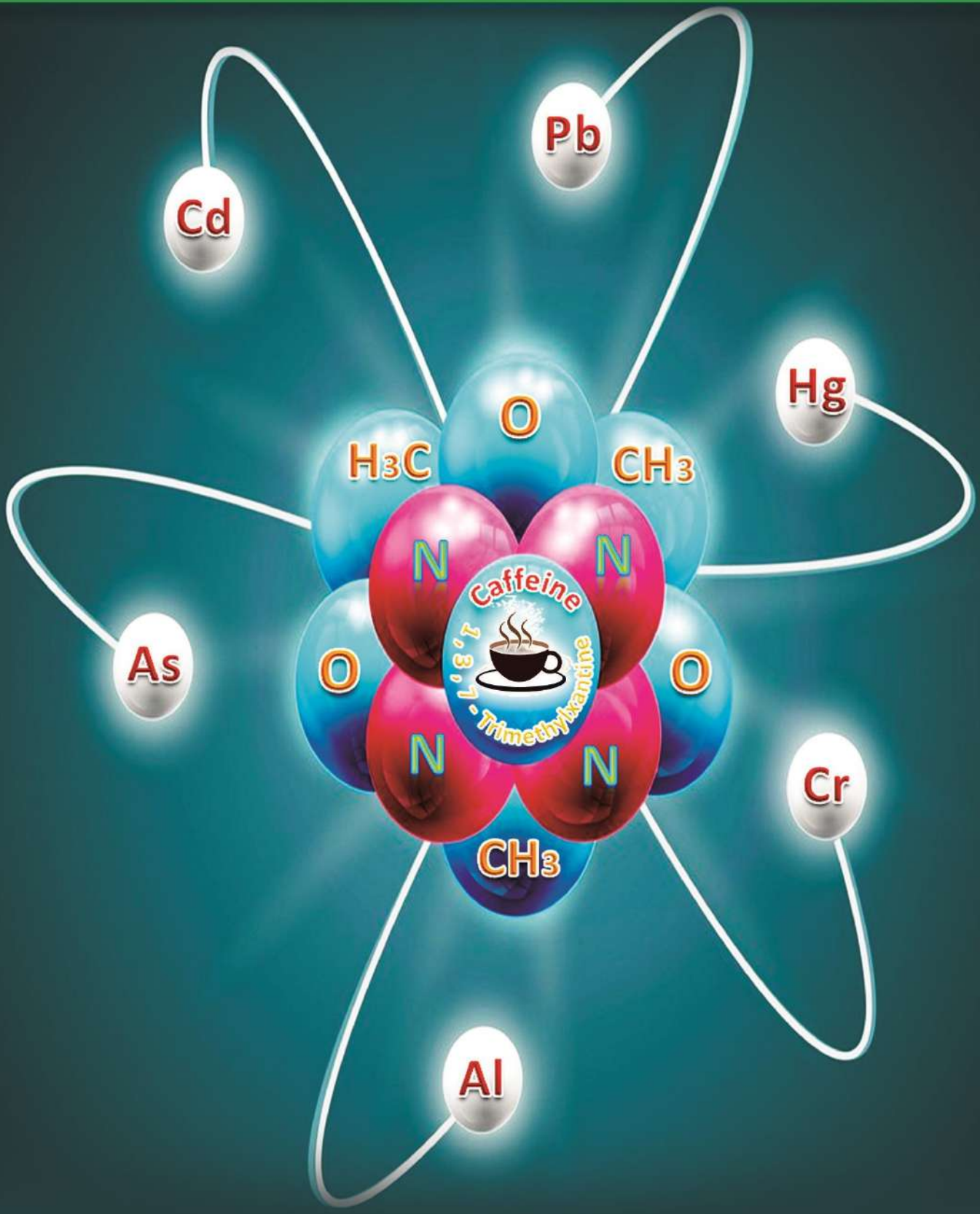
CAFFEINE (1,3,7-TRIMETHYLXANTHINE) AS A MODULATOR OF HEAVY METALS



Dr. Shamsundar D. Gulbhile

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"I find the Power of Dreams with the Help of Study and Research"

This Book Dedicated to ...

My Grand Father
Late. Dewaji Bapurao Gulbhile

&
My Parents
Shri. Dhondiram Dewaji Gulbhile
Sau. Ratan Dhondiram Gulbhile

They Always Guide, Support, Inspire & Teach Me.....

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Dr. Gulbhile Shamsundar Dhondiram

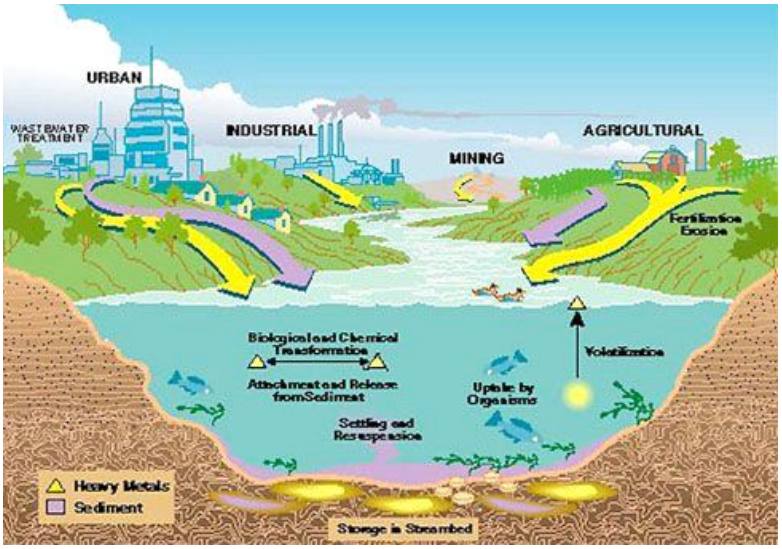
PREFACE

Progresses in industrial revolution and agriculture during the modern developments in the latter half of the 19th century, has exploited the natural resources indiscriminately leading to the uneven distribution of toxic compounds in natural bodies causing the pollution.

Source of Images: Internet

The global problem of pollution by heavy metals in the environment is increasing due to the human activities. Metals form almost two thirds of the chemical elements listed in the periodic table. Typically, elemental metals have near luster (reflected light glow), are solid at room temperature, can loss electrons to form a positive ion and readily conduct heat electricity. There are about 40 heavy metals, though again the exact number depends on definitions used. One definition, based on

specific gravity, excludes metal that another definition includes, based on atomic number. There are so many different definitions that the term lacks precise chemical meaning. Many chemists simply assert cyclonically that a heavy metal is "a metal that behaves in a heavy metal manner". Some heavy metals that are typically monitored in environmental surveys are listed.

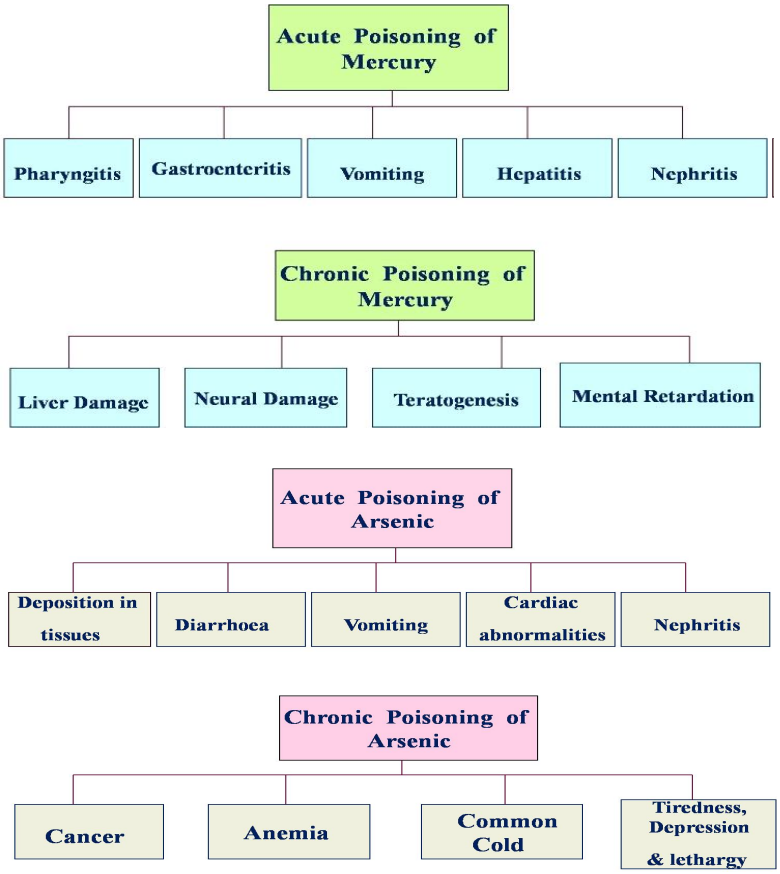


Source of Image: Internet

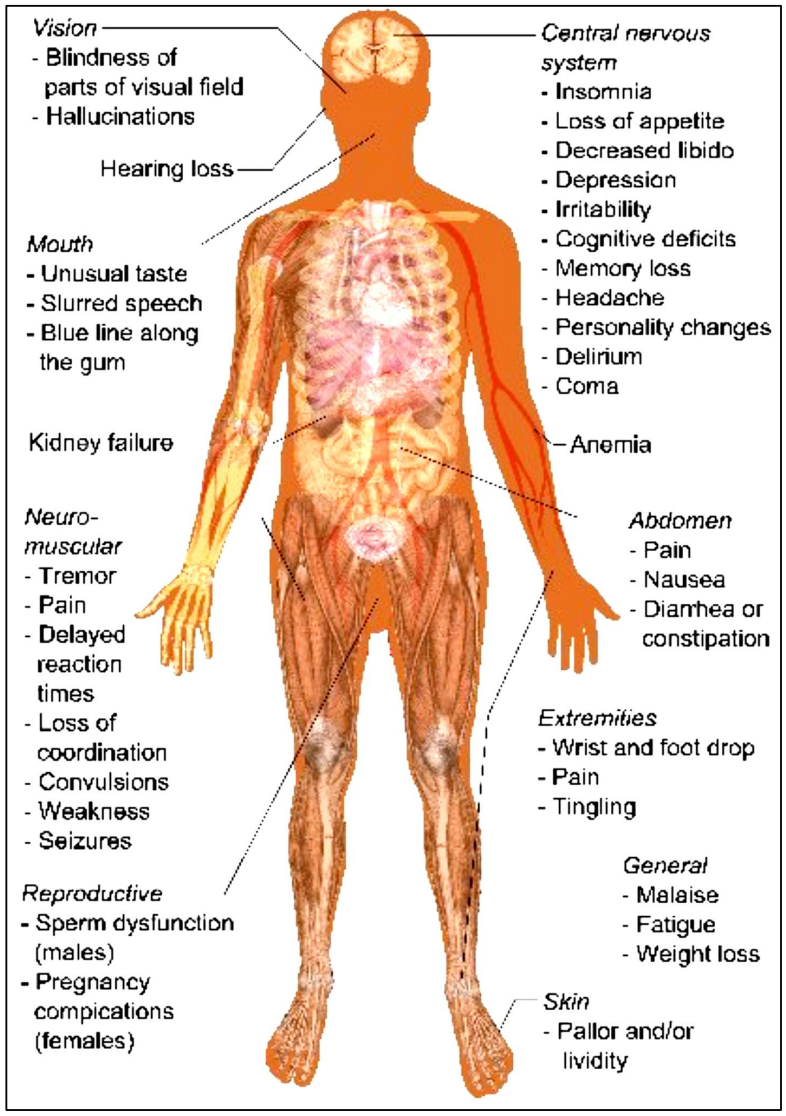
Heavy metals are widely distributed in the Earth's Crust. Most have a rather patchy distribution worldwide, with scattered pockets of higher concentrations. Heavy metals weathered from natural rock formations spread widely in the environment occurring in particulate or dissolved form in soils, rivers, lade seawater and sea floor sediments.

Fate of Heavy Metals:-

A metal is regarded to be toxic if it impairs growth, reproduction and metabolism of organisms, when supplied above a certain concentration. Uptake of heavy metal by living organism causes the death. Mercury and Arsenic are recognized as toxic contaminants of our environment. These highly toxic heavy metals such as mercury and arsenic enter into the body of living organism including man through non-vegetarian and vegetarian diet and drinking water and accumulate in the tissues. Mainly heavy metals react with protein and disturb the physiological activities, hence increasing level of heavy metals cause risk of life in the different ways. A main problem of the in toxic effect of heavy metals is that they are very difficult to remove from the body of animal, because they are usually bound to some legends. The heavy metals bind to the cell membrane. Therefore, they are very difficult to remove from cell membrane.



Mercury poisoning shows the symptoms such as weakness, loss of appetite, loosening of teeth, insomnia, irritability, loss of memory, indigestion, diarrhea etc. and arsenic poisoning make people tried, depressed and lethargic. Arsenic poisoning is difficult to diagnose. The great challenge is for removing Mercury and Arsenic from water and body of animals, and from global environment. After various works on the detoxification of heavy metals, the chelation therapy is useful way to detoxify the heavy metals.



Source of Image: Internet

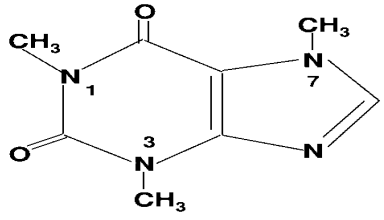
Use of the Chelators:-

Chelators are particular substances that bind to heavy metals and speeds their elimination. The united states of public health service, in

collaboration with the National Institutes of Health, organized a study of EDTA Chelation in 1981and reported that EDTA Chelation therapy for arteriosclerosis should be considered experimental and without substantial incidence to support its clinical use. Most of the clinical reports, documenting appropriation of EDTA chelation for lead intoxication, originated in the early 1950s (Foreman *et al.*, 1953). According to the reports of American Heart Association, side effects of EDTA includes anemia, blood clotting, bone marrow damage, fever, insulin shock, irregular heartbeat, kidney damage, joint pain, difficult and painful urination etc.

Reasons of the Selection Caffeine for Detoxification:-

Heavy metal contents in water were found to be much reduced after addition of coffee, dissolved heavy metal ions are positively charged and coffee contain uncharged and negatively charged molecules, the metal ions might be taken out of solution by binding to negatively charged molecules of the coffee ground. Caffeine has oxygen at ortho and para position which can form a chelate with heavy metal. It can have the capacity to remove the heavy metals from the body of livings and prevent the damage of tissues.



Structure of Caffeine – 1, 3, 7– Trimethylxanthine

Caffeine is found to have antioxidant activity; this antioxidant activity of caffeine can protect the damage of tissues. Caffeine molecule is having a site that usually binds a divalent cation Ca^{++} and blocks the activity of Ca^{++} dependent enzyme. Mike McLaughlin of CSIRO Australia in February 2000 has proved that coffee has the capacity to bind with heavy metals. The caffeine being water soluble and common cheaper beverage, caffeine will be cheapest preventive and curative medicine. The caffeine increases the rate of urine formation and molecule of caffeine being small is easily excreted.

Reasons of the Selection of *Lamellidens corrianus* as an Experimental Model:-

Classification

Animalia
Mollusca
Bivalvia
Unionoidea
Unionidae
Lamellidens
Lamellidens corrianus
(Lea 1834)

For the present research work, *L. corrianus* is selected as an experimental model because the body wall, gill, digestive glands and Mantle of this animal are soft through which substance diffuses easily as compared to thicker skin. This animal is filter feeder, because all above-mentioned organs always both in toxicant contaminated water, large surface of the body is exposed to water at particular strength, so that there is no problem of feeding or injecting the doses and the effect will be prominent at low doses. The animals are easily available in large number and are easy to rear in the laboratory.

For the present investigation four chapters

are structured, which deals with different aspects as mentioned below:

The first chapter, Histomorphological study deals with the histomorphology of tissues such as gills, testis and digestive glands of the bivalves exposed to acute concentration of mercuric chloride and sodium arsenate with and without caffeine up to 96 hrs and latter recovery with and without caffeine.

The histological study revealed that the gill lamellae, testicular follicles and hepatic lobules were badly affected by heavy metals in proportion to the period of exposure. The histological alterations in the structure of gills, testis and digestive glands by heavy metal ions were minimum in presence of caffeine. The bivalves pre-exposed to different heavy metals showed fast recovery with respect to histomorphological structures of each tissue as compared to those, which recovered naturally. The photo plates of histomorphological changes are presented in the chapter. The results are discussed with the citation of literature and the references supporting the work are cited at the end. The probable role of caffeine is mentioned.

The second chapter deals with biochemical estimation of protein, collagen, ascorbic acid, DNA and RNA from gill, testis and digestive glands of control and experimental bivalves at various periods of exposure. It was observed that after acute exposure of mercuric chloride and sodium arsenate there was decrease in the protein, ascorbic acid, collagen, DNA and RNA content in various tissues of experimental bivalves as compared to those of control bivalve. The protein, collagen, ascorbic acid,

DNA and RNA contents were least affected in heavy metal salt with caffeine-exposed bivalves as compared to those exposed to only heavy metal salts. The bivalves showed fast recovery of tissue protein, collagen, ascorbic acid, DNA and RNA in presence of caffeine than those allowed curing naturally. The effect of mercuric chloride was found to be more drastic as compared to sodium arsenate. The results are discussed with the citation of literature and probable role of caffeine.

