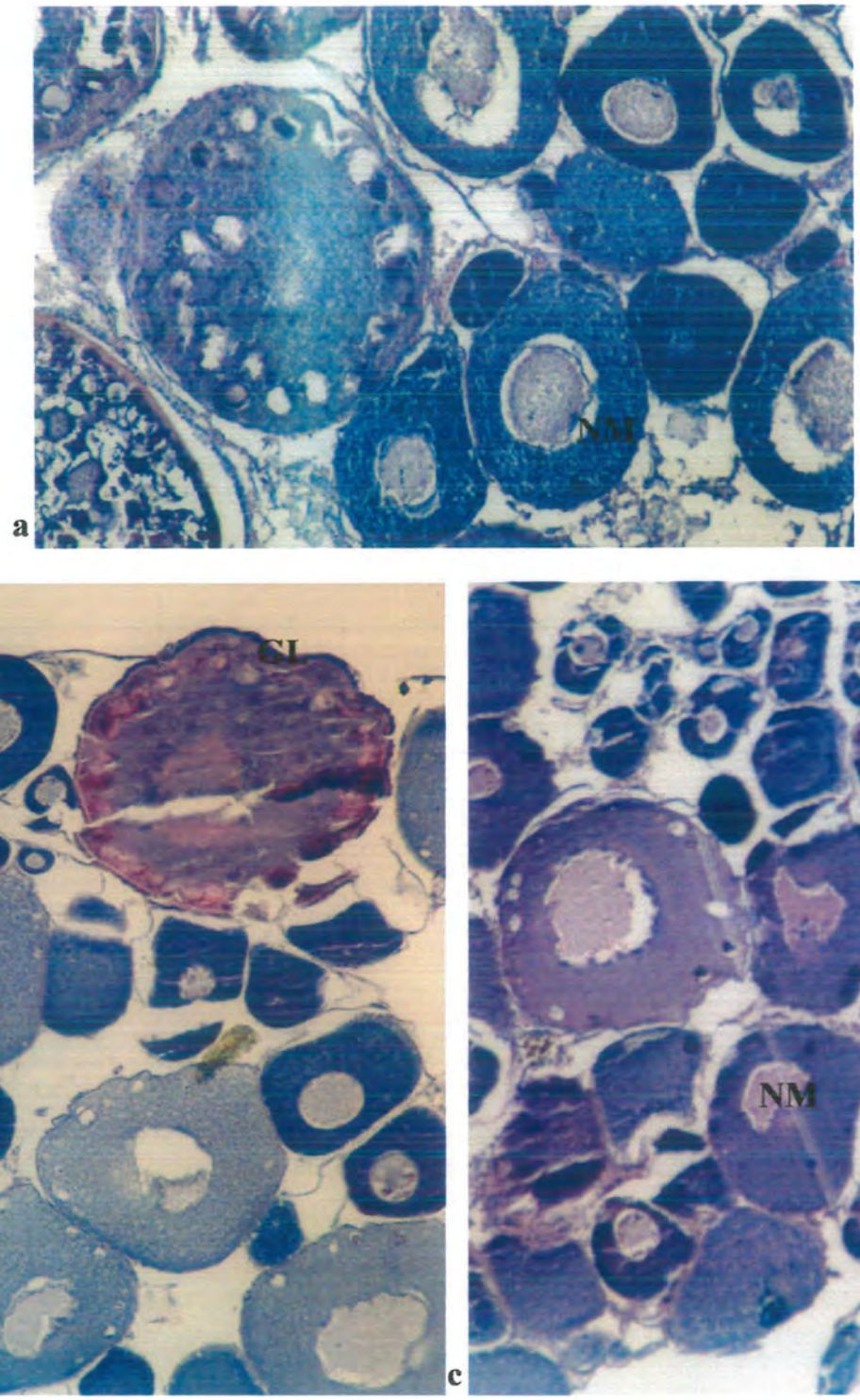
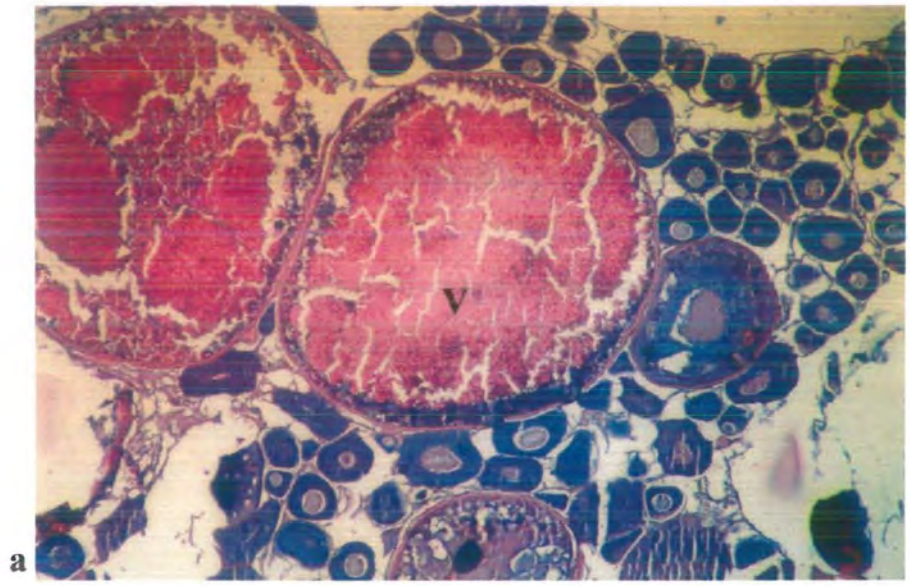