The third chapter deals with Enzyme study. The enzyme activity of alkaline phosphatase and acid phosphatase from tissues such as gills, testis, and digestive glands of control and experimental bivalve at various periods of exposure were estimated. It was observed that after acute exposure to mercuric chloride and sodium arsenate, there was increased acid and alkaline phosphatase activity in various tissues of experimental as compared to those of control bivalves. The alkaline phosphatase and acid phosphatase activities were not severely altered in heavy metal salt with caffeine than those exposed to only heavy metal salts. The bivalve showed recovery of tissues alkaline phosphatase and acid phosphatase activity in presence of caffeine and in normal water. The results are given in the tables with percent change over control and the results of statistical tests.

The fourth and last chapter deals with the Bioaccumulation. The heavy metal contents in tissue were found to be increased in the tissue of bivalves, which were exposed to heavy metals. The mercury and arsenic contents in the tissues of the bivalves were less in heavy metal salt with caffeine

than those exposed to only heavy metal salts. The heavy metals in the pre exposed bivalves showed decreased heavy metal content during recovery however the rate of the removal of the mercury and arsenic was more in the presence of caffeine.

The references regarding the related work done by the different authors are cited at the end of this book.

The author has brought out this research book with a specific motivation and direction for advanced research. This book will be useful for the UG, PG, Ph.D. and M.Phil. students, teachers, laboratory assistants and other stakeholders who willing do research. I am sincerely hope that, all stakeholders of this book would accept this idea with an open mind and encourage me for further literature.

- Author

CHAPTER : 1
HISTOPATHOLOGICAL STUDY

INTRODUCTION

Water is the vital resource necessary for life of all the organisms. The fresh water ecosystems like rivers, lakes, ponds, wells, reservoirs etc. provide us drinking water and also provide food by supporting plants and animals in and around them.

Due to the industrial revolution in the later half of the 19th century, the population explosion, modernization in industries, urbanization and mainly unwillingness and negligence by man, these natural resources are being exploited indiscriminately leading to their pollution.

Towards the water pollution problem, scientists have drawn the attention in the end of 19th century, and significant awareness has been developed in recent days. The problems of pollution are different in water bodies. Usually in ponds and lakes, the water contains high quantities of pollutants.

Generally, water is contaminated by toxic heavy metals and pesticides. Heavy metals are

elements having atomic weight between 63.546 and 200.590 (Kennish, 1992) and a specific gravity greater than 4.0 (Connell *et. al.*, 1984). Heavy metals are among the most dangerous contaminants. All heavy metal exits in the surface waters in colloidal, particulate, and dissolved phases, although dissolved concentrations are generally low (Kennish, 1992). The colloidal and particulate metal may be found in 1) hydroxides, oxides, silicates, or sulfides or 2) adsorbed to clay, silica, or organic mater. The soluble forms are generally ions or unionized organometalic chelates or complexes. The solubility of heavy metals in surface water is predominately controlled by the pH of water, the type and concentration of ligands on which the metal could be absorbed, the oxidation state of the mineral components and the redox environment of the system (Connell *et al.*, 1984).

According to Nieboer and Richardson (1980), a metal is regarded to be toxic if it impairs growth, reproduction and metabolism of organisms when supplied above a certain concentration. Living organisms require trace amounts of some heavy metals, including cobalt, copper, iron, manganese, molybdenum, vanadium, strontium and zinc. Excessive levels of essential metals however can be determined to the organism. Non-essential heavy metals of particular concern to surface water systems are cadmium, chromium, mercury, lead, arsenic, and antimony (Kennish, 1992).

Wood (1974) classified metals into three categories [1] Non critical, for e.g. sodium, potassium, calcium, magnesium and iron, [2] Toxic but very rare, or very insoluble e.g. rare metals like thorium and [3] very toxic, soluble and relatively accessible e.g. selenium, arsenic, zinc, mercury,

lead, copper, cobalt, nickel etc. The metals in the 3rd group are highly toxic.

Arsenic, cadmium, cobalt, chromium, copper, iron, mercury, maganese, molybdenum, nickel, lead, vanadium and zinc (Lisk, 1972) show deleterious effects on the water quality, soil quality, enters plants and animals through the food chain, and finally reaches in man in toxic concentration.

Mercury posses a great risk to humans especially in the form of methyl mercury, when mercury enters water, it is often transformed by microorganisms into the toxic methyl mercury. Symptoms of acute poisoning are pharyngitis, gastroenteritis, vomiting, nephritis, hepatitis and circulatory collapse while chronic poisoning may cause liver damage, neural damage, and teratogenesis (USEPA, 1987).

Regular and frequent intake of mercury causes cancer. Mother ingesting large amount of methyl mercury give birth to babies suffering from palsy, convulsions, and mental retardation (Matsumoto *et al.,* 1965). The experimental work indicates that mercury compounds are potent inhibitors of cell division and segregates the chromosomes (Ramel, 1967).

Exposure of children to phenyl mercuric propionate in house paint may cause Acrodynia (pink disease). The interval between mercury exposure and symptoms may vary from one week to several months (Hirschman, *et al.*, 1963).

A new general approach to the mechanism of action of heavy metals has been pioneered by Rothstein (1959). It is based on the assumption that the cell membrane is the first point of attack by heavy metals. The studies show that heavy metals bind to the cell membrane and the first

detectable change in cellular function are due to changes in the membrane either in inhibition of active transport process or in an increase in passive permeability. This membrane concept and the relevant studies up to 1960 have been the subject of a general review (Passow, *et al.*, 1961).

The relationship of toxic effects to the rates of release of organic mercury from organomercurial compounds has not been studied extensively. Chronic exposure to phenyl mercury acetate causes kidney damage in experimental animals (Fitzhugh *et al.*, 1950). Because this compound is rapidly degraded to inorganic mercury it is likely that the inorganic effects (Gage, 1964). Very little inorganic mercury is found in the brain, the target organ for alkyl mercury compounds (Gage, 1964, Norseth and Clarkson, 1971).

Arsenic ingestion can cause severe toxicity through ingestion of contaminated food and water causing, vomiting, diarrhoea, and cardiac abnormalities (Viessman and Hammer, 1985). After acute exposure the deposition in tissues is, in the order, liver, kidney, intestine, spleen, and lung (Dubois K.B., and F.M.K., 1959). Arsenic appears in hair about 2 weeks after the first exposure, where it is bound to the sulfide linkages in keratin, chronic exposure leads to accumulation in hair, bone, and skin (Joseph, 1971). Arsenic may be found in high concentrations in the hair; year after cessation of exposure and after most of the metal has been removed from the soft tissues (Gaddum, 1959).

Acute poisoning occurs because arsenic, especially in the form of As_2O_3 which is readily available, practically tasteless, has the appearance of sugar, and is quickly absorbed from the gastrointestinal tract. Oral intake is followed by a

symptomatic period of about 30 min. (Gaddum, 1959). The throat and stomach pain, vomiting may ensure, which can develop the life risk. Depressed urine flow is characteristic of acute arsenic intoxication. Death usually results in 1 to 3 days after arsenic poisoning due to nephritis. Dubois K.B., and F.M.K., (1959) proved that, diarrhoea and vomiting occur, but are less pronounced than in case of acute poisoning. The mucous membranes are affected, giving rise to symptoms of the common cold, the horny layer of the skin is stimulated, leading to the appearance of dark brown scales (Fenn *et al.,* 1968).

Increase of high level of arsenic in drinking water increases the risk of cancer of lung, skin, liver and the bladder of the population. Hyden *et al.*, (1997) reported that many well water have arsenic concentrations in the range of 100-800 ppb. Continued exposure to arsine gas generally results in symptoms similar to the picture of arsenic poisoning. However, a destruction of red cells takes place, resulting in a steady level of anemia. Skin keratoses result from prolonged exposure to arsenic and may become malignant. It seems unlikely that arsenic causes cancer in other tissues. Heavy metals are known to interfere with functional groups of micro molecules, the presence of heavy metal above threshold level results in irrevocable alterations in the microenvironment of the cell. Therefore, a continuos series of investigations have been performed using aquatic animals (Shastry and Sunita, 1984; Singh and Sahai, 1984).

Heavy metals enter the system of aquatic organisms via three main pathways. 1) Free metal ions and metal ions adsorbed on the particles that are absorbed through respiratory surface (e.g. gills)

readily diffused into the blood stream. 2) Free metal ions that are absorbed by the body surface are passively diffused into the blood stream. 3) Metals that are adsorbed on to food and particulates may be ingested; as well as there free ions are ingested with water (Connell *et al.*, 1984).

Mercury and Arsenic are the most potent and versatile pollutants. Their physiological hazards lie in the fact that they are not organ specific like some of the organic pesticides eg. The organophosphates, which are neuro inhibitors and organochlorine compounds like DDT; which primarily accumulates in the gonads. Heavy metals are hepatotoxic agents, which induce neoplasmic lesions in various tissues and cause general histogical damages. Histopathological changes in the hepatopancreas due to industrial pollutant and chlorinated hydrocarbons are on record (Eller, 1971; Bhattacharya *et al.*, 1975; Mukharjee and Bhattacharya, 1975; Dubale and Shah 1979). Saxena (1981) has reported neoplasia in the kidney of fresh water teleost fish, *Channa punctatus* due to cadmium exposure.

Shrivastava and Maurya (1991) studied the history of gill and intestine of fish *Mystus vittatus* exposed to chromium stress under the scanning electron microscope. Usharani (1986) reported acute and sub lethal effect of cadmium on histology of kidney of *Tilapia mossambica*.

The histopathological studies show that heavy metal caused tissue damage in aquatic organisms has been previously noted in a variety of animals by many workers, Vernberg and Vernberg (1972) in crabs exposed to mercury, Nimmo, *et al.*, (1971) in shrimps exposed to cadmium, Shriwastawa *et al.*, (1982a and 1982b)

in fish exposed to chromium, Ghate and Mulherkar (1977) in two species of prawns exposed to copper, and Khangarot and Somani (1980) in the fish *Punctius sophore* exposed to mercury are note worthy.

All scientists have proved that the heavy metal increases the risk of life in various ways leading to the death of organisms. Heavy metals are very difficult to remove from body; the damage of tissues caused by heavy metals may be recovered. Various antioxidants are used for recovery or reduce the damage of tissue due to heavy metals. Vitamin C and E are common antioxidants in the diet.

The caffeine molecule is a bitter alkaloid, which contributes to both acidity as well as the bitter properties of coffee. Chemically caffeine is 1, 3, 7-Trimethylxanthine and is structurally related to uric acid. It gets metabolized in the body by the biochemical processes of demethylation and oxidation. The main urinary metabolites are 1-methylxanthine, 1-methyluric acid, and an acetylated uracil derivative.

The most profound action of methylxanthine, the major metabolite of caffeine in the human body is to bind to the adenosine receptors of cells, as the structure of this chemical compound is very similar to 'adenosine', which is a chemical narturally produced by the nerve cells.

Caffeine has similar effect on several organ systems. They differ mainly in their relative potencies. Caffeine has an ability to produce central stimulation and thus is usually classified as central nervous system stimulant. However, it has other important effects, such as relaxation of smooth muscles (particularly in the brachioles and certain arterioles), stimulation of both cardiac and skeletal muscle and diuresis.

Caffeine protects the damage of tissues chemicals and genetic materials of organisms from the heavy metals generated free oxygen radicals. MSH calcium ion relase channel protein due to caffeine stimulation of malignant hyperthermia susceptible sarcoplasmic reticulum was reported by Shomer and Nickelson (1994).

The present research work, on the study of caffeine suplimentation on Mercury and Arsenic induced altrations on the histopathological study of Gill, Testis, and Digestive glands of an experimental model animal, the fresh water bivalve, *Lamellidens corrianus* has been carried out to study the efficacy of caffeine in heavy metal induced alterations.

MATERIALS AND METHODS

The selected model animals, the freshwater bivalves, *Lamellidens corrianus* were collected from the Paithan dam at Paithan Tal. Dist. Aurangabad (M.S.). After collection, the bivalves were acclimatized in the laboratory condition at room temparature for 2-3 days. The active acclimatized bivalves of approximately same size were selected for experiment.

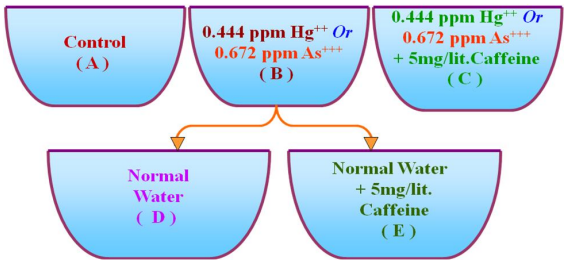
Before starting the experiment, these bivalves were divided into five groups such as A, B and C.

1. 'A' group bivalves were maintained as control.
2. 'B' group bivalves were exposed to acute dose ($LC_{50/2}$) of heavy metal salts, mercuric chloride (0.6ppm) and sodium arsenate (0.9ppm).
3. 'C' group bivalves were exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate with caffeine (5 mg/lit).

   Bivalves from B group were divided into D and E group after 4 day acute exposure.

4. 'D' group bivalves pre-exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate were allowed for self cure in normal water while,
5. 'E' group bivalves pre-exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate were exposed to caffeine (5mg/lit.) for rapid recovery from tissue damage.

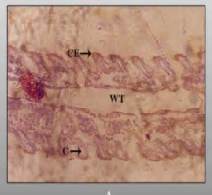
Experimental Setup:-



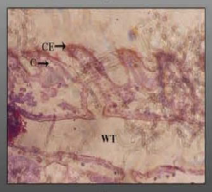
The experimental bivalves of A, B and C group were dissected after 24hrs and 96hrs and from D and E group of recovery after 2 days and 4 days and tissues such as gill, testis and digestive gland were fixed in Bouin's fluid, for 24hrs washed and dehydrated in alcohol grades, cleared in toluene and embedded in parafin wax (58-60°C). Prior to fixation gonads were screened by smear, techniques and only testis were fixed.

Prepared blocks of tissues were cut at the thickness of 6ì and stained with Mallory tripple stain. Stained slides with serial sections were examined under light microscope for histopathological impact. Gills, testes and digestive glands of bivalves from all groups i.e. control, exposed and recovery were screened and data is presented [Photomicroplates 1) A to 9) B.] for comparison.

PLATE 1



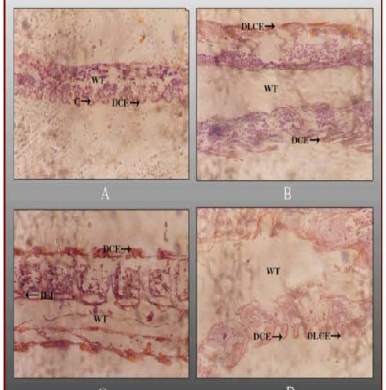
A



B

A) PHOTOMICROGRAPH OF NORMAL GILL OF *L.CORRIANUS* X100
B) PHOTOMICROGRAPH OF NORMAL GILL OF *L.CORRIANUS* X200

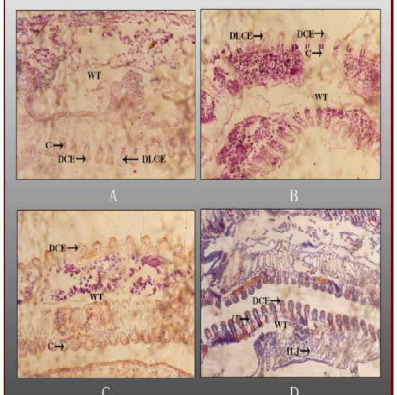
PLATE 2A



PHOTOMICROGRAPH OF GILL OF *L. CORRIANUS* ON EXPOSURE TO,

A) Mercuric chloride for 24 hrs
B) Mercuric chloride + Caffeine for 24 hrs
C) Mercuric chloride for 96 hrs
D) Mercuric chloride + Caffeine for 96 hrs
(Magnification x 100)

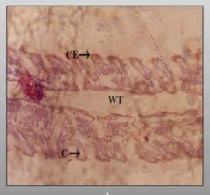
PLATE 2B



PHOTOMICROGRAPH OF GILL OF *L.CORRIANUS* DURING RECOVERY AFTER EXPOSURE TO MERCURIC CHLORIDE ON EXPOSURE TO,

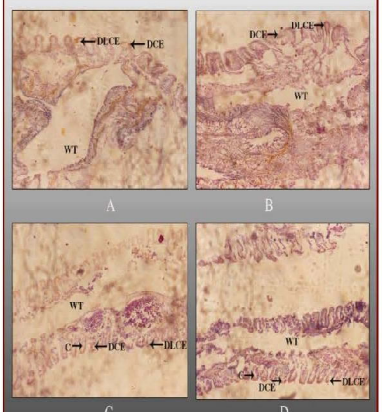
A) Normal water for 2 days
B) Caffeine for 2 days
C) Normal water for 4 days
D) Caffeine for 4 days
(Magnification x 100)

PLATE 1



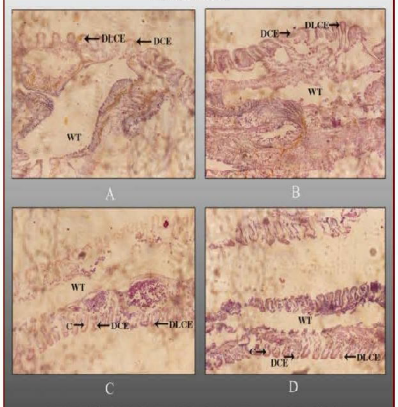
A) PHOTOMICROGRAPH OF NORMAL GILL OF *L.CORRIANUS* X100
B) PHOTOMICROGRAPH OF NORMAL GILL OF *L.CORRIANUS* X200