Fig 19:Photomicrographs of section of control and treated ovary of *Cyprinion watsoni* **a.** Control, showing follicles with well - defined nuclear membrane (NM) 164.97 X. **b.** (exposed to copper, 0.08 ppm for 21 days, showing wrinkling of developing granulosa layer (GL) of stage IV follicles 168.2 X. **c.** Follicles of same (treated) ovary, showing disintegration of nuclear membrane (NM) 163.5 X.



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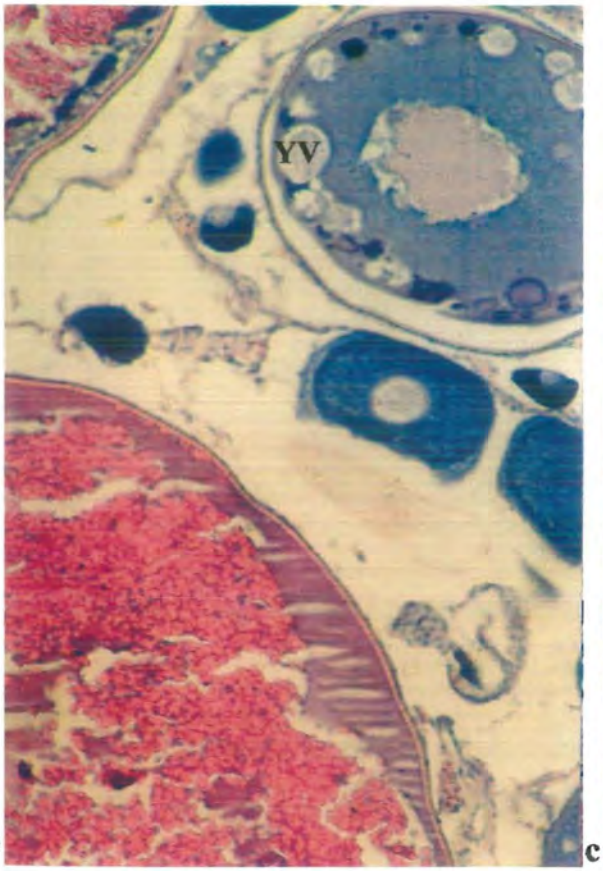

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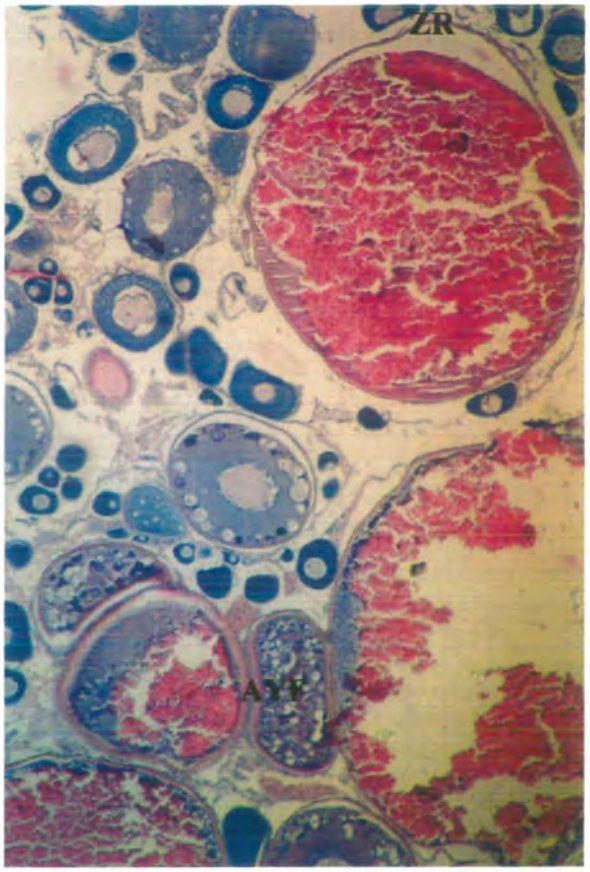