PLATE 3A



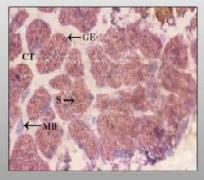
PHOTOMICROGRAPH OF GILL OF *L. CORRINUS* ON EXPOSURE TO,
A) Sodium arsenate for 24 hrs
B) Sodium arsenate+Caffeine for 24 hrs
C) Sodium arsenate for 96 hrs
D) Sodium arsenate +Caffeine for 96 hrs
(Magnification x 100)

PLATE 3B

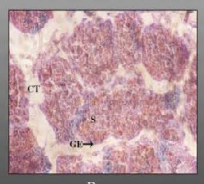


PHOTOMICROGRAPH OF GILL OF ***L.CORRIANUS*** DURING RECOVERY AFTER PRE- EXPOSURE TO SODIUM ARSENATE ON EXPOSURE TO,
A) Normal water for 2 days
B) Caffeine for 2 days
C) Normal water for 4 days
D) Caffeine for 4 days
(Magnification x 100)

PLATE 4



A

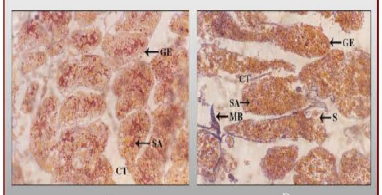


B

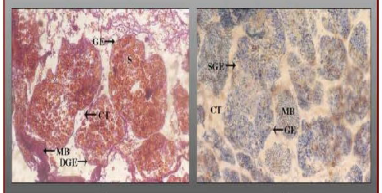
A) PHOTOMICROGRAPH OF NORMAL TESTIS OF *L.CORRIANUS* X100

B) PHOTOMICROGRAPH OF NORMAL TESTIS OF *L.CORRIANUS* X200

PLATE 5A



A B

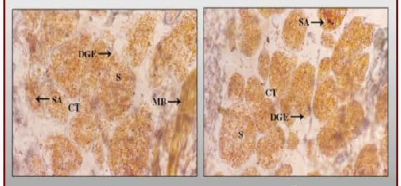


C D

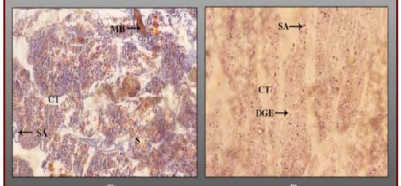
PHOTOMICROGRAPH OF TESTIS OF *L.CORRIANUS* ON EXPOSURE TO,

A) Mercurie chloride for 24 hrs
B) Mercurie chloride + Caffeine for 24 hrs
C) Mercurie chloride for 96 hrs
D) Mercurie chloride + Caffeine for 96 hrs
(Magnification x 100)

PLATE 5B



A B

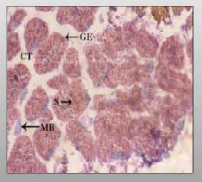


C D

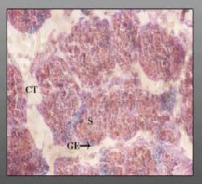
PHOTOMICROGRAPH OF TESTIS OF *L. CORRIANUS* DURING RECOVERY AFTER PRE-EXPOSURE TO MERCURIC CHLORIDE ON EXPOSURE TO,

A) Normal water for 2 days
B) Caffeine for 2 days
C) Normal water for 4 days
D) Caffeine for 4 days
(Magnification x 100)

PLATE 4



A

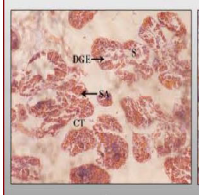


B

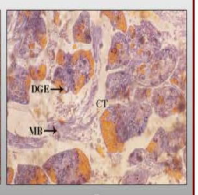
A) PHOTOMICROGRAPH OF NORMAL TESTIS OF *L.CORRIANUS* X100

B) PHOTOMICROGRAPH OF NORMAL TESTIS OF *L. CORRIANUS* X200

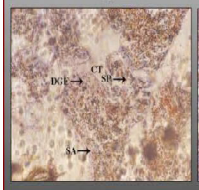
PLATE 6A



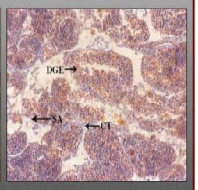
A



B



C

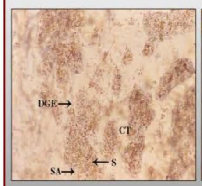


D

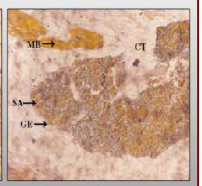
PHOTOMICROGRAPH OF *L. CORRINUS* ON EXPOSURE TO,

A) Sodium arsenate for 24 hrs
B) Sodium arsenate+Caffeine for 24 hrs
C) Sodium arsenate for 96 hrs
D) Sodium arsenate +Caffeine for 96 hrs
(Magnification x 100)

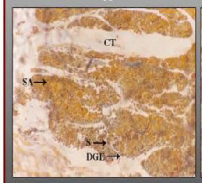
PLATE 6B



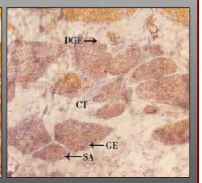
A



B



C

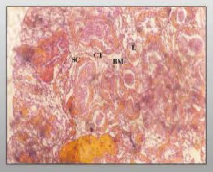


D

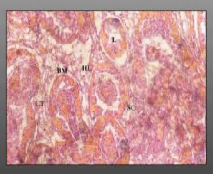
PHOTOMICROGRAPH OF TESTIS OF *L. CORRIANUS* DURING RECOVERY AFTER PRE-EXPOSURE TO SODIUM ARSENATE ON EXPOSURE TO,

A) Normal water for 2 days
B) Caffeine for 2 days
C) Normal water for 4 days
D) Caffeine for 4 days
(Magnification x 100)

PLATE 7



A

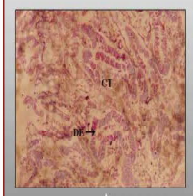


B

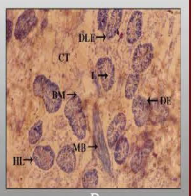
A) PHOTOMICROGRAPH OF NORMAL DIGESTIVE GLAND OF *L. CORRINUS*
X100

B) PHOTOMICROGRAPH OF NORMAL DIGESTIVE GLAND OF *L. CORRINUS*
X200

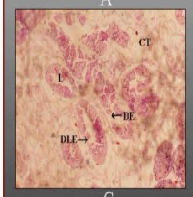
PLATE 8A



A



B



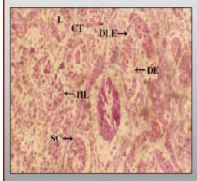
C

D

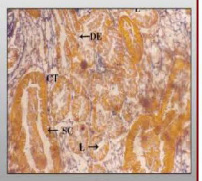
PHOTOMICROGRAPH OF DIGESTIVE GLAND OF *L.CORRIANUS* ON EXPOSURE TO,

A) Mercuric chloride for 24 hrs
B) Mercuric chloride + Caffeine for 24 hrs
C) Mercuric chloride for 96 hrs
D) Mercuric chloride + Caffeine for 96 hrs
(Magnification x 100)

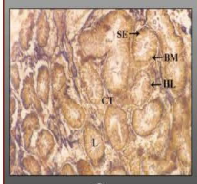
PLATE 8B



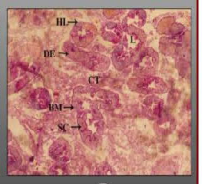
A



B



C

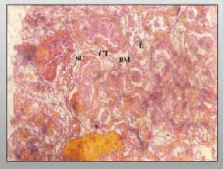


D

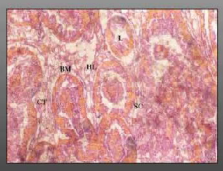
PHOTOMICROGRAPH OF DIGESTIVE GLAND OF *L. CORRIANUS* DURING RECOVERY AFTER PRE EXPOSURE TO MERCURIC CHLORIDE ON EXPOSURE TO,

A) Normal water for 2 days
B) Caffeine for 2 days
C) Normal water for 4 days
D) Caffeine for 4 days
(Magnification x 100)

PLATE 7



A

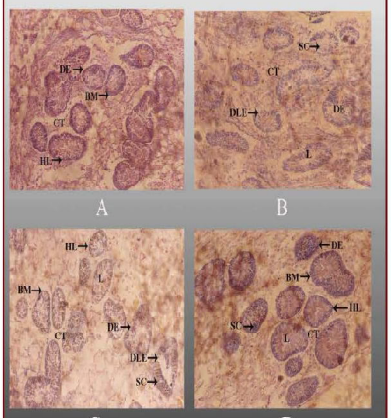


B

A) PHOTOMICROGRAPH OF NORMAL DIGESTIVE GLAND OF *L. CORRINUS* X100

B) PHOTOMICROGRAPH OF NORMAL DIGESTIVE GLAND OF *L. CORRINUS* X200

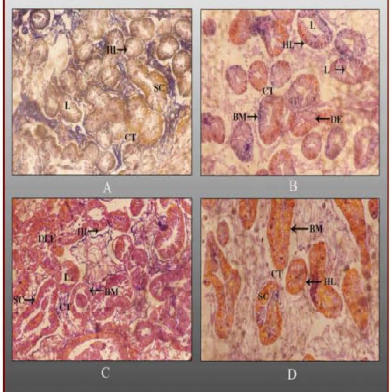
PLATE 9A



PHOTOMICROGRAPH OF DIGESTIVE GLAND OF *L. CORRINUS* ON EXPOSURE TO,

A) Sodium arsenate for 24 hrs
B) Sodium arsenate+Caffeine for 24 hrs
C) Sodium arsenate for 96 hrs
D) Sodium arsenate +Caffeine for 96 hrs
(Magnification x 100)

PLATE 9B



PHOTOMICROGRAPH OF DIGESTIVE GLAND OF *L. CORRIANUS* DURING RECOVERY AFTER PRE EXPOSURE TO SODIUM ARSENATE ON EXPOSURE TO,

A) Normal water for 2 days
B) Caffeine for 2 days
C) Normal water for 4 days
D) Caffeine for 4 days
(Magnification x 100)

OBSERVATIONS AND RESULTS

The effect of mercury and arsenic on gills, testis and digestive glands of *Lamellidens corrianus* after exposure to 0.444 ppm Hg^{++} and 0.672 ppm As^{+++} with and without caffeine and during recovery has been shown in the plate No. 1) A to 9) B of the photomicrographs.

The histological structure of these tissues from different groups indicates the effect of acute exposure to mercury and arsenic, Hg^{++} and As^{+++} with caffeine and recovery.

Bivalves are filter–feeders. As they pump the water, the gills filter out particles and remaining suspended material from the water. Gill is the main respiratory organ in the aquatic animals, which is directly exposed to the chemicals and contaminants or heavy metals present in water.

Histological Structure of Gill:-

Histological structure of gill shows mainly gill lamellae with the ciliated epithelium, lamellar junctions, chitious rods and water tubes as shown in Photomicroplate 1) A and 1) B.

The histological changes in the Gills of *Lamellidens corrianus* exposed to acute concentration of mercury (0.444 ppm) with and without caffeine for 96 hrs and during recovery are shown in photomicroplates 2) A and 2) B.

Histological changes after exposure to acute concentration of arsenic (0.672 ppm) with and without caffeine for 96 hrs and during recovery are shown in photomicroplates 3) A and 3) B.

Histological structure of Testis:-

Gonad is the main organ of reproductive metabolism, which expresses the heavy metal

impact on both testicular follicles and ovarian follicles. In the present investigation, impact of mercury and arsenic on testicular follicles is studied because the testicular follicles have similar histological structure through the maturity period while ovary is very dynamic having ovarian follicles and ova at different development stages and hence it is difficult to predict the response of the toxicant.

In the testis, mainly connective tissue, muscle bands, germinal epithelium, spermatogenic stages and sperm aggregates were focused, as shown in photomicroplate (4 A) and (4 B).

The histological changes in the testis of *Lamellidens corrianus* exposed to acute concentration of mercury (0.444 ppm) with and without caffeine for 96 hrs and during recovery are shown in photomicroplate 5(a) and 5(B) while after exposure to acute concentration of arsenic (0.672 ppm) with and without caffeine for 96 hrs and during recovery are given in photomicroplate 6(a) and 6 (b).

Histological structure of digestive glands (Hepatopancreas):-

Digestive gland is a main organ of storage of metabolic reserves, which provide a source of energy during the period of physiological stress in addition to its secretion for the digestion of food. The hepatopancreas shows hepatic lobules, with columnar cells and secretory cells, both resting on basement membrane. The lobules of the gland are bound together by the thin connective tissue layer as shown in photomicroplate 7 (a) and 7 (B).

The histological changes in the digestive gland of *Lamellidens corrianus* exposed to mercury (0.444 ppm) with and without caffeine for 96 hrs

and during recovery are shown in photomicroplate 8 (a) and 8 (B), and exposed to arsenic (0.672 ppm) with and without caffeine for 96 hrs and during recovery are presented in photomicroplate 9 (a) and 9 (B).

After mercuric chloride exposure, the lamellae of gills showed various changes, such as rupture of the ciliated epithelium, increase in the size of lamellae, increase in space between the inter lamellar junction and increase in space between the water tubes and inter lamellar junctions. Normal structure of gills are totally damaged or disturbed due to the mercuric chloride showing fusion and atrophy of secondary gill lamellae, displacement and necrosis of outer layer of gill lamellar epithelium.

After 24 hrs and 96 hrs of exposure to mercuric chloride, the change in the cytoarchitecture of gills of *Lamellidens corrianus* were more severe as compared to the gills of those bivalves, exposed to mercuric chloride with caffeine.

During the period of recovery, the gills in normal water showed space between inter lamellar junctions and water tubes, and damaged ciliated epithelium while the recovery was faster in gills of caffeine exposed bivalves with respect after 2 days and 4 days. During recovery, the nature of damage of lamellar ciliated epithelium was less than mercuric chloride exposed gill lamellae.

Caffeine exposed bivalves showed that the caffeine increased the rate of recovery and recovered from the damage early as compared to the normal recovery in water indicating its role in detoxification of mercury.

After sodium arsenate exposure, the lamellae of gills showed various changes, such as rupture of the ciliated epithelium, increase in the size of lamellae, increase in space between the inter lamellar junctions and increase in space between the water tubes and inter lamellar junctions. Normal structure of gills is totally damaged or disturbed showing the fusion and atrophy of secondary gill lamellae, displacement and necrosis of outer layer of gill lamellar epithelium due to the sodium arsenate.

After 24 hrs and 96 hrs of exposure to sodium arsenate, the change in the cytoarchitecture of gills of *Lamellidens corrianus* were severe as compared to the gills of those bivalves exposed to sodium arsenate with caffeine.

During the period of recovery, the gills in normal water showed space between inter lamellar junctions and water tubes, and damaged ciliated epithelium, while the recovery was faster in gills of caffeine exposed bivalves with respect to these changes after 2 days and 4 days.

During recovery, the nature of damage of lamellar ciliated epithelium was less than sodium arsenate exposed gill lamellae. Caffeine exposed bivalves showed that the caffeine increased the rate of recovery and reduced the damage as compared to the normal recovery in water indicating its role in detoxification of arsenic.

After mercuric chloride exposure, testis of *Lamellidens corrianus* showed disturbed testicular follicles, excessive degeneration of germinal epithelium of testicular follicles, distortion of spermatids and pyknosis of spermatogenic cells,

and the sperm aggregations. There was pronounced thinning of interlobular connective tissue. Normal structure of testis was totally damaged or disturbed due to the mercuric chloride.

After 24 hrs and 96 hrs of exposure to mercuric chloride, the changes in the cytoarchitecture of testis of *Lamellidens corrianus* were more severe as compared to the testis of those exposed to mercuric chloride with caffeine.

During the recovery of the testis of *Lamellidens corrianus*, in normal water distorted testicular follicles and sperm aggregations recovered to some extent, while caffeine induced the faster recovery in the testis of mercury exposed bivalves, after 2 days and 4 days. During the recovery, caffeine exposed bivalves showed increased the rate of recovery and reduced the damage as compared to the normal recovery in water.

After sodium arsenate exposure, testis of *Lamellidens corrianus* showed disturbed testicular follicles, excessive degeneration of germinal epithelium of testicular follicles, distortion of spermatids and pyknosis of spermatogenic cells, and the sperms aggregations. There was pronounced thinning of inter lobular connective tissues. Normal structure of testis was totally damaged or disturbed due to the sodium arsenate.

After 24 and 96 hrs of exposure to sodium arsenate, the change in the microscopic structure of testis of *Lamellidens corrianus* were more severe as compared to the testis of those exposed to sodium arsenate with caffeine.

During the recovery of the testis of

Lamellidens corrianus in normal water distorted testicular follicles and sperm aggregations recovered to some extent while caffeine induced the faster recovery in the testis of arsenic exposed bivalves, after 2 days and 4 days. The severity of damage of testicular follicles was less than sodium arsenate exposed testis with caffeine.

Caffeine exposed bivalves showed that, the caffeine increased the rate of recovery, reduced the damage as compared to the normal recovery in water.

On exposure to mercuric chloride, the hepatic lobules of digestive gland (Hepatopancreas) showed swelling. The basement membrane was ruptured, separation of epithelium from the basement membrane, enlargement of columnar epithelial cell and secretory cells, necrosis and vacuolization of cells, increase in the size of lumen, loss of cytoplasm and degeneration of nucleus of cells were observed after exposure to Hg^{++}.

After 24 and 96 hrs of exposure to mercuric chloride, the changes in the cytoarchitecture of hepatic lobules of *Lamellidens corrianus* was more severe as compared to the hepatic lobules of those exposed to mercuric chloride with caffeine.

The number of damaged hepatic lobules was less in those exposed to mercury with caffeine as compared to those exposed to only mercury. During the recovery, the hepatic lobules of hepatopancreas in normal water showed space between epithelium and its basement membrane, the damaged epithelial cells, increased size of lumen and loss of cytoplasm in cells while the recovery was faster in digestive gland of caffeine exposed bivalves after 2

and 4 days. The increase of recovered hepatic lobules in hepatopancreas of caffeine exposed bivalves indicates that the caffeine increased the rate of recovery as compared to normal water on exposure to sodium arsenate, the hepatic lobules of digestive gland (hepatopancreas) showed swelling. The basement membrane was ruptured, separation of epithelium from the basement membrane, enlargement of columnar epithelial cells and secretory cells, necrosis and vacuolization of cells, increase in the size of lumen, loss of cytoplasm and degeneration of nucleus of cells were observed after exposure to As^{+++}.

After 24 and 96 hrs of exposure to sodium arsenate, the changes in the cytoarchitecture of hepatic lobules of *Lamellidens corrianus* was more severe as compared to the hepatic lobules of those exposed to sodium arsenate with caffeine.

The number of damaged hepatic lobules was less in those exposed to arsenic with caffeine as compared to those exposed to only arsenic.

During the recovery, the hepatic lobules of hepatopancreas in normal water showed space between epithelium and its basement membrane, the damaged epithelial cells, increased size of lumen of loss of cytoplasm in cells while the recovery was faster in digestive gland of caffeine exposed bivalves after 2 and 4 days. The increase of recovered hepatic lobules in hepatopancreas of caffeine-exposed bivalves indicates that the caffeine increased the rate of recovery as compared to normal water.

The nature of damage in gills, testis, and digestive gland observed in mercuric chloride exposed bivalves was more as compared with those

exposed to sodium arsenate. Simultaneous use of caffeine showed protection by caffeine on heavy metal induced alterations. Faster recovery was observed in all tissues on exposure to caffeine.

The present investigation indicates that, caffeine has a protective and curative role in the mercury and arsenic inducted alterations. The histological structure of gills, testes and digestive gland showed less damage in the presence of caffeine and the recovery from the mercury and arsenic induced alterations in the structure of gills, testes and digestive glands were faster in presence of caffeine.

DISCUSSION

The histopathological study shows that, alterations caused by mercury and arsenic in various tissues is tissue specific and time dependent.

Histopathological studies were also useful in evaluating the pollution level of pesticides, since trace amount of these chemicals which do not bring animal mortality over a given period, were capable of producing considerable organ damage (Kumar and Pant, 1984). Number of workers has reported the degenerative changes in selected tissues in response to pollutants by various toxicants (Eller, 1971; Bhattacharya *et al.*, 1975; Anees, 1978; Dubale and Shah, 1979 Goel and Garg, 1980; Ram and Satyanesan, 1984; Banerjee and Bhattacharya, 1997).

Histological approach is the most valuable tool for assessing the action of toxicant at tissue level and for its manifestation of structural and functional changes (Sprague, 1971).

Histopathological abnormalities caused due to toxicity of heavy metals in animals have been reported earlier (Khalid Shareef *et al.*, 1986; Srivatsava *et al.*, 1982).

The effect of various compounds of the heavy metals is mainly studied in invertebrates, amphibians and mammals (Laborda *et al.*, 1986 and Joshi and Patil, 1995).

Gills function as the major route for the uptake of heavy metals as they are the most permeable regions of the body (Victor, 1993a; 1994). Mahajan and Zambare (2001) studied the effect of copper and mercury on the fresh water bivalve *Corbicula striatella* and observed that the gills are highly affected due to continuous exposure to toxicants. The heavy metals cause severe damage to gill surface and reduce oxygen uptake capacity of respiratory organs (Nonnotte *et al.,* 1993). Prasad *et al.,* (2000) observed the damage of the gill tissue marked by curling of secondary lamellae, rupture of gill rackers, displacement and necrosis of outer layer of lamellar epithelium due to the exposure to toxicants.

Heavy metals interferes the respriratoy mechanism by disrupting the structure of gills in crustaceans and fishes (Jones, 1975; Victor *et al.*, 1985; Victor, 1993 a and b; 1994, Vogen *et al.,* 2001). The overall factors influencing the accumulation of metals is absorption of ions by membrane, interference in gills and the uptake or diffusion by active or passive mechanisms (Carpentieri and George, 1981). The effect of chromium stress on gill and intestine of *Mystus vittatus* for prolonged period of 75 days was

observed by Shrivastava and Maurya (1991). The histological changes observed in the organs were the normal irregular concentric ridges on primary gill lamellae and smooth surface of secondary gill lamellae, changes to symmetrical concentric ridges resulting into crevices and furrows on the surface.

Khare and Singh (2002) observed that after short-term exposure to copper sulphate, gill arch remained unaffected, while in the case of lead nitrate slight damage was seen in the cartilagenous and muscular part of the gill arch. After long term exposure of copper and lead, severe damage was seen in the gill arch.

Most of the chondroblast cells were found in shrunken condition. The damage was more severe in the case of lead nitrate in comparison to copper sulphate. Gupta and Rajbanshi (1979) observed the degeneration of blood cells, blood capillaries and cartilagenous cells in the gills of *Heteropneustes fossilis*.

After long term exposure to copper sulphate, primary gill lamellae showed hyperplasia at certain places while on exposure to lead nitrate, heavy necrosis was noticed by various reporters such as, Daoust *et al.,* (1984); Wagh *et al.*, (1985); Versteeg and Giesy (1986); Thatheyus *et al.*, (1992) and Gupta and Rajbanshi (1995). Matei *et al.*, (1993) have also reported degeneration, hypertrophy and hyperplasia in the secondary gill lamellae. Vijayalakshmi and Tilak (1996) reported that sublethal concentration of toxicants induced fusion and atrophy of secondary gill lamellae.

Gill injuries such as separation of gill epithelium, fusion of secondary lamellae,

degeneration, of epithelial cells, nuclei and dissolution of the basement membrane have also been reported in the fresh water fish, *Aplocheius* and *Puntisus sophore* after the treatment of heavy metals like Cu and Hg (Khangarot and Somani, 1980 and Ghate, 1981). Kshemkalyani *et al.*, (1990) reported that histopathological changes in the gill and liver of fish *Hepidocephalus guntae* (Ham), after 96 hrs of exposure showed swelling of secondary gill lamellae, loss of epithelial cells in the gills and swelling of nuclei and necrosis of liver cells. Sultana and Sharief (2004) observed that *Tilapia mossambica* revealed extensive damage in the internal gill architecture of treated fish as compared to gills of control fish, where there are degenerative changes, swelling, fusion, atrophy and degeneration of secondary gill lamellae with bulging at tips. Reduction in the size of primary and secondary gill lamellae and necrosis of tissue in the copper, lead and zinc treated fishes were observed in contrast to control fish. Similar results were observed by Paulose (1989); Padgaonkar and Parab (1994); Anitha Kumari (1998); Dhanapakim *et al.*,(1998) and Sakthivel and Gaikwad (2000).

The histological changes in testis were studied by very few investigators. The present investigation mainly showed the sperm aggregations in the both mercuric chloride and sodium arsenate exposed bivalves, *Lamellidens corriaus*. In ova the histopathological changes were observed by many authors in many other crustaceans namely *Macrobrachium idea* in response to cadmium toxicity (Victor *et al.*, 1985), *Caridina rajadhani* in response to

organophosphorous pesticides (Victor and Sarojini, 1985) and in *Oziotelphusa senex* in response to cythion (Victor, 1989). Devi (1996) reported the histopathological changes in *Uca triangularis* with urea and naphthalene. Suresh (2001) also observed such changes like swelling of oocytes, vacuolization in oocytes, degeneration of oolemma, loss of normal shape of Oocyte, necrosis, fusion of adjacent pocytes, pyknosis in oplasm and nucleus, atresia, turgidity in ovary disorganized Ooplasm, hyper chromatic nuclei, necrotic oocytes, and fibrosis of ovaries.

Patil and Dhande (2000), on exposure to mercuric chloride, cadmium chloride and cupric chloride observed excessive degeneration of seminiferous tubules in teleost, C*hanna punctutus.* After 10 days of treatment, distortion of spermatids and pyknosis of spermatogenetic cells was observed.

Binding of heavy metal ions with sulfahydril and hydroxyl sites of membrane (Nilsson, 1970) in testis might have caused morphological destruction in all the heavy metal treated fishes. This could have also been due to biomagnifications or more toxic biodegraded form of the heavy metal compounds. Ejection of sperms from the lumen of seminiferous tubules could be a compensatory mechanism due to stress. In the present study, exposure to mercury and arsenic showed that in *L. corrianus,* mercury and arsenic damaged the normal structure of testis, aggregated the sperms and changed the shape of testicular follicles. Mercuric chloride induced profound histological changes in the liver a *Channa punctatus* such as

necrosis, vacuolation and degeneration of hepatocytes (Sastry *et al.*, 1979). The functioning of vertebrate liver is comparable with the hepatopancreas of molluscs.

According to Lucky and Venugopal (1977) the primary effect of mercury in cells appears to be it's binding with sulfahydryl group of surface membrane proteins. Kumar and Pant, (1981) studied histopathological effect of acute toxic level of copper and zinc on gill, liver and kidney of *P.conchonius* and they suggested that possible interaction between mucus and heavy metals resulting into formation of coagulated mass of mucus on gill surface interferes the process of gaseous exchange. Muley (1990) studied the fluoride-induced changes in the gonads and hepatopancreas of fresh water mussel, *Indonaia caeruleus* and observed severe damage to the gonads and hepatopancreas and severity of effect was dose dependent. The tubules were disfigured and lumen size was enlarged, disorganization and extensive vacuolation in the cytoplasm of cells were observed. Victor et *al.*, (1990) observed histopathological changes in the hepatopancreas of *P. hydrodromous* in response to cythion resulting in reduction in the height of tubular epithelium, enlargement of lumen, vacuolization and atrophy.

The histopathological changes indicated that the animals were not able to digest and store food properly and hence lack of nutrients resulted in the atrophy of hepatopancreas (Victor *et al.,* 1990a). Suresh, (2001) observed disorganised condition of hepatopancreas in *U. annulipes* in response to cadmium and mercury. Bhavan and Geraldine

(2000) observed extensive vacuolation in the cells of hepatopancreas in *U. triangularis* on exposure to urea and naphthalene.

In present study, caffeine was used to reduce the stress of heavy metals, or intoxification of heavy metals as it has antioxidant property. The caffeine can protect tissues from the damage due to heavy metal induced free radicals. Hosaka *et al.*, (2001) has observed the inhibition of hepatocarcinogenesis by caffeine in Ag1 rats treated with 2-acetylaminoflurene and has proposed that caffeine inhibited hepatocarcinogenesis induced by 2-acetylaminoflure. Mahajan and Zambare (2005) observed that caffeine have the capacity to reduce the heavy metals, mercuric chloride, arsenic trioxide and lead nitrate in snail, *Bellamya bengalennsis*. They suggested that caffeine have the capacity to reduce the tissue damage and protect the hepatopancreas. Chung Fung-Lung (1999) reported that caffeine when given in drinking water at a concentration identical to that found in 2% tea was able to inhibit lung tumors induced by 4 (methylnitrosamino)-1-(3-pyridyl)-1butanol (NNK). Shomer and Nickelson (1994) also suggested caffeine stimulation of malignant hypothermia susceptible sacroplasmic reticulum Ca_2^+ release, and suggested that caffeine sensitivity of malignant hypothermia susceptible (MHS) skeletal muscle fiber bundles is due to an altered caffeine sensitivity of MHS calcium ion release channel protein.

Puming *et al.*, (2001) studied suppression of lipopolysaccharide induced liver injury by various types of tea and coffee in D-galactosamine sensitized rats and suggested that caffeine

containing beverages generally suppress, lipopolyasaccharide induced liver injury according to their caffeine content. Inhibition of ATM and ATR kinase activities by the radio sensitizing agent, the caffeine and suggested that the radio sensitizing effects of caffeine are related to inhibition of the protein kinase activities of ATM and ATR and that both proteins are relevant targets for the development of novel anticancer agents (Sarkaria *et al.*, 1999). Plasskett *et al.*, (2001) reported that caffeine increases endurance and attenuates force sensations during the first 10-20 second of concentration. The rapidity of this effect suggests that caffeine exerts its effects naturally.

Caffeine has been found to increase glutathione synthetase and reduced glutathione in liver and lungs of mouse (Shelar *et al.*, 2002). Bunn and Poyton (1996), observed that, VEGF gene expression was found to be relatively unaffected by BAPTA-AM. To avoid these hazards and to exclude the possibility that cytosolic calcium chelation is balanced by an increased inward leak, they repeated the experiment with another membrane permanent Ca^{++} chelating agent, EGTAAM, in the absence of extra cellular calcium ions. This treatment lead to an apparent accumulation of HIF-1 in parallel with an increase in VEGE secretion, surprisingly; EPO production was dose dependent and significantly reduced. These data may indicate that elevated HIF-1 levels are not sufficient to derive hypoxia induced EPO gene expression.

For removal of unwanted metal ions from the body, the application of chelation technique is used.

Calcium EDTA is administered intravenously for the removal of excess of metal ions from the body. However, EDTA is a very strong chelator, which chelates any micronutrient and causes the micronutrient deficiency. It is also very toxic and causes liver and kidney damage.

Oxygen at second and sixth position of caffeine probably forms the chelate with the metal and hence caffeine-metal-chelate complex can reduce the activity of metal and complex can be excreted out as it has low molecular weight.

Present investigation showed that, the caffeine has the capacity to protect the damage of tissues against the mercury and arsenic induced toxic impact.

SUMMARY

- *The effect of heavy metals, mercury and arsenic without and with caffeine and during the recovery on gill, testis and digestive glands of freshwater bivalve, Lamellidens corrianus were studied histologically and the observations were recorded.*
- *After acute treatment of mercury and arsenic, the histomorphology of gill, testis and digestive glands showed that the gill lamellae, testicular follicles, hepatic lobules were badly affected by heavy metals in proportion to the period of exposure.*
- *After acute treatment of mercury and arsenic along with caffeine, the change in the cytoarchitacture of gill, testis and digestive glands were less severe as compared to the gill, testis and digestive glands of those exposed to only heavy metals.*
- *After 96 hrs exposure to heavy metals, the caffeine treated bivalves recovered faster as compared to those of normal water.*

CHAPTER : 2
BIOCHEMICAL STUDY

INTRODUCTION

Water contaminants have become a hazard to human health and became environmental problem in India as well as in many parts of world. Heavy metal pollution of water is a major environment problem facing the modern world (Dushenkov *et al.*, 1995). The global heavy metal pollutants are increasing in the environment due to increase in human activities. Industries of various kinds expel several million tons of waste each year. These dumps when come in contact with water flows or rainfalls generate leachates. Migration of chemicals through ground and surface water source in turn affects human health (Means *et al.*, 1978). Moreover, it is gaining importance day by day due to its obvious impact on human health through the food chain (Prasad, 1997).

The danger of heavy metals is aggravated by their almost infinite persistence in the environment because they cannot be destroyed biologically but are only transformed from oxidation state or organic complex to another. In addition, they are highly toxic for both higher organisms and microorganisms (Garbisu and Alkorta, 2001). Contaminants present in water source are microbial, inorganic and organic chemicals. In the last century, it was established that the introduction of the chlorinated water caused a large drop in the mortality from the infectious diseases (Mughal, 1992).Chlorination produces many compounds containing chlorine and lower bromine. Some of which have been shown to be carcinogenic, mutagenic or teratogenic in animal studies (Abbas and Fisher, 1997).

Contamination of water bodies with metal viz., mercury, arsenic, cadmium, chromium, lead, iron, copper had been a cause of considerable concern due to wide spread use in industrialization and unplanned urbanization. The world Health organization reported the industrial use of mercury and its general toxic effect on human and animal systems (WHO, 1990). Mercury in any chemical form denatures proteins, inactivates enzymes and causes severe disruption of any tissue, with which it comes into contact in sufficient concentration (WHO, 1991).

Mercury in the form of methyl mercury is a well-known human neurotoxin (Clarkson, 1994). Das and Patnaik (1980) studied the acute and sub acute toxicity of mercuric chloride to the air breathing fish *Heteropneustis fossilis*. This fish has been found to be more resistant to mercury.

Macronutrients intakes such as a fat intake have also been co-related with methyl mercury toxicity (Meltzer *et al.*, 1994). The main sources of mercury in the diet, such as fish and marine mammals, are also rich sources of Se (Cuvin-Aralar and Furness, 1991).