c

Fig 20: Photomicrographs of section of control and treated ovary of *Cyprinion watsoni*. **a.** Control, showing variety of follicles and vitellogenic follicle (V) 64.85 X. **b.** (exposed to copper, 0.08 ppm for 28 days), increased vacuolation in peripheral yolk (YV) 163.5 X. **c.** Another treated follicle, showing separation of zona radiata (ZR) from inner yolk and increased atresia of yolk follicles (AYF) 65.42 X.

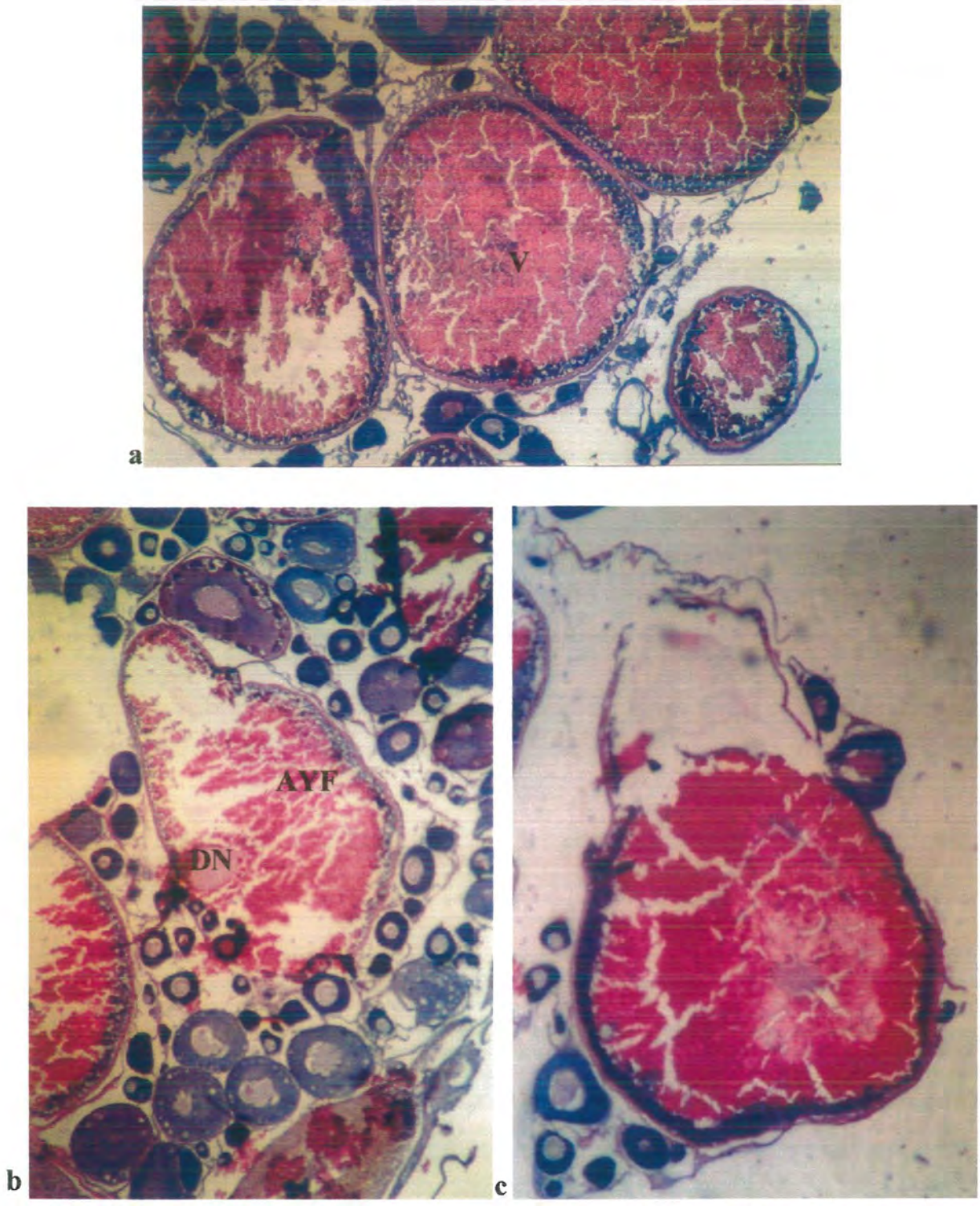


Fig 21: Photomicrographs of section of control and treated ovary of *Cyprinion watsoni* .**a.** Control, showing vitellogenic follicle (stage V) 65.4 X. **b.** (exposed to copper, 0.08 ppm for 28 days), showing atresia of yolk follicles (AYF), degenerating nucleus (DN) 65.4 X. **c.** same (treated) ovary, showing clumped pattern of yolk 65.4 X.

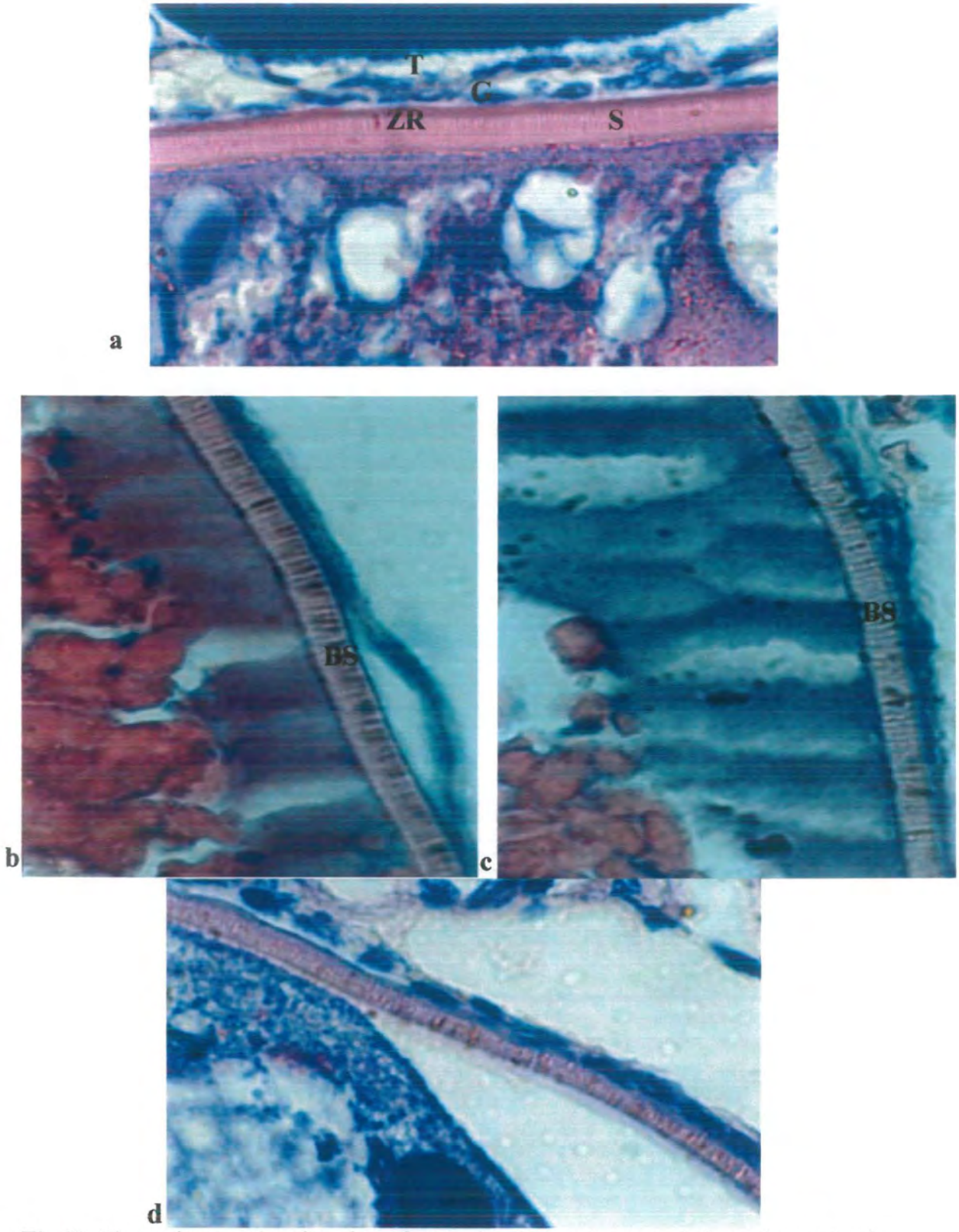
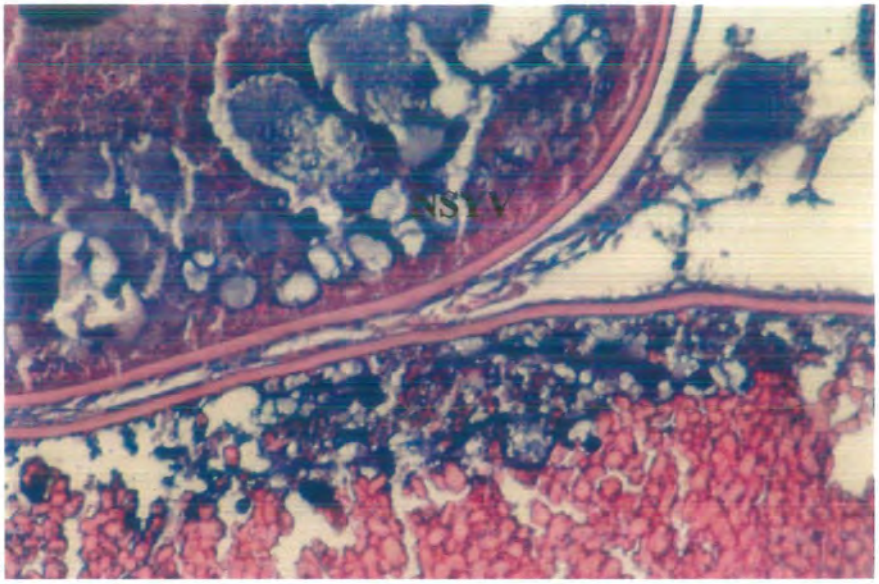