The toxicity of mercury is related to the chemical form. Liquid mercury appears to have little effect but mercury vapour is readily absorbed into the blood stream producing brain damage. Mercury as a toxic metal came to the limelight after the incidence of "Minamata disease" in 1953-60 in Japan. Nearly 111 causes of mercury poisoning were reported among persons who had eaten mercury-contaminated fish from Minamata Bay. Among them, about 45 died. Genetic defects had been observed in 20 babies whose mothers had eaten seafood from the bay. Therefore, the mercury is the most toxic heavy metal and serious incidence has been resulted from mercury poisoning.

Arsenic is naturally occurring element as a compound of underground rock and soil and finds its way to ground water and in food chains through the flow of energy from lower trophic to higher trophic level. The presence of arsenic in drinking water has been of great concern. Source of arsenic pollution is from the discharge of anthropogenic activities, geogenic nature and industrial applications. Arsenic shows the adverse health affect on the human in both acute and chronic manner. It is distributed in many organs such as lungs, liver, kidney and skin etc. after its intake.

Arsenic contamination has been found in many areas in West Bengal (India), Bangladesh and several other countries. The chemistry of arsenic

in aquatic system is quite complicated, however, in ground water the arsenate (H_3 AsO_4, H_2 AsO_4^{-1}, H AsO_4^{-2}) and arsenite (H_3 AsO_3, H_2 AsO_3^{-1}, $HAsO_3^{-2}$) species are more predominant.

Arsenic can be remediated by oxidation, coagulation, sedimentation, filtration, adsorption, ion change and reverse osmosis from biological materials (Johnson and Heijnen, 2001). Coagulation involves the removal of colloidal (0.001 -100m) stable particles and co-precipitation occurs when arsenic forms an insoluble complex with coagulant. Lastly, it causes death. Chelation therapy for metal ion toxicity has been reported by Sharma (1995). There are number of metal chelators, which are used for the remediation of metal toxicity (Hammond, 1971; Friedheim *et al.*, 1978 Graziano *et al.*, 1985).

Caffeine is found to have antioxidant activity. This antioxidant activity of caffeine can protect the damage of tissues biochemical and genetic material of organisms from the heavy metal generated free oxygen radicals. Mike McLaughlin of CSIRO Australia (2000) has proved that caffeine has the capacity to bind with heavy metals. Heavy metal contents of water were found to be much reduced after addition of caffeine. Dissolved heavy metal ions are positively charged and coffee contains uncharged and negatively charged molecules. The metal ions might be taken out of solution of binding to negatively charged molecules in the coffee ground.

Caffeine molecule is having a site that usually binds divalent cations Ca^{++} and affects activity of Ca^{++} dependent enzymes. Caffeine chelate with heavy metals can be easily excreted out by the

biological system. Molecular weight of caffeine is 194.2 daltons thus caffeine metal chelate will be having less than 500Dalton molecular weight and hence it can easily pass through membranes. Therefore, caffeine can have the capacity to remove the heavy metals from the body of living organisms and prevent the damage of tissues and can indirectly save the life of living organisms. Secondly, it is known that caffeine increases the rate of urine formation.

Nafisi *et al.*, (2002) reported the binding constants of Ca^{2+} and Mg^{2+} with caffeine to be 29.8 and 22.4 M^{-1} respectively. Kolayly *et al.* (2004) found that the eight metal ions, CA^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+} and Cr^{2+} investigated, formed complexes with caffeine in varying capacities but these were very weak in strength when compared to EDTA. EDTA shows 10^{10} fold higher metal binding activity compared to caffeine.

Protein:-

Protein is an important organic constituent, which play important role in metabolism of organisms and metabolic activities. It is an integral part of cell membrane. Harper *et al.*, (1978) showed and reported that, the proteins are among the most abundant biological macromolecules and are extremely versatile in their function and interaction during protein metabolism in protein, amino acids, enzymes and co-enzymes. Deshmukh and Lomte (1998) studied the biochemical content of protein in different tissues such as mantle, foot, gill, digestive gland and whole body of freshwater bivalve, *Parreysia corrugata* after acute and chronic exposure to copper sulphate. The biochemical variations in protein content of *Pila globosa* after

exposing to pesticide were studied by Ramanna Rao and Ramamurthi (1978). Katticaram *et al.*, (1995) studied the copper induced alterations in total carbohydrates and protein levels in the bivalve, *Sunetta scripta*. Lomte and Alam (1982) studied the biochemical composition of *Bellamya (Viviparous) bengalensis* after treatment with the pesticides. Mahajan (2005) studied the biochemical changes induced by heavy metals, lead, mercury and arsenic in the protein content on the gastropod, *Bellamya (Viviparous) bengalensis.* The biochemical variations in *Mytilus edulis* were studied by William, (1969).

Collagen:-

Collagen is largely responsible for maintaining the functional integrity of the myocardium, which allows interdigitation and transmission of force between contracting myocytes. Medugorac and Jacob (1993) reported that, to correlate the collagen content with myocardial mechanical parameters, the knowledge of collagen type distribution in normal and diseased cardiac tissue is essential. The precise magnitude of the changes in collagen parameters may be of great significance to understand the mechanism of cardiac hypertrophy, heart failure, or pathogenesis of other cardiac diseases.

Increase in the stability of collagen occurs due to increase in the number of intra and intermolecular cross linkages. Enhanced cross-linking of collagen in extra cellular space restricts the transport of metabolites and useful gases to concerned tissues leading to physiological incompetence of the organs (Kohn, 1978).

The alteration of collagen phenotypes may

be responsible for compromised function in hypertensive heart disease (Thiedmann *et al.*, 1983). Michael *et al.* (1980) reported that in kidney, the successful formation of a relatively protein free ultra filtrate by the glomeroulus involves a complex interplay of structural, biochemical and homodynamic factors. Kidney has its own pattern of collagen type distribution. Ohyama *et al.*, (1990) reported that any imbalanced in the extracellular matrix or alterations in the metabolism of collagen in a pathological condition such as glomerulosclerosis lead to significantly reduced glomerular function.

Jendryczko and Drozdz (1987) proposed that lithium treatment is an established practice with mania although it reportedly has detrimental long-term effects due to the induction of a collagen like syndrome. They also reported the lower activity of superoxide dismutase, the antioxidative copper zinc dependent enzyme, in rats treated with Lithium salt.

The tertiary structure and the properties of collagen are maintained due to disulphide linkages. The heavy metal usually binds with —SH groups of proteins. Mercury and arsenic being highly reactive can bind the --SH groups of collagen and alter its structure. The collagen is the major structural protein found in all basement membranes and connective tissues. If the structural integrity of the basal membranes is disturbed, due to alteration in collagen structure, the epithelia resting on the basement membrane can puff out or function poorly due to poor base or poor supply of nutrients. It is therefore necessary to study the interaction of heavy metals with collagen.

Ascorbic Acid:-

Ascorbic acid is an "enediol - lactone" of an acid with a configuration similar to that of the sugar, L-glucose. The vitamin C is L ascorbic acid while D- ascorbic acid is antiscorbutic. Ascorbic acid plays a very important role in tissue synthesis and growth process and obviously mediates rapid tissue repair in diseased condition. Glucose and hexose are utilized for the biosynthesis of ascorbic acid.

Halver, (1972) reported that the ascorbic acid plays a major role in the tissue synthesis and growth processes. Ascorbic acid is necessary in the formation and maintenance of collagen, the basis of connective tissue, which is formed in skin, ligaments, cartilage, vertebral discs, joint linings, capillary walls, bones and teeth. Collagen and vitamin C, are needed to give support and shape to the body, to help wound heal and to maintain healthy blood vessels. Siddiqui (1967) observed the levels of ascorbic acid in liver, gonad and serum of *Ophiocephalus punctatus* during summer season. Chitra and Ramana Rao (1977) reported that low temperature induced a variety of changes in blood glycogen and ascorbic acid. Chinoy and Garg (1978) reported that ascorbic acid by virtue of possessing reducing properties is known to act as radio protective agent in several tissues including reproductive organs by preventing radiation induced oxidation.

Mahajan (2005) studied the alteration in the ascorbic acid contents in snail, *Bellamiya bengalensis* after exposure to lead, mercury and arsenic. Zambare (1991) studied the ascorbic acid levels in response to pollutants in various tissues

of freshwater bivalve, *Corbicula striatella*. Pardeshi and Zambare (2005) studied the ascorbic acid contents of different body components in relation to reproduction of the freshwater bivalve, *Parreysia cylindrica*.

Talwar (1980) and Chatterjee *et al.*, (1995) reported that the ascorbic acid protect the mammalian tissues against oxidative damage both at the intracellular and extra cellular levels. Waykar *et al.*, (2001) studied the changes in the ascorbic acid content during acute and chronic exposure to cypermethrin in mantle, gill, foot, digestive gland and whole body tissues of the bivalves, *Parreysia cylindrica*.

The ascorbic acid content in the tissue of fresh water bivalve molluscs has received little attention. Considering the multiple role of ascorbic acid and paucity of information on its level in the tissue of freshwater bivalves (Kachole *et al.,* 1977), this essential vitamin C is drastically affected and altered by various environmental pollutants (Bhusari, 1987; Jadhav *et al.*, 1996). Halver (1972) stated that the ascorbic acid plays major role in tissue synthesis and growth processes. Ascorbic acid maintains prolyl hydroxylase in an active form. Collagen contains large amount of hydroxyproline, which is synthesized by prolyl hydroxylase from proline. The collagen forms the main component of the basement membranes of epithelia. Therefore, ascorbic acid is necessary for the synthesis of collagen.

DNA:-

Heavy metals enter into the body of organism through the respiratory organs like gill, lung, etc. and through food and drinking water. It is

hazardous to aquatic ecosystem and disturbs the food chain. This disturbed phenomenon has been expressed in the biochemical contents of tissues of animals.

Nucleic acid contents are considered as an index of capacity of an organism for protein synthesis. Different hormones and stress conditions may exert control over synthesis, activity and break down of nucleic acids. The nucleic acid contents can cause alterations in genetical information and genome functioning so it is important to investigate the levels of DNA and RNA periodically in different tissues of the organisms undergoing stress conditions.

DNA (Deoxyribose nucleic acid) contents can be the index of capacity of an organism for protein synthesis in the different stress conditions affected by heavy metals or any toxic metals or pesticides. Structural changes in the DNA can be monitored using biochemical methods and usually low quantitative changes are observed on heavy metals exposure. DNA strand scission can also be sensitively monitored, and even more importantly, the specific nucleotide position cleaved can be pin pointed by biochemical methods. This methodology has been applied successfully in monitoring both the efficiency of DNA strand scission by metal complexes and the specific sites cleaved, and where the complexes are specifically bound on the helical strand.

The binding of metal ions and their complex to DNA or, more generally to nucleotides, there are several different coordination sites available. The metal centers can bind to the negatively charged oxygen atoms of (poly) phosphate group or the

nitrogen and oxygen atoms of purine and those containing large JL systems as ligands should also be able to inter catalane between two base pairs, possibly even in a sequence specific fashion. Lastly, coordinated ligands with, for example, amine or hydroxyl functions may form hydrogen bonds with protein acceptor components of the polynucleotides.

Interactions of metal ions or metal complexes with nucleic acid play an important role:

1. In sustaining the conformation such as DNA or RNA through electrostatic effects,
2. In the nucleic acid metabolism particularly in phosphoryl transfer,
3. In the regulation, replication and transcription of genetic information,
4. For efforts directed at specific DNA cleavage with synthetic probes and
5. For metal induced mutagenesis.

Such mutations can be due to genometric distortions of the DNA through unphysiological cross linking or to the stabilization or a wrong nucleobase tautomer complex nucleic acid inter actions and their physiological consequences can thus be quite varied, even in the extensively studied platinum compounds they are far from being fully under stood.

The platinum complexes act as anticancer agents by interacting with DNA and trans platinum bind most strongly to RNA than to DNA and least strongly to proteins. When the activity was assessed as the ability to suppress the synthesis of DNA, RNA and protein, only the synthesis of DNA was suppressed.

The selectivity of platinum complexes in attacking tumor cells rather than normal cells, even

through there is little or no preferential uptake of platinum in tumour cells, has led to the suggestion that cancer cells are deficient in some DNA repair mechanisms. DNA is constantly being damaged but various repair proteins can recognize the damaged segment and cause the repair. Platinum specifically binds DNA at the minor groove.

Detmar and Andrea (1992) studied that cobalt is an essential trace element for mammalian nutrition, but also is classified as carcinogenic with the fidelity of DNA synthesis. Regarding anti and co-mutagenic mechanisms, the evidence for interference of Co (II) with DNA repair processes is known.

An excellent example of the work at the London St. Mary's Branch and the San Diego Branch, found that in a normal cell P-53 expression levels in response to stress and DNA damaging agents caused cell cycle arrest and apoptosis before the damage to the DNA of their nuclei causing oncogenesis. Bryan *et al.*, (1986) suggested the relationship between copper and nucleo-proteins and copper related changes in DNA cleavage. The excess copper, as ionic copper, reportedly reduces DNA damage when introduced into at cite tumors.

A severe copper depletion has been recorded because of a major burn (Brian *et al.*, 1987) and production of several organics as Ceruloplasmin (Danks *et al.*, 1986) and Cytochrome C-552 (Merchant and Bogorad, 1987b) has been demonstrated to be genetically controlled but the genetic expression is dictated by copper availability to the cells. Even certain nucleoproteins are copper rich although it is not known, if the metals complexes to protein, to the nucleic acid, or exists

in a protein copper nucleic acid complex (Bryan *et al.*, 1985). The design of a double stranded DNA cleavage agent with two-polyamine metal binding arms, which they suggested might be able to deliver metal activated chemistry to one or both DNA strands. Black *et al.*,(1996) found significant DNA strand breakage in different tissues from *Anodonta grandis* exposed to lead.

Arsenic is known to cause DNA damage and related events, such as DNA protein cross-links, micronuclei etc. (Schaumloffel and Gebel, 1998), DNA strand breaks (Lynn *et al.*, 1998; Liu and Jan, 2000), or alterations in DNA repair enzymes (Hartwing, 1998). Supper oxide scavengers such as Cu, Zn-SOD suppress arsenic induced DNA damage (Hartwing, 1998; Lynn *et al.*, 1998; Liu and Jan, 2000).

Tong Lu *et al.*, (2001) studied that approximately 60 genes (10%) were differentially expressed in arsenic exposed human livers as compared with those of controls, damage was also observed due to involved arsenic in the DNA of respective cells.

Low-dose exposures to methyl mercury are not likely to cause cancer in humans. Data on effects related to mutation formation (Changes in DNA) indicates that methyl mercury could increase frequencies of mutation in human eggs and sperm.

Excess copper, as ionic copper, produces DNA damage when introduced into as cite tumors. Zhao *et al.*,(1997) studied the association of arsenic induced malignant transformation with DNA hypomethylation and aberrant gene expression. Beyersmann (1994) studied that the carcinogenicity and genotoxicity of cadmium, chromium, cobalt and nickel strongly depend on their chemical ligands

(speciation), which modulate them with the exception of hexavalent chromium; carcinogenic metal compounds are only weakly genotoxic. These effects are interpreted by the interference of the toxic metal ions with biochemical functions of magnesium, calcium and zinc ions.

RNA:-

Heavy metals also interact with RNA polymerases. Severe effects are expressed as such in DNA metal binding. RNA polymerase must bind site specifically to its DNA template, binds its nucleotide and primer substrates, and form a new phosphodiester bond in elongating the growing RNA. Zinc ion appears to be essential to the functioning to RNA polymerases and DNA topoisomerases, (Giedroc and Coleman, 1989).

Eukaryatic RNA polymerases I, II and III are involved in the synthesis of ribosomal, messenger and transfer RNAs, respectively. The DNA dependent RNA polymerases I (Falchuk *et al.*,1977), II (Falchuk *et al.,*1976) and III (Wandzilak and Benson, 1977) of the unicellular eukaryote *Euglena gracilis* have all been showed to be zinc metallo enzyme, each binding about 2 gram atoms of zinc.

The role of RNA is to help protein synthesis in the cytoplasm hence depletion of RNA level also resulted decreased rate of protein synthesis (Rao *et al.*, 1990). Similar decreased amount of RNA levels was observed by Asfia and Vasantha (1986) in *Clarius batracus*, by Lomte and Patil (1989) in *Mythima* (*Pseudoletia seperata* and by Choudhari *et al.*,(1993) in *Thiara lineata* under different toxic stress. The cellular degradation, rapid histolysis and decreased rate of protein synthesis are the possible reasons.

Ester Saball *et al.*,(2000) observed the total

tissue m-RNA of liver and kidneys of control and $HgCl_2$ treated rats. Tong Lu *et al.*,(2001) observed that 10% genes, mostly related to cell cycle regulation, apoptosis, DNA damage response etc. were differentially expressed in the form of RNA and such abnormal RNA are vulnerable to RNA are attack. Ermachenko *et al.*,(1987) observed reduction in weight of testis and prostate, increased RNA concentration in testis, solerosis of leyding cells and increase in the number of normal spermatozoa after inhalation of $CuCl_2$ for three months. Copper has, for example been reported to stimulate RNA synthesis in germinating wheat seeds (Khan and Fizza, 1986). Rao *et al.*,(1998) studied the RNA levels in various tissues of freshwater crab *Barytelphusa cunicularis* when exposed to Fluoride.

Thus, proteins collagen ascorbic acid, DNA and RNA levels in the tissues after exposure to heavy metals can be considered as the indices for stress.

MATERIALS AND METHODS

Selected experimental model animals, the freshwater bivalve, *Lamellidens corrianus* were collected from the Paithan dam at Paithan Tq. Paithan, Dist. Aurangabad (M.S.). After collection, bivalves were acclimatized in the laboratory condition at room temperature for 2-3 days. The healthy and active acclimatized bivalves of approximately same size were selected for experiment.