Fig 22: Photomicrographs of section of control and treated ovary of *Cyprinion watsoni.* **a.** Control, showing striations (S) of zona radiata (ZR), theca (T) and granulosa cells (G) 1543.11 X. **b, c;** (exposed to copper, 0.08 ppm for 28 days), showing blackish striations (BS) of zona radiata 1201.77 X. **d.** Other follicle of same (treated) ovary showing gradual separation of zona radiata 1450.66 X.



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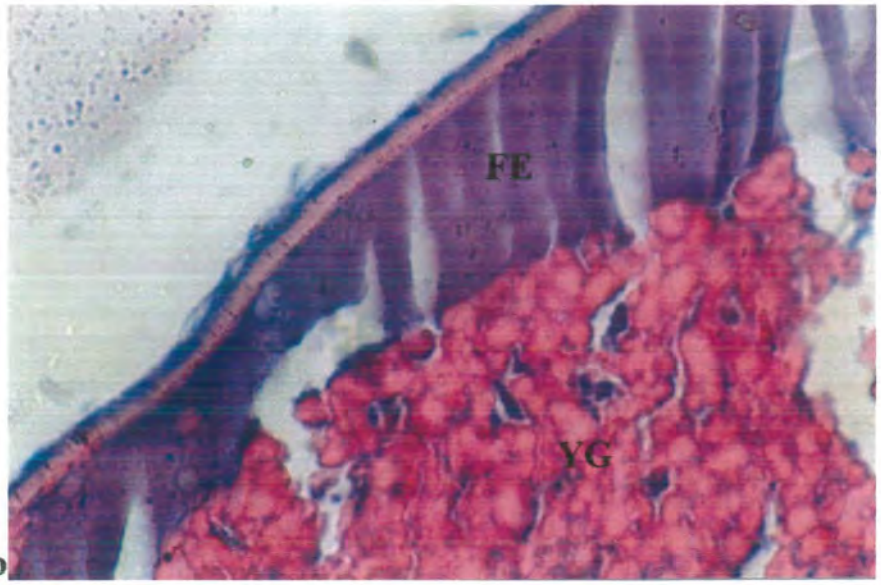

b

Fig 23:Photomicrographs of section of control and treated ovary of *Cyprinion watsoni* **a.** Control, showing normal non-staining yolk vacuoles (NSYV) 163.5 X. **b.** (exposed to copper, 0.08 ppm for 28 days), showing complete disappearance of non-staining yolk vacuoles replaced by fiber like tissue (FE) of follicular layer and clumping of stained yolk globules (YG) 327.11 sX.

DISCUSSION

DISCUSSION

Main objective of the present study was to investigate the potential effects of exposure to heavy metal copper on several reproductive parameters with the ovary considered as the most probable target organ of fish (*Cyprinion watsoni*), keeping in view the follicular development at various stages, follicle numbers, atresia, gonadosomatic index (GSI) and condition factor (K).