Before starting the experiment, these bivalves were divided into three groups such as A, B and C.

1. A group bivalves were kept as control,

(2) B group bivalves were exposed to acute dose ($LC_{50/2}$) of heavy metal salts, mercuric chloride

(0.6 ppm equivalent to 0.444 ppm Hg^{++}) and sodium arsenate (0.9 ppm equivalent to 0.672 ppm As^{+++})

(2) C group bivalves were exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate with caffeine (5 mg/L.).

After 4 days bivalves from group B were divided into two groups D and E

(4) D group bivalves pre exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate were allowed to cure in normal dechlorinated water

(5) E group bivalves pre exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate were exposed to caffeine (5 mg./l).

The experimental bivalves of A, B and C group were dissected after 24 hrs and 96 hrs and from D and E groups of recovery after 2 days and 4 days. Testis, gills and digestive glands from all five groups of bivalves were dried at 80°C in an oven until constant weight was obtained. The dried powders of these different tissues of control and experimental animals were used for estimations of their protein, collagen, ascorbic acid, DNA and RNA contents.

Total proteins were estimated by Lowry's method (Lowry *et al.*, 1951) using bovine serum albumen as standard from each powder. Collagen contents were estimated by the method of Wossner (Wossner, 1963) using Chloramin-T. Ascorbic acid contents were estimated by the method of Roe J.H. (1967) using hydrazine reagent. DNA contents were estimated by Diphenylamine method (Schender, 1967) while RNA contents were estimated by using Orcinol reagent (Dischel, 1955).

The results are presented in the table 2.1.1 to 2.5.2 as percent changes of three repeats and are expressed as percentage of dry weight. Standard deviation and student 't' test of significance are

calculated and expressed in respective tables.

Table No.2.1.1 : Protein content in selected tissues of *Lamellidens corrianus* after acute exposure to Hg^{++} without and with caffeine and during recovery. (Values represent % in dry weight)

Treatment		Tissue	24 hrs	96 hrs	Recovery	
					2days	4days
Control (A)		Gill	64.77 ± 1.567	64.16 ± 1.256		
		Testis	52.33 ± 1.525	51.67 ± 1.216		
		Digestive Glands	54.81 ± 1.489	52.94 ± 1.319		
0.444 ppm Hg^{++} (B)		Gill	53.56 ± 2.740 ❖❖ (-17.307)	45.47 ± 1.759❖❖❖ (-29.130)		
		Testis	41.73 ± 2.389 ❖❖ (-20.256)	38.61 ± 2.056❖❖❖ (-25.275)		
		Digestive Glands	43.60 ± 1.956 ❖❖ (-20.452)	37.38 ± 1.891❖❖❖ (-29.391)		
0.444 ppm Hg^{++} + 5mg/lit. Caffeine (C)		Gill	56.68 ±1.752❖❖ (-12.490)	49.82 ± 1.625❖❖❖ (-22.350)		
		Testis	46.08 ± 1.689❖❖ (-11.943)	41.11 ± 2.060❖❖ (-20.437)		
		Digestive Glands	49.21 ± 2.690 ❖ (-10.217)	45.34 ± 1.750❖❖ (-14.345)		
After 96hrs Exposure to 0.444 ppm Hg^{++}	Normal Water (D)	Gill			47.95 ± 1.859 NS (+5.454)	49.82 ± 1.756■ (+9.566)
		Testis			40.48 ± 2.789 NS (+4.843)	42.35 ± 2.169NS (+9.686)
		Digestive Glands			37.99 ± 2.089 NS (+1.631)	39.24 ± 2.962NS (+4.975)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			48.59 ± 2.065 NS (+6.861)	50.46 ± 1.430■ (+10.974)
		Testis			41.73 ±2.168 NS (+8.080)	44.22 ± 1.578■ (+14.529)
		Digestive Glands			40.48 ± 2.196 NS (+8.293)	43.60 ± 1.258■■ (+16.639)

Table No.2.1.2 : Protein content in selected tissues of *Lamellidens corrianus* after acute exposure to As^{+++} without and with caffeine and during recovery.(Values represent % in dry weight)

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	64.77 ± 1.567	64.16 ± 1.256		
		Testis	52.33 ± 1.525	51.67 ± 1.216		
		Digestive Glands	54.81 ± 1.489	52.94 ± 1.319		
0.672 ppm As^{+++} (B)		Gill	50.46 ± 2.560 ❖❖ (-22.093)	44.85 ± 1.562❖❖❖ (-30.096)		
		Testis	38.61 ± 2.287❖❖❖ (-26.218)	36.74 ± 2.034❖❖❖ (-28.922)		
		Digestive Glands	41.11 ± 1.894❖❖❖ (-24.995)	34.87 ± 1.739❖❖❖ (-34.378)		
0.672 ppm As^{+++} + 5mg/lit. Caffeine (C)		Gill	54.20 ± 1.695❖❖ (-16.319)	46.08 ± 1.618❖❖❖ (-28.179)		
		Testis	45.47 ± 1.213❖❖ (-13.109)	37.99 ± 2.020❖❖❖ (-26.504)		
		Digestive Glands	49.82 ± 1.975❖❖ (-9.104)	42.98 ± 1.695❖❖ (-18.813)		
After 96hrs Exposure to 0.672 ppm As^{+++}	Normal Water (D)	Gill			46.08 ± 1.746 NS (+2.742)	47.43 ± 1.639 NS (+5.551)
		Testis			39.24 ± 2.579 NS (+6.804)	41.11 ± 2.148 NS (+11.894)
		Digestive Glands			37.38 ± 2.036 NS (+7.198)	39.24 ± 2.786 NS (+12.532)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			47.47 ± 2.137 NS (+5.841)	48.59 ± 1.613■ (+8.338)
		Testis			40.48 ± 2.043 NS (+10.179)	44.85 ± 2.154■■ (+22.074)
		Digestive Glands			39.24 ± 2.063■ (+12.532)	42.98 ± 1.984■■ (+23.257)

Values in the ( ) brackets indicate percent change over control, N.S.-Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

Table No.2.2.1: Collagen content in selected tissues of *Lamellidens corrianus* after acute exposure to Hg^{++} without and with caffeine and during recovery. (Values represent percentage in dry weight)

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	2.427 ± 0.06	2.434 ± 0.07		
		Testis	3.276 ± 0.09	3.261 ± 0.12		
		Digestive Glands	3.934 ± 0.08	3.755 ± 0.10		
0.444 ppm Hg^{++} (B)		Gill	1.516 ± 0.08❖❖❖ (-37.536)	1.365 ± 0.05❖❖❖ (-43.919)		
		Testis	2.358 ± 0.17❖❖❖ (-28.021)	2.033 ± 0.12❖❖❖ (-37.657)		
		Digestive Glands	2.593 ± 0.15❖❖❖ (-34.087)	2.845 ± 0.10❖❖❖ (-24.234)		
0.444 ppm Hg^{++} + 5mg/lit. Caffeine (C)		Gill	1.744 ± 0.10❖❖❖ (-28.141)	1.934 ± 0.06❖❖❖ (-20.542)		
		Testis	2.800 ± 0.08❖❖ (-14.529)	2.617 ± 0.10❖❖ (-19.748)		
		Digestive Glands	2.958 ± 0.07❖❖❖ (-24.809)	3.072 ± 0.12❖❖ (-18.189)		
After 96hrs Exposure to 0.444 ppm Hg^{++}	Normal Water (D)	Gill			1.479 ± 0.06 NS (+8.351)	1.593 ± 0.05 NS (+16.703)
		Testis			2.254 ± 0.12 NS (+10.870)	2.468 ± 0.10 NS (+21.396)
		Digestive Glands			2.958 ± 0.10 NS (+3.971)	2.996 ± 0.11 NS (+5.307)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			1.707 ± 0.05■■ (+25.054)	2.162 ± 0.09■■■ (+58.388)
		Testis			2.654 ± 0.08■■ (+30.545)	2.768 ± 0.12■■ (+36.153)
		Digestive Glands			3.186 ± 0.10■ (+11.985)	3.260 ± 0.08■■ (+14.586)

Table No.2.2.2: Collagen content in selected tissues of *Lamellidens corrianus* after acute exposure to As^{+++} without and with caffeine and during recovery.(Values represent % in dry weight)

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	2.427 ± 0.08	2.434 ± 0.07		
		Testis	3.276 ± 0.16	3.261 ± 0.17		
		Digestive Glands	3.934 ± 0.12	3.755 ± 0.14		
0.672 ppm As^{+++} (B)		Gill	2.176 ± 0.07❖ (-10.341)	1.707 ± 0.05❖❖❖ (-29.868)		
		Testis	2.448 ± 0.10❖❖ (-25.274)	2.019 ± 0.12❖❖❖ (-38.086)		
		Digestive Glands	2.707 ± 0.09❖❖❖ (-31.189)	2.300 ± 0.12❖❖❖ (-38.748)		
0.672 ppm As^{+++} + 5mg/lit. Caffeine (C)		Gill	2.389 ± 0.14 NS (-1.565)	2.313 ± 0.11❖❖❖ (-4.971)		
		Testis	2.864 ± 0.08❖ (-12.576)	2.759 ± 0.12❖❖ (-15.394)		
		Digestive Glands	2.934 ± 0.08❖❖❖ (-25.419)	2.731 ± 0.10❖❖❖ (-27.270)		
After 96hrs Exposure to 0.672 ppm As^{+++}	Normal Water (D)	Gill			1.820 ± 0.07 NS (+6.619)	1.934 ± 0.09■ (+13.298)
		Testis			2.276 ± 0.10 ■ (+12.729)	2.527 ± 0.09■■ (+25.160)
		Digestive Glands			2.389 ± 0.13 NS (+3.869)	2.589 ± 0.12 NS (+12.565)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			1.920 ± 0.06■■ (+12.478)	2.072 ± 0.10■■ (+21.382)
		Testis			2.458 ± 0.10■■ (+21.743)	2.631 ± 0.12■■ (+30.312)
		Digestive Glands			2.645 ± 0.09■ (+15)	2.851 ± 0.10■■ (+23.695)

Values in the ( ) brackets indicate percent change over control, N.S.-Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

Table No.2.3.1: Ascorbic acid content in selected tissues of *Lamellidens corrianus* after acute exposure to Hg^{++} without and with caffeine and during recovery.(Values represent % in dry weight)

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	0.849 ± 0.0471	0.855 ± 0.0412		
		Testis	1.038 ± 0.0816	1.037 ± 0.0508		
		Digestive Glands	0.859 ± 0.0501	0.876 ± 0.0895		
0.444 ppm Hg^{++} (B)		Gill	0.151 ±0.0816❖❖❖ (-82.221)	0.373±0.0371❖❖❖ (-56.374)		
		Testis	0.593 ±0.0432❖❖ (-42.870)	0.312±0.0395❖❖❖ (-69.913)		
		Digestive Glands	0.373 ±0.0489❖❖❖ (-56.577)	0.412 ±0.0508❖❖ (-52.968)		
0.444 ppm Hg^{++} + 5mg/lit. Caffeine (C)		Gill	0.286 ±0.0916❖❖❖ (-66.313)	0.664 ±0.0716 NS (-22.339)		
		Testis	1.812 ±0.0716❖❖❖ (-74.566)	0.513 ±0.0926❖❖ (-50.530)		
		Digestive Glands	0.513 ±0.0876❖❖ (-40.279)	0.543 ±0.0686❖❖ (-38.013)		
After 96hrs Exposure to 0.444 ppm Hg^{++}	Normal Water (D)	Gill			0.415 ±0.0411 NS (+11.260)	0.456 ±0.0101■ (+22.252)
		Testis			0.472 ±0.0919■ (+51.282)	0.516 ±0.0716 NS (+65.384)
		Digestive Glands			0.564 ±0.0416■ (+36.893)	0.615 ±0.0489■■ (+48.543)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			0.510 ±0.0391■ (+36.729)	0.631 ±0.0525■■ (+69.168)
		Testis			0.505 ±0.0444■ (+61.858)	0.625 ±0.0381 NS (+100.320)
		Digestive Glands			0.589 ±0.0425■■ (+42.961)	0.621 ±0.0399■■ (+50.728)

Table No.2.3.2: Ascorbic acid content in selected tissues of *Lamellidens corrianus* after acute exposure to As^{+++}without and with caffeine and during recovery.(Values represent % in dry weight)

Treatment		Tissue	48hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	0.849 ±0.0471	0.855 ±0.0561		
		Testis	1.038 ±0.0615	1.037 ±0.0372		
		Digestive Glands	0.859 ±0.0671	0.876 ±0.0913		
0.672 ppm As^{+++} (B)		Gill	0.621 ±0.0715❖❖ (-26.855)	0.513 ±0.0926❖❖ (-40)		
		Testis	0.755 ±0.0616❖❖ (-27.263)	0.604 ±0.0726❖❖❖ (-41.755)		
		Digestive Glands	0.592 ±0.0519❖❖ (-31.082)	0.453 ±0.0718❖❖ (-48.287)		
0.672ppm As^{+++}+ 5mg/lit. Caffeine (C)		Gill	0.693 ±0.0372❖ (-18.374)	0.514 ±0.0578❖❖ (-39.883)		
		Testis	0.936 ±0.0616 NS (-9.826)	0.714 ±0.0381❖❖❖ (-31.147)		
		Digestive Glands	0.566 ±0.0354❖❖ (-34.109)	0.544 ±0.0374❖❖ (-37.899)		
After 96hrs Exposure to 0.672 ppm As^{+++}	Normal Water (D)	Gill			0.589 ±0.0489 NS (+14.814)	0.625 ±0.0581 NS (+21.832)
		Testis			0.694 ±0.0946 NS (+14.900)	0.785 ±0.0271■ (+29.966)
		Digestive Glands			0.583 ±0.0776 NS (+28.697)	0.785 ±0.0132■■ (+73.289)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			0.602 ±0.0373 NS (+17.348)	0.712 ±0.0571■ (+38.791)
		Testis			0.725 ±0.0849 NS (+20.033)	0.815 ±0.0341■■ (+34.933)
		Digestive Glands			0.602 ±0.0385■ (+32.891)	0.618 ±0.0497■ (+36.423)

Values in the ( ) brackets indicate percent change over control, N.S.-Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

Table No. 2.4.1: DNA content in selected tissues of *Lamellidens corrianus* after acute exposure to Hg^{++} without and with caffeine and during recovery. (Values represent % in dry weight)

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	1.218 ± 0.0362	1.220 ± 0.0298		
		Testis	2.410 ± 0.0789	2.300 ± 0.0472		
		Digestive Glands	2.218 ± 0.0487	2.113 ± 0.0762		
0.444 ppm Hg^{++} (B)		Gill	1.163 ± 0.0756 NS (-4.515)	1.055 ± 0.0367❖❖ (-13.520)		
		Testis	1612 ±0.0426❖❖❖ (-33.112)	1.163 ±0.0382❖❖❖ (-49.434)		
		Digestive Glands	1.718 ±0.0479❖❖❖ (-22.542)	1.558 ±0.0496❖❖❖ (-26.265)		
0.444 ppm Hg^{++} + 5mg/lit. Caffeine (C)		Gill	1.189 ± 0.0816 NS (-2.380)	1.109 ± 0.0621❖ (-9.098)		
		Testis	2.057 ± 0.0634❖❖ (-14.647)	1.461 ±0.0716❖❖❖ (-36.478)		
		Digestive Glands	1.905 ± 0.0794❖❖ (-14.111)	1.758 ± 0.0577❖❖ (-16.800)		
After 96hrs Exposure to 0.444 ppm Hg^{++}	Normal Water (D)	Gill			1.136 ± 0.0398NS (+7.677)	1.153 ± 0.010■ (+9.289)
		Testis			1.461 ± 0.0876■■■ (+25.623)	1.735 ± 0.0672■■■ (+49.183)
		Digestive Glands			1.678 ± 0.0386■ (+7.702)	1.753 ± 0.0416■■ (+12.516)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			1.169 ± 0.0367■ (+10.805)	1.195 ± 0.0432■ (+13.270)
		Testis			1.579 ± 0.0438■■■ (+35.769)	1.901 ± 0.0372■■■ (+63.456)
		Digestive Glands			1.795 ± 0.0416■■ (+15.211)	1.952 ± 0.0383■■■ (+25.258)

Table No. 2.4.2: DNA content in selected tissues of *Lamellidens corrianus* after acute exposure to As^{+++} without and with caffeine and during recovery.(Values represent % in dry weight)