The use of *Cyprinion watsoni* as a test organism is ecologically relevant in hilly areas like Islamabad owing to their abundance in fresh water ecosystem. Furthermore, it is small, easy to handle in aquaria, and suitable for use in many disciplines e.g. Developmental Biology, Reproductive Physiology, Endocrinology and Ecotoxicology (Shaikh and Jalali, 1986).

Behavioral changes as shown by *Cyprinion watsoni* due to exposure to copper sulphate were typical sign of uneasiness and stress. After addition of copper sulphate solution the first observation was uneasiness of fish and they moved towards the bottom of the aquarium, moving very fast in circular motion. Similar observations have been reported in *Cyprinion watsoni* after Copper sulphate treatment (Shah, 2002). Their studies showed that copper sulphate treatment (0.03 mg Cu/L) caused little change in fish behaviour, while 0.06 mg Cu/L caused increased swimming activity and breathing movements. On the other hand due to high dose (0.12 mg Cu/L), fish became lethargic and lost equilibrium.

Similar observations have been reported in catfish (Heteropneustu fossililis) after chromium chloride treatment (Dhaked, *et al.*, 1993) and in Cirrhina mirgala after zinc treatment (Sharma and Sharma, 1995). In an early study, Vogal (1959) has shown that gold fish exposed to 1 mg/l of copper develops Wilson disease.

Stress and uneasiness may be due to depletion of energy in the body of animal. A drop in the metabolic production of cellular energy in the form of high-energy bond in Bluegill sunfish (Lepomis macrochirus) on exposure to copper has been reported (Ellgaard and Guillot, 1988). Decreased and increased glucose levels on cadmium exposure have been reported in Hetropneustes fossilis and Labeo rohita

respectively (Das and Banerjee, 1980). The varying levels of blood glucose are indicative of abnormal carbohydrate metabolism and are possibly the result of impaired hormonal control (Andersson, *et al*, 1988). The release of corticosteroid hormones in Sockeye salmon (Oncorhyncus nerka), when treated with copper has been reported (Donaldson and Dye, 1975).

Collier, (1992) has investigated that size of fish is an important determinant of reproductive success in fish. After 7, 14 and 21 days of copper treatment neither fish length nor body weight showed any significant change ($P > 0.05$). Sehgal and Saxena (1986) showed body weight of female treated fish (Lebistes reticulates) was no affected by 278-mg/L zinc. But after 28 days of copper treatment both fish length and body weight of treated fish significantly decreased ($P<0.05$). Again it may be due to depletion of energy in the body of animal as mentioned earlier (Ellgaard and Guillot, 1988). Ovarian weight of *Cyprinion watsoni* showed no significant difference ($P>0.05$) after 7, 14 and 21 days of copper treatment but it decreased significantly ($P<0.01$) after 28 days of treatment in fish exposed to copper. Sehgal and Saxena (1986) have also showed decreased ovarian weight in their study on the toxicity of 278mg/L zinc for 20 days in Lebistes reticulates. Copper caused no significant difference ($P>0.05$) in ovarian length and breadth after 7, 14 and 21 days of copper treatment. But after 28 days of copper treatment, ovarian length and breadth of treated fish was significantly decreased ($P<0.05$) compared to control fish. However, the impact of these changes in ovarian size (length and breadth) and ovarian weight on the reproductive fitness of effected fish is not clear but it is likely that these will tend to produce altered size and number of eggs that has been associated with lower growth and survival rates in other fish species (Buckley, 1991), because low ovarian weight may be associated with suppressed ovarian development in adult female fish.

A condition factor (K) was determined in both the control and treated animals. So the influence of emaciation on ovarian development could be distinguished from any potential effect of contaminant exposure. In our study condition factor of fish exposed to copper in all groups showed no significant difference ($P>0.05$), compared to control fish. So the condition factor is a generalized indicator of the overall health of a fish and can reflect the integrate effect of nutritional status and metabolic stress.

Adams *et al*, (1992) studied the effect of contaminations on condition factor of fish in river that was contaminated with the effluent of pulp mill. Comparison of contaminated and uncontaminated water fish shows that condition factor was highly significant ($P<0.05$) in contaminated water fish than uncontaminated water fish. These results are not consistent with the present study. However our findings were in agreement with the previous findings (Jhonson *et al*, 1997), in which no significant intersite difference was found in condition factor (length, weight relationship) in English sole (Pleuronectes ventulus).