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	1.218 ± 0.0389	1.220 ± 0.0471		
		Testis	2.410 ± 0.0582	2.300 ± 0.0354		
		Digestive Glands	2.218 ± 0.0589	2.113 ± 0.0892		
0.672 ppm As^{+++} (B)		Gill	1.031 ± 0.0710NS (-15.353)	0.854 ± 0.0913❖❖ (-30)		
		Testis	1.624 ±0.0600❖❖❖ (-32.614)	1.236 ±0.0693❖❖❖ (-46.260)		
		Digestive Glands	2.002 ± 0.0468❖❖ (-9.738)	1.653 ± 0.0663❖❖ (-21.769)		
0.672 ppm As^{+++}+ 5mg/lit. Caffeine (C)		Gill	1.135 ± 0.0345❖❖ (-6.814)	0.972 ± 0.0564❖❖ (-20.327)		
		Testis	2.030 ± 0.0613❖❖ (-15.767)	1.473 ±0.0375❖❖❖ (-35.956)		
		Digestive Gland	2.083 ± 0.0349❖ (-6.086)	1.961 ± 0.0368NS (-7.193)		
After 96hrs Exposure to 0.672 ppm As^{+++}	Normal Water (D)	Gill			0.865 ± 0.0473NS (+1.346)	0.931 ± 0.0552NS (+9.016)
		Testis			1.315 ± 0.0935NS (+6.391)	1.437 ± 0.0254■■ (+16.242)
		Digestive Glands			1.721 ± 0.0753NS (+4.113)	1.906 ± 0.0118■■ (+15.305)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			0.952 ± 0.0364NS (+11.475)	1.127 ± 0.0571■ (+31.967)
		Testis			1.624 ± 0.0834■■ (+31.391)	1.977± 0.0332■■■ (+59.951)
		Digestive Glands			1.831 ± 0.0376■■ (+10.768)	1.993 ± 0.0482■■ (+20.568)

Values in the ( ) brackets indicate percent change over control, N.S.- Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

Table No. 2.5.1: RNA content in selected tissues of *Lamellidens corrianus* after acute exposure to Hg^{++} without and with caffeine and during recovery. (Values represent % in dry weight)

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	19.418 ±1.347	19.521 ±1.438		
		Testis	4.417 ±0.743	4.571 ±0.612		
		Digestive Glands	8.607 ±0.813	8.767 ±0.758		
0.444 ppm Hg^{++} (B)		Gill	15.235 ±1.236❖ (-21.541)	13.563 ±1.127❖❖ (-30.875)		
		Testis	3.692 ±0.215NS (-16.413)	3.077 ±0.195❖ (-32.684)		
		Digestive Glands	6.190 ±0.637❖ (-28.081)	4.660±0.475❖❖ (-46.846)		
0.444 ppm Hg^{++} + 5mg/lit. Caffeine (C)		Gill	17.418 ±1.342NS (-10.299)	15.447 ±1.087❖ (-21.273)		
		Testis	4.145 ±0.426NS (-6.158)	4.015 ±0.376NS (-12.163)		
		Digestive Glands	7.031 ±0.522❖ (-18.310)	5.336 ±0.508❖❖ (-39.135)		
After 96hrs Exposure to 0.444 ppm Hg^{++}	Normal Water (D)	Gill			15.767 ±1.146NS (+16.250)	16.418 ±1.285■ (+21.049)
		Testis			4.052 ±0.647NS (+31.686)	4.312 ±0.849NS (+40.136)
		Digestive Glands			5.265 ±0.896NS (+12.986)	6.735 ±0.976■ (+44.527)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			16.563 ±0.979■ (+22.119)	18.592 ±1.018■■ (+37.078)
		Testis			4.480 ±0.346■■ (+45.596)	4.680 ±0.549■■ (+52.096)
		Digestive Glands			6.135 ±0.793■ (+6.792)	7.672 ±0.857■■ (+64.635)

Table No.2.5.2: RNA content in selected tissues of *Lamellidens corrianus* after acute exposure to As^{+++} without and with caffeine and during recovery.(Values represent % in dry weight)

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	19.418 ±1.386	19.512 ±1.239		
		Testis	4.417 ±0.806	4.571 ±0.563		
		Digestive Glands	8.607 ±0.921	8.767 ±0.694		
0.672 ppm As^{+++} (B)		Gill	15.194 ±1.359❖ (-21.175)	14.079 ±1.253❖❖ (-27.844)		
		Testis	3.478 ±0.208NS (-21.258)	2.986 ±0.187❖❖ (-34.675)		
		Digestive Glands	5.969 ±0.586❖ (-30.649)	4.321 ±0.438❖ (-50.712)		
0.672 ppm As^{+++}+ 5mg/lit. Caffeine (C)		Gill	17.364 ±1.163NS (-10.577)	15.738 ±1.122❖ (-19.341)		
		Testis	4.237 ±0.534NS (-4.075)	3.405 ±0.295❖❖ (-25.508)		
		Digestive Gland	6.865 ±0.612NS (-20.239)	5.749 ±0.486❖❖ (-34.424)		
After 96hrs Exposure to 0.672 ppm As^{+++}	Normal Water (D)	Gill			15.342 ±1.096NS (+8.970)	16.357 ±1.263NS (+16.180)
		Testis			3.578 ±0.573NS (+19.825)	3.904 ±0.652NS (+30.745)
		Digestive Glands			4.783 ±0.792NS (+10.691)	5.075 ±0.864NS (+17.449)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			16.185 ±0.964NS (+14.958)	17.542 ±0.996■ (+24.596)
		Testis			4.276 ±0.327■■ (+43.201)	5.613 ±0.842■■ (+7.977)
		Digestive Glands			5.194 ±0.756■ (+20.203)	7.089 ±0.907■ (+64.059)

Values in the ( ) brackets indicate percent change over control, N.S.- Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

OBSERVATIONS AND RESULTS

Protein, collagen, ascorbic acid, DNA and RNA contents were estimated in the gills, testis and digestive glands of freshwater bivalve *L. corrianus*, from the control, mercury (0.444 ppm Hg^{++}) and arsenic (0.672 ppm As^{+++}) exposed bivalves after 24 hrs and 96 hrs with and without caffeine. And during recovery with and without caffeine from 2 days and 4 days exposed bivalves respectively and the data obtained for each biochemical with respective time of exposure from all five groups of bivalves is given in the tables 2.1.1 to 2.5.2. The results are given in table with percent changes over control and results of statistical test.

Changes in Protein Contents:-

Table 2.1.1 and 2.1.2 indicate changes in protein level of different tissues of *L. corrianus* on acute exposure to mercury (0.444 ppm Hg^{++}) and arsenic (0.672 ppm As^{+++}). Protein contents after acute exposure to mercury and arsenic, were decreased in gills, testis and digestive glands of experimental bivalves as compared to those of control bivalves and bivalves exposed to heavy metal with caffeine showed less variations as compared to those of only metal exposure. The bivalves showed faster recovery of tissue protein level in presence of caffeine than those allowed curing naturally.

Changes in Collagen Contents:-

Table 2.2.1 and 2.2.2 indicate changes in collagen level of different tissues of *L. corrianus* on acute exposure to mercury and arsenic. Collagen contents after acute exposure of mercury and arsenic were decreased as compared to those of

control bivalves. The collagen contents were higher in heavy metal salt with caffeine-exposed bivalves as compared to those exposed to only heavy metal salts. The bivalves showed faster recovery of tissues collagen level in presence of caffeine than those allowed curing naturally.

Changes in Ascorbic Acid Contents:-

Tables 2.3.1 and 2.3.2 indicate changes in ascorbic acid level of different tissues of *L. corrianus* on acute exposure to mercury and arsenic with and without caffeine and during recovery. It was observed that after acute exposure of mercury and arsenic there was decrease in the ascorbic acid content in gill, testis and digestive gland of experimental bivalves as compared to those of control bivalves. The ascorbic acid contents were higher in heavy metal with caffeine-exposed bivalves as compared to those exposed to only heavy metal salts. The bivalves showed the faster recovery to tissue ascorbic acid level in presence of caffeine than those allowed curing naturally.

Changes in DNA Contents:-

Table 2.4.1 and 2.4.2 indicate changes in DNA level of different tissues of *L. corrianus* on acute exposure to mercury and arsenic with and without caffeine and during recovery. It was observed that after acute exposure of mercury and arsenic, there was decrease in the level of DNA content in various tissues of experimental bivalves as compared to those of control bivalves. The DNA contents were higher in heavy metal salt with caffeine-exposed bivalves as compared to those exposed to only heavy metal salts. The bivalves showed the faster rate of recovery of tissue DNA level in presence of caffeine

than those allowed curing naturally.

Changes in RNA Contents:-

Tables 2.5.1 and 2.5.2 indicate changes in RNA level of different tissues of *L. corrianus* on acute exposure to mercury and arsenic with and without caffeine and during recovery. It was observed that after acute exposure of mercury and arsenic there was decrease in the RNA content in gill, testis and digestive gland of experimental bivalves as compared to that of control bivalves. The depletion in RNA contents was poor in heavy metal with caffeine-exposed bivalves as compared to those exposed to only heavy metal salts. The bivalves showed the faster recovery of tissue RNA level in presence of caffeine than those allowed curing naturally.

The results given in the table also show increased or decreased in all biochemical's with respect to time of exposure and results of 't' test at significant levels $P < 0.001$, $P < 0.01$, or $P < 0.05$ for acute exposure of mercury and arsenic with and without caffeine and during recovery. Significant differences at different levels indicate the toxic effects of the concerned heavy metal and the preventive and curative effect of the caffeine.

DISCUSSION

Aquatic invertebrates naturally accumulate abnormally high amount of heavy metals. The effects of these heavy metals on the normal function of cells, tissues and organs are deleterious due to accumulative toxicity. Mercury and arsenic are hazardous when accumulated even at trace level in the system of all living organisms.

The results of biochemical estimations of

protein, collagen, ascorbic acid, DNA and RNA on acute exposure to mercury (0.444 ppm Hg^{++}) and arsenic (0.672 ppm As^{+++}) showed drastic changes in the physiology of freshwater bivalve, *L. corrianus*.

Mercury and arsenic exposed bivalves showed decrease in the protein, collagen, ascorbic acid, DNA and RNA contents. The exposure of mercury and arsenic with caffeine showed less decrease in the contents of said biochemicals as compare to those of respective heavy metal exposures bivalves. The faster recovery was observed after exposure to caffeine as compared to those recovered naturally in normal water.

Changes in Protein Contents:-

Protein is a key substance to show the effects of heavy metals. Proteins respond for better survival by either increasing or decreasing their levels. So, protein assessment can be considered as a diagnostic tool to determine the physiological responses of the cells and organs.

Protein is an important organic constituent that plays a crucial role in metabolism. Being the integral part of cell membrane, intracellular and extracellular passages are linked through to it. Interactions occurring during protein metabolism in protein, amino acids, enzymes and co- enzymes were studied by Harper *et al.*,(1978). The depletion of tissue protein was due to diversification of energy to meet the impending energy demand under toxic stress (Vincent *et al.*, 1995).

Present investigation clearly showed that after acute exposure to mercury and arsenic protein levels were decreased in gills, testis and digestive glands. The protein contents were significantly

reduced in heavy metal exposed bivalves however the percent decrease in the levels of protein in bivalves exposed to heavy metal with caffeine was less as compared to those exposed to only respective heavy metal at a specific period of exposure.

Sekeri *et al.*, (1968) studied that all enzymes are proteins in nature and they control subcellular functions. In the metabolism of protein many enzymes, co-enzymes intermediate protein and amino acids are involved.

Nagpure and Zambare (2005) observed that on acute and chronic exposure to tetracycline and chloramphenicol, *L. corrianus* showed decrease in protein levels, in proportion with the period of exposure. Muley and Mane (1995) observed that, the freshwater bivalve *L. marginalis* when exposed to sub lethal dose of endosulfan, showed depletion of protein content of almost all tissues undertaken for study. The decrease in protein content may be due to altered size of pores in membrane (Abel, 1974) or diminished protein synthesis (Reddy, 1979). Lomte and Alam (1982) observed the decline in protein level in *Bellamiya bengalensis* after pesticide stress. Ramana Rao and Ramamurthi (1978) reported the depletion in protein content in the tissues of *Pila globosa* after exposure to pesticides. The decrease in average total protein content of tissue after treatment suggests enhancement of proteolysis to meet the high energy demands under heavy metal or other stress.

Mahajan and Zambare (2005) observed a significant decrease in the protein content in various tissues of experimental snails *Bellamiya bengalensis* as compared to that of control. The

protein contents were more in heavy metal salt with caffeine-exposed snails as compared to those exposed to only heavy metal salts.

Impact of heavy metal exposure showed the decreased levels of protein in various animals in aquatic environment. The heavy metals denature the proteins. Mahajan and Zambare (2001) showed that after acute and chronic exposure to $HgCl_2$ and $CuSO_4$, protein contents in different tissues of *Corbicula striatella* were found to be highly depleted and maximum protein depletion was found in foot of $HgCl_2$ treated animals but in $CuSO_4$ treated animal decrease was slight.

Rao *et al.*,(1994) recorded that the content of sperm protein in cauda epididymis reduced significantly on exposure to mercury for 60 days, Nagabhushanam *et al.*,(1987) observed decrease of total proteins in hepatopancreas and its increase in gonad and muscles of prawn, *Macro brachium kistenensis* after acute exposure to pesticides.

Khan *et al.*, (2001) found that the mussels, *P. viridis* when exposed to zinc chloride at 1/10th LC_0 and LC_{50} concentrations showed variation in protein content. The decrease in the protein content can be due to anaerobic metabolism**.** Protein content of brain, liver, kidney and gills of *Heteropneustes fossilis* on exposure to sublethal concentration of mercuric chloride were significantly. Exposure of fish to mercuric chloride + chabazite improved the protein content in comparison to fish of group II. When fish was exposed to chabazite, only, protein contents were found to be increased in comparison to their respective control.

Sastry and Gupta (1978) emphasized that over all decrease in protein content was probably due to enzyme inhibition, which plays an important role in protein synthesis. Rao *et al.*, (1987) found decrease in protein levels in the hepatopancreas of *Indonaia caerules* on exposure to fluorides.

Changes in the Collagen Contents:-

The year 2004 also marks the fifty years of the "Madras Triple Helix", the molecular model proposed for the fibrous protein collagen by Prof. Gopalasamudram Narayana Ramchandran (08 October, 1922 - 07 April, 2001), Physicist turned outstanding structural biologist, and his group. About one quarter of all of the protein in our body is collagen. Collagen is a major structural protein, forming molecular cables that strength the tendon and vast, resilient sheets that support the skin and internal organs. Though collagen is a relatively simple protein, its structure remained a mystery until 1954.

Collagen is the body's most important structural substance. It is the ground substance, or cement, that supports and holds the tissues and organs together. The substance in the bones provides the toughness and flexibility and prevents brittleness. Without it, the body would just disintegrate or dissolve away. It is the substance that strengthens the arteries and veins, supports the muscles, toughens the ligaments and bones, supplies the scar tissue for healing wounds and keeps the youthful skin tissues soft, firm, supple and wrinkle free.

The disturbance or alteration in collagen

formation causes the fearful effects of scurvy, the brittle bones that fracture on the slightest impact, the weakened arteries that rupture and cause hemorrhage, the incapacitating muscle weakness, the affected joints that are too painful to move, the teeth that fall out, and the wounds and sores that never heal. Sub optimal amounts of ascorbic acid over prolonged periods during the collagen, may be the factor in later life that causes the high incidence of arthritis and joint diseases, broken hips, the heart and vascular diseases that cause sudden death, and the strokes that bring on senility. Collagen is intimately connected with the entire aging process.

Effect of heavy metal on collagen content was studied by very few investigators. The present investigation shows the decrease in collagen level in the freshwater bivalves, *L. corrianus* on exposure to acute concentration of mercury and arsenic as compared with control bivalves.

Rajashree and Puvanakrishnan (2000) found that total collagen contents in heart and kidney decreased significantly until 8 day of dexamethasone treatment in the rat. Kidney has its own pattern of collagen type distribution (Michael *et al.*, 1980). Sauk *et al.*, (1992) studied that lead inhibits secretion of osteonectin /SPARC without significantly altering collagen or HsP47 production in Osteoblast like ROS1712.8 cells. Lupton *et al.,* (1985) investigated that cutaneous mercury granulomas are rarely encountered. Clinically they pose difficulty in diagnosis when there is no clear history of penetrating injury by objects containing metallic mercury. Metallic

mercury in tissue sections appears as dark, opaque globules, usually spherical in shape and of varying sizes and number. A zone of collagen necrosis often surrounds the mercury globules.

Any imbalance in the extracellular matrix or alterations in the metabolism of collagen in a pathological condition such as glomerulosclerosis lead to significantly reduced glomerular function (Ohyama *et al.*, 1990). The amount of insoluble collagen is decreased after 14 days of treatment with cortisone (Rajashree and Puvanakrishnan, 1999). Ohyama *et al.*, (1990) reported that the synthesis of low molecular weight collagen is affected most dramatically. Reduction of type IV collagen protein and mRNA by dexamethasone on basement membrane collagens (found in the basement membrane) are most affected. Thus, they suggested that the interstitial and basement membrane collagens have been coordinately down regulated by dexamothasene. Mukharjee *et al.*, (1990) found the decrease in type I and Type III ratio in 10 week old spontaneously hypertensive rats. The dramatic decrease in the type I:III ratio, observed in their study, emphasizes that the type of collage may play an important role in my Ocardial dysfunction. The decrease in type I:III ratio of collagen in kidney was observed by Rajashree and Puvanakhrishna (2000). Bratoma and Patnaik (1983) studied that increase in total collagen content of tendon also suggests either increased rate of synthesis and or low rate of degradation of collagen on exposure to low temperature regime. The effect of hypothermia on collagens of different tissues of Garden lizard was not uniform.

Presumably the differences were observed in the response of tissue reflect, the differences in the type of cross-links present in each issue and the initial status of collagen at the beginning of the experiment.