Gonadosomatic index is an indicator of the level of gonadal development. A gonadosomatic index of fish was determined. Copper caused no significant difference ($P>0.05$) in index after 7, 14 and 21 days of copper treatment. But after 28 days of copper treatment, GSI of treated fish was significantly decreased ($P<0.05$) compared to control fish. Dhawan and Kaur, (1997) observed concentration dependent decline in GSI in Cyprinus carpio and Cirrhina mirgala. They have reported that exposure of Cyprinus carpio to 56 and 100μg/L heavy metal zinc and Cirrhina mirgala to 560 and 1000 μg/L of heavy metal zinc for 60 days cause dose dependent decline.

Martin Diaz, *et al* (2005) reported decrease in GSI value, when female red swamp cray fish, (Procambarus clarkii) exposed to higher concentration of heavy metal zinc (3000μg/L), while low concentration of heavy metal zinc (1000μg/L) was unable to affect GSI of female cray fish. Similarly when female red swamp cray fish exposed to heavy metal cadmium (10 μg/L and 30μg/L), the GSI values were low, but not significantly different to the control animals.

The stimulation or inhibition of GSI has been directly related to stimulation or inhibition of ovarian maturation (Martin Diaz, 2005). In present study the reduction in GSI values observed, for *Cyprinion watsoni* exposed to heavy metal copper (after 28 days of treatment) showed significant decrease when compared with the control. It could be due to an increase or decrease in total body weights of fish. Sehgal *et al*, (1984) reported lowered gonadal activity in $HgCl_2$ exposed fish.

In *Cyprinion watsoni* before gonadal recrudescence began, the ovaries contained mostly follicles (oocytes) in primary growth stage (stage I follicle). Some pf these had entered the perinucleolar stage (Stage II follicles). As gonadal

development progressed, cortical alveoli began to appear near the follicle (oocyte) membrane (stage III and IV follicles). Subsequent to this, yolk accumulation became evident in the ooplasm (stage V follicle). Similar ovarian developmental patterns were previously reported by Shaikh and Jalali, (1986).

In our study after 7 days of copper treatment no significant effect ($P>0.05$) were noticed on follicle numbers of different follicular stages in treated and control groups. Follicle numbers of stage I and II remained unaffected ($P>0.05$) but follicle numbers of stage III, IV and V were significantly decreased ($P<0.05$) in treated group compared to control after 14 days of copper treatment. Except stage I, follicle numbers of all stages were significantly decreased ($P<0.05$) in treated group compared to control group after 21 days of copper treatment. All follicle stages are adversely affected after 28 days of copper treatment, follicle numbers in all stages were significantly decreased ($P<0.05$) in treated group compared to control. Kumar and Pant (1984) reported in their study on teleost fish (Puntius conchonius Ham) exposed to sublethal poisoning of copper, zinc and lead that follicle number of stage III significantly decreased in treated group compared to control after 2 and 3 month copper treatment but no significant effect was observed on other follicular stages and after 4 month of copper treatment follicle numbers of stage III and stage V were significantly decreased in treated groups.

The histological abnormalities and low percentage of mature follicles (oocytes) in 278 mg/L zinc treated fish (Lebister reticulates) has also been reported by Sehgal and Saxena (1986). In present observations, the processes of atresia in control *Cyprinion watsoni* was generally similar to that described by Shaikh and Jalali (1986) and in other teleost species (Lambert, 1970). Mild atresia of yolked follicles has been reported in many other teleost species and appears to be a normal part of the reproductive process (Babu and Nair, 1983).

In present study atresia of both yolk and non-yolked follicles were observed. When fish (*Cyprinion watsoni*) was exposed to heavy metal copper, numbers of atretic follicles were significantly increased ($P<0.05$) in treated group compared to control group after 7, 14, 21 and 28 days of copper treatment. Similar results were reported by Kumar and Pant, (1984) in their study on teleost fish (Puntius conchonius

Ham) exposed to sublethal poisoning of heavy metals (copper, zinc and lead). According to them number of atretic follicles significantly increased in copper treated group after 2, 3 and 4 month of copper treatment, similarly heavy metals (zinc and lead) showed significant increase in atretic follicles in treated fish compared to control fish.

The mode of action of heavy metals on the gonads of fish is not well understood. Decrease in follicle number and increase in number of atretic follicles in treated group may be due to inhibition of pituitary activity. Ram and Sathyanesan, (1983) has attributed the mercury induced pathological changes in the ovary and testes of Channa punctatus to the inhibition of activity of pituitary gonadotrophs and somatotrophs. Our findings though chiefly of morphological significance, do suggest a direct effect of heavy metal copper on the female gonad to produce pathological changes, besides their possible action through the pituitary. It seemed increased atresia in copper treated *Cyprinion watsoni* due to inhibition of gonadotropin production, it seemed realistic because there was also decrease in number of follicles (oocytes) of all stages.

The change in oocyte size can also be related with follicular diameter. In present study follicular diameter of Stage III was significantly decreased ($P<0.05$) in treated compared to control after 7, 14, 21 and 28 days of treatment. While follicular diameters of stage I, II and V were significantly decreased ($P<0.05$) in treated compared to control after 14, 21 and 28 days of copper treatment. But follicular diameter of stage IV was significantly decreased in treated fish compared to control after 28 days of treatment. As observed earlier by Dey and Bhattacharya, (1989) acute changes in diameter of different stages of follicles (oocyte) by treatment with elsan (211ppb), mercury (16.7ppb) and ammonia (15.65ppm) in a fish (Channa punctatus) but no remarked changes were observed in the diameter of stage I follicle with respect to control. However, they studied decrease in diameter of stage II and III follicles (oocytes), which is similar to present study. Decreased follicular diameter has also been studied by Dhawan and Kaur, (1997) in their study, when Cyprinus carpio and Cirrhina mirgala exposed to 56 and 560 μg/L concentrations of heavy metal zinc. Massanyi, *et al* (2005) reported that follicular diameter was significantly decreased in

treated rabbits compared to control after administration of heavy metal cadmium to rabbits. In present study heavy metal copper adversely effect nuclear diameter of follicles. Nuclear diameter of stage I and IV was significantly decreased ($P<0.05$) in treated fish compared to control fish after 7, 14, 21 and 28 days of copper treatment. While nuclear diameter of stage II and III was significantly decreased ($P<0.05$) in treated group compared to control group after 14, 21 and 28 days of copper treatment. But stage V follicle diameter was adversely affected ($P<0.05$) in treated after 21 and 28 days of copper treatment. Decrease in nuclear diameter of follicles was observed by Khan, (2001), when fish (Tilapia nilotica) was exposed to heavy metal zinc.

In present findings, heavy metal copper adversely effect nucleoli number of follicles. Nucleoli numbers of stage III, IV and V follicles (oocyte) of treated fish decreased significantly ($P<0.05$) compared to control fish after 7, 14, 21, 28 and 14, 21 days of copper treatment respectively. Similar results were reported by Khan, (2001), when fish (Tilapia nilotica) was exposed to heavy metal zinc, there was dose dependent significant decrease in nucleoli number of stage III and IV follicle. In our study, nucleoli number of stage I decreased significantly ($P<0.05$) after 21and 28 days of copper treatment and significant decrease in nucleoli number of stage II follicle after 7, 21 and 28 days of copper treatment. According to Khan, (2001) nucleoli number of stage I and II increased in treated fish (Tilapia nilotica) compared to control.

The histological examination also reveals that copper causes adverse effect on theca, granulosa and zona radiata of follicles of fish (*Cyprinion watsoni*). In present study separation of follicular layer from inner yolk material, clumping of yolk globules, disintegrating of nuclear membrane, wrinkling of developing granulosa layer, increased vacuolation in yolk, decrease in thickness of zona radiata, conversion of zona radiata striations into blackish striations, complete disappearance of non staining yolk vacuoles and replaced by thick condensed striations of yolk were observed in stage IV and V follicles of treated fish after 28 days. Conversion of zona radiata striations into blackish striations, complete disappearance of non staining yolk vacuoles and replaced by thick condensed striations of yolk may be due to some biochemical changes in copper treated groups. While disintegration of developing

granulosa layer and nuclear membrane was observed in stage III and IV follicles of treated fish after 7, 14, 21 and 28 days of copper treatment. The result of present study suggests that exposure to heavy metals in the environment may have a pronounced effect on the ovarian developmental process in *Cyprinion watsoni*. These findings are consistent with several reports in the literature linking disrupted or inhibited ovarian development to contaminant exposure in other fish species (Johnson et al., 1988; Murugesan and Hanfia, 1992).

Heavy metal copper has drastic effect on ovarian histology of fish after 28 days of treatment so it can effect population of fish, *Cyprinion watsoni* in future.

In conclusion the present findings suggest that we must be aware of the effects of copper pollution, because the accelerating industrial and agricultural development coupled with rapidly increasing population in the developing countries, exerts considerable pressure on ecosystems. The recent increase in chemical production and its usage in Asian countries is a real threat, which can destroy nature. So it is need of time to make policies for the regulation of environment, which can be accomplished if it is based on the knowledge of environmental toxicology in this region. Such research is extremely important, if we have to understand the potential impact of degradation of freshwater fish population. Studies that will enhance our ability to identify species, which may be at risk for contaminated induced reproductive impairment are critical to successful conservation and management of freshwater fish population.

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