Hansson *et al.*, (2005) observed that exposure to mercuric chloride during the induction phase and after the onset of collagen induced arthritis, enhances immune autoimmune responses and exacerbates the disease in DBA/1 mice. They suggested that, production of collagen specific IgG antibodies is considered the hallmark of humoral immune responses associated with collagen-induced arthritis. Thirty four days after secondary immunization with collagen type II, all of the collagen induced arthritis mice documented in regardless of whether they were treated with mercury, demonstrated levels of IgG1 antibody against chicken collagen II that were similar and much higher than those of control animals administrated mercury or saline alone. By day 62, these IgG1 antibody levels in collagen induced arthritis mice had decreased. They clearly stated that the influence of chronic exposure to sub toxic dose of mercuric chloride on the development of collagen induced autoimmune murine model of chronic graft versus host disease (GVHD) such results were suggested by Via et al., (2000).

Copper plays an important role in the production and maintenance of connective tissue. Copper deficiency in young dogs was associated with poor collagen cross-linking. Changes in plasma copper concentrations because of traumatic bone injury and partly because of the inflammation,

partly because of the role of copper in collagen formation during bone repair. Interestingly, serum copper values of patients with osteoporosis do not differ significantly from normal patients. Lithium treatment is an established practice with mania although it reportedly has detrimental long-term effect due to the induction of a collagen like syndrome (Jendryczko and Drozdz, 1985).

Basement membranes are composed of type IV collagen, laminin, HSPGs, fibronectin and entactin. Type IV collagen is composed of six genetically distinct genes. Type IV collagen may contain two isoforms in human basement membranes (Paulsson, 1992) or more in other forms. Type IV collagen promotes cell adhesion, migration, differentiation and growth (Paulsson, 1992), and play crucial role in endothelial cell proliferation and behavior during angeogenesis.

The impact of decreased collagen levels in the tissues may be due to binding of heavy metals to disulphide linkages that maintains the triple helical tertiary structure of collagen. The abnormal collagen thus formed may be digested by the collagenase enzyme and hence the collagen contents were decreased. Alterations in the basement membranes of epithelia due to the changes in the collagen can alter the extra cellular matrix-cell interactions and the receptor cells of the epithelia resulting into poor functioning of epithelia. The hepatopancreas, testis and gills have major epithelial structures whose physiological status can be altered due to variation in the collagen levels and its structure.

Changes in the Ascorbic Acid Contents:-

Ascorbic acid is an “enedial lactone” of an

acid with a configuration similar to that of the sugar L-glucose. The vitamin C is L-ascorbic acid while D-ascorbic acid is antiscorbutic. Glucose and hexoses are utilized for the biosynthesis of ascorbic acid.

The present investigation results showed that after acute exposure to mercuric chloride and sodium arsenate there was decrease in ascorbic acid content in freshwater bivalve, *L. corrianus*, in various tissues of experimental bivalves as compared to that of control bivalve. The ascorbic acid contents increased in heavy metal with caffeine exposed bivalves as compared with only heavy metal salts.

In the bivalve, *Parreysia cylindrica*, ascorbic acid content was found to be decreased in whole body, mantle, gill, digestive gland and elevated in foot after acute and chronic treatment of cypermethrin (Waykar *et al.,* 2001). Jadhav *et al.*, (1996) found the decrease in ascorbic acid levels of various tissues of the bivalve, *Corbicula striatella* during acute and chronic exposure to carbaryl. Ali *et al*. (1983) proposed that, changes in the environment cause alteration in the ascorbic acid content. Different pollutant stress has its impact on the concentration of ascorbic acid. Seymour (1981b) reported that the levels of ascorbic acid in the ovaries of maturing crustacean carp *Carassius carassis* decreased after injection of pituitary extract. Dedemeyer (1969) observed that the stress-induced release of cortisol occurred colomitant with a decrease in the ascorbic acid in the kidney of salmonids.

Zambare (1991) in *Corbicula striatella* found that the ascorbic acid, content were higher during

high gametogenic activity. Shanta and Motelica (1963) suggested that the concentration of ascorbic acid in tissue depends on physiological state of fish, *Mugil dussumieri*. Giroud *et al.*, (1968) in teleost fishes reported the changes in ascorbic acid content after heavy metal stress in snails. The domination might be due to decreased biosynthesis and increased catabolism as was observed by Banerjee and Basu (1975). Kachole *et al.*, (1977) observed that in *Parreysia cylindrica* the ascorbic acid might have induced hepatic mixed function of oxidase system and played important role of bio-transformation of toxic substances into non toxic.

Changes in the DNA Contents:-

DNA content, the index of capacity of an organism for protein synthesis in the different stress conditions was affected by heavy metals or any toxic metals or pesticides. Copper ions introduced into asides tumors penetrate the nucleic acid (DNA) and damage it, causing incardinating of the chromatin structure, copper associates with DNA at higher copper concentrations. Simultaneous changes in Ca, Mg, Fe, Cu and Zn concentrations in cultured human lymphocytes affected thymidine incorporation and surface antigens of human T and B lymphocytes (Carpentieri, 1987; Carpentieri *et al.*, 1987).

Black *et al.*, (1996) observed significant DNA strand breakage in the foot tissue from *Anodonta grandis* exposed to lead. They suggested that a threshold effect for DNA damage and repair resulting from Pb exposure were by repair of DNA strand breaks that may occur only if certain body burden or exposure duration has been achieved.

Tong Lu *et al.*, (2001) observed that approximately 60 genes (10%) were differentially expressed in arsenic exposed human livers compared to controls. The differentially expressed genes induced those involved in cell cycle regulation, apoptosis, DNA damage response, and intermediate filaments. The observed gene alterations appear to be reflective of hepatic degenerative lesions seen in the arsenic exposed patients. This array analysis revealed important patterns of aberrant gene expression occurring with arsenic exposure in human liver. Aberrant expressions of several genes were consistent with the results of array analysis of chronic arsenic exposed mouse livers and chronic arsenic-transformed rat liver cells.

They suggested that clearly a variety of gene expression changes might play an integral role in arsenic hepatotoxicity and possibly carcinogenesis. The metals may be carcinogenic because of their ability to generate reactive oxygen species and other reactive intermediates or react directly with DNA. In deed, several transition metals can generate reactive oxygen species in biologic fluids at physiologic pH, Moreover Cr (VI) is a better carcinogen than Cr (III) and yet Cr (III) is much better in DNA binding reactions than nickel or Cr (VI). In addition the high radioactive metals iron and copper, which also bind to DNA more avidly than does Ni (II) are only weakly carcinogenic.

Another explanation may be based on the known ability of carcinogenic metals to facilitate DNA damage through inhibition of DNA repair enzymes or binding to histones. In both cases, it is

assumed that the ultimate target of free radicals or metals is DNA and that the mechanisms of carcinogenesis must include genotoxic effects. However, metal such as Ni (II) is only weakly genotoxic and mutagenic. Rao *et al.* (1998) studied the effect of Fluoride toxicity on the nucleic acid contents of freshwater crab, *Barytelphusa cunicularis*. They observed that the level of DNA in muscles and hepatopancreas were found to be elevated initially and then a gradual decrease was noted in gills, testes and ovaries.

The decreased levels of DNA and RNA were observed by various investigators, Lomte and Patil (1989) in *Mythima seperata*. The cellular degradation rapid histolysis and decreased rate of protein synthesis are possible reasons behind this. As compared and supported by above literature, the present investigation of the acute exposure of mercury and arsenic to bivalves *L. corrianus*, showed decreased DNA contents compared with control bivalves, and those exposed to heavy metals with caffeine. The bivalves showed faster recovery due to caffeine, as compared with those recovered in natural water.

Aurintricarboxylic acid (ATA), a polyanionic form probably interferes with the electrostatic interaction between DNA and RNA in their interaction with nuclei acid binding proteins. The synthetic deutero ATA fraction was capable of inhibiting the degradation of yeast RNA by bovine pancreatic RNase and thus molecules that interact by electrostatic interactions can affect the rate of RNA catabolism.

Changes in the RNA Contents:-

RNA polymerase binds the binding site especially to its DNA template, binds its nucleotide and primer substrates, forms a new phosphodiester bond and elongates the growing RNA. In the present investigation, the RNA contents were decreased due to the acute exposure of mercury and arsenic. Rao *et al.* (1998) in *B. cunicularis* observed decreased level of RNA on heavy metal stress.

The present investigation shows the interaction of heavy metals and RNA. It shows that the heavy metals reduced the RNA contents and after the recovery with caffeine the RNA, contents were increased. The decrease in RNA on exposure to mercury and arsenic may be due to damage in DNA, poor rate of synthesis of enzymes necessary for transcription or increased catabolism of RNA due to their abnormalities on binding to heavy metal or abnormal.

Changes in Protein, Collagen, Ascorbic Acid, DNA and RNA Contents in the Recovery due to the Caffeine Exposure:-

Detoxification can be used as a beneficial curative measure and as a tool to increase overall health and vitality. Detoxification treatment has become one of the cornerstones of alternative medicine. Detoxification therapies are having increasing importance and popularity.

Caffeine has capacity to bind with heavy metals. Heavy metal content of water was reduced after addition of coffee (Mick McLaughlin 2000 of CSIRO, Australia). Caffeine binds divalent cations of calcium in *Ferrete* ventricular muscles (Leoty *et al.*, 2001).

Kolayly *et al.*, (2004) studied the binding

capacities of caffeine with different micronutrients. According to him, the binding strength of the caffeine is weaker than that of the EDTA. Since all nitrogen, groups in caffeine are blocked by methylation, metals probably forms complexes with second and sixth organ of caffeine. Physical properties of bond energies suggest that oxygen metal bonds are stronger than sulfur metal bonds hence the caffeine molecules can drag the heavy metal that is bound to the protein.

Caffeine is well-known nervous system stimulant but besides it, it is now found that it has antioxidant activity. This activity of caffeine can protect the damage of tissue chemicals and genetic materials from heavy metal generated free oxygen radicals.

In present investigation due to heavy metals, mercury and arsenic, there was decrease in all biochemical contents and during the recovery, there was increase in the all biochemical contents in all tissues. In presence of caffeine, the decrease rate in biochemicals was low and in presence of caffeine, recovery rate was faster. Therefore, the caffeine has the protective and curative role in repair of tissue damage caused due to the exposure to heavy metals. Very little work has been carried on the recovery of tissue damage and mainly caffeine's protective role in the tissue damage by heavy metals.

Chelation therapy is the application of Chelation techniques for the therapeutic or preventive effects of removing unwanted metal ions from the body combination therapy. Dimercaprol (Bal) is used for metal intoxication as a cheater to

remove arsenic, mercury, cadmium and lead poisoning. EDTA is useful antidote in lead poisoning and lead encephalopathy. EDTA is administered intravenously, or intramuscularly (Chelationtherapy, journal of Envt. Engg. Vol. 127, No.3).

Mahajan (2005) studied that alterations caused due to mercury, arsenic and lead in acute and chronic exposed snails, *B. bengalensis* decreased the contents, such as protein, lipid, glycogen, ascorbic acid, which was improved due to the caffeine exposure**.** Mapengo (1990) studied that the fusion temperatures of caffeine–1, 3-(CD3)2, caffeine–3, 7-(CD3) 2, Caffeine 1, 3, 7-(CD3) 3 were 0.4 - 1.7°C) higher than for caffeine, indicating a higher degree of hydrogen bonding in the crystalline forms of these compounds. Plaskett and Cafarelli, (2001) studied that caffeine has known exogenic effects, some of which have been observed to increase the T lymphocytes. The radio sensitizing effects of caffeine is associated with the disruption of DNA damage responsive cell cycle checkpoints. The caffeine might inhibit one or more components in an ATM dependent Checkpoint medium pathway in DNA damaged cells. Caffeine inhibits the catalytic activity of ATM and the related kinase and DNA damage as was studied by Sarkaria *et al.,* (1999).

Harish et al. (2000) observed that the effect of caffeine as a reflective DNA synthesis inhibitor or given as pre-inter and post treatments on the ethyl methano sulphonate (EMS) induced adaptive responses in vivo mouse bone marrows cells was studied in order to understand the influence of

caffeine on the adaptive response. Matsumura et al (2000) studied that lack of Ca_2^+ and ATP dependent priming stage in caffeine induced exocytosis in bovine adrenal chromaffin cells in comparison with Ca^{++}. They suggested that the ATP requiring priming stage is lacking in the process of caffeine-induced exocytosis in bovine adrenal chromaffin cells.

Effect of caffeine and zinc on DNA and protein synthesis of neonatal rat cardiac mussel cell in culture was studied by Kanemuru *et al.*, (1992) and they found that the effect of caffeine (0.2^{-2} mM) inhibited both DNA and protein synthesis of the cells. Addition of EDTA in the growth medium inhibited both DNA and protein synthesis without caffeine and in the presence of lower concentration of caffeine (0.2 mM) in the growth medium, 10 micron of zinc concentration reversed DNA synthesis, which was inhibited by the chelating agent (EDTA). A higher concentration of caffeine (2mM) in the growth medium was completely abolished sensitivity of cardiac myocytes to zinc. Additional zinc supplementation to the growth medium of cardiac myocytes, effect of caffeine may be associated with the zinc dependent enzymes involved in DNA synthesis and caffeine zinc chelate formed makes zinc unavailable for these enzymes. Thus the impact of heavy metals on several biochemicals, if considered as a tool for studying the toxic level, caffeine reduces the toxic stress and hence has a preventive and curative property against the heavy metal induced tissue alterations. The rapid recovery by caffeine also suggests it's chelate formation and the removal of the chelate

complex from the body and thus the reduction of the metal stress.

SUMMARY

- *The present investigation showed the role of caffeine in heavy metal induced biochemical alterations in an experiment model, the freshwater bivalve, Lamellidens corrianus.*
- *The biochemical contents such as protein, collagen, ascorbic acid, DNA and RNA in various tissues like gill, testis and digestive glands of freshwater bivalves; Lamellidens corrianus were studied after acute exposures to mercury (0.444ppm Hg^{++}) and arsenic (0.672ppm As^{+++}) with and without caffeine and during recovery.*
- *The protein, collagen, ascorbic acid, DNA and RNA content in gill, testis and digestive glands were found to be significantly decreased after acute treatment by heavy metal salts.*
- *The protein, collagen, ascorbic acid, DNA and RNA contents were more in gill, testis and digestive glands of freshwater bivalves, Lamellidens corrianus when exposed to heavy metal salts with caffeine as compared to those exposed to only heavy metal.*
- *After 96 hrs exposure to heavy metal salts, The bivalves showed fast recovery of tissue biochemical contents in presence of caffeine than those allowed to cure naturally.*
- *The results indicate the detoxifying effect of caffeine on heavy metal induced alterations.*

❑❑❑

CHAPTER : 3
ENZYME STUDY

INTRODUCTION

The heavy metals enter into the body of animals including man through the non-vegetarian and vegetarian diet and drinking water and accumulate in the tissues. In air breathers, metal particles can also enter in respiratory organs where they are absorbed. Various studies confirm that heavy metals can directly influence the behavior by impairing metal and neurological function influencing neurotransmitter production and utilization and altering numerous metabolic body processes. Heavy or toxic metals are trace metals with a density at least five times that of water. As such, they are stable elements meaning they cannot be metabolized by the body and hence they bio-accumulate, usually react with proteins, interfere physiological activities, and thus increases the risk

of life in various ways. They are difficult to remove from body.

Heavy metals have high biological activity and have a tendency to accumulate in organisms, making adverse effects possible at very low levels of exposure. Many enzymes are metalloenzymes, which need a specific metal as co-factor for its activity. Some non-target highly reactive metals can bind with these enzymes and thus reduce the activity of that enzyme. Secondly, some heavy metals bind at –SH, groups of enzymes thus the tertiary structure of enzyme is affected and the enzyme looses its enzyme activity.

Among the heavy metals mercury, arsenic, lead, and cadmium are mostly non-essential elements. Much more disturbance in metabolism results into death of an organism (Burton *et al.*, 1972; Alam and Lomte, 1984) due to such metals.

The study of enzymes creates special interest because it lies just on the borderline where biological and physical sciences met. On the other hand, enzymes are of supreme importance in biology. Life depends on complex network of chemical reactions brought about by specific enzymes, and any modification of the enzyme pattern may have far-reaching consequences for the living organisms. The mechanism of enzymes is itself one of the most facinating fields of scientific investigation.

Alkaline Phosphatase:-

Hiromu (1969) reported that this enzyme helps in the metabolism and transphosphorylation. Acid phosphatase, being a lysosomal enzyme, hydrolyses phosphate esters in acidic medium De

Duve *et al.*, (1955) studied the enzyme is a non-specific phosphomonoesterase that shows maximum activity at pH values greater than 8. It also catalyzes the transfer of phosphoryl groups. These reactions involve the formation of a phosphoryl intermediate and the hydrolyzed substrate. The phosphoenzyme may transfer the phosphoryl group to water or to an acceptor molecule to give a new phosphoester.

$$E + Rop \Leftrightarrow E.Rop \Leftrightarrow E\text{-}P + Ro^{-}$$

$$E\text{-}P \Leftrightarrow E.P \Leftrightarrow E + Pi$$

Where, E-P represents the phosphoenzyme bound by covalent bond and E.P a non-covalent complex, in which phosphate is a co-ordinate to the zinc. The phosphoenzyme may be formed from either direction.

Alkaline phosphatase is known to be involved in mineralization, possibly through hydrolysis of organic phosphates to raise the product $[Ca^{2+}]$ [Pi] to a level where precipitation occurs.

Functional significances of alkaline phosphatase were studied first by Moog (1946). Its localization in plasma membrane perhaps played an important role in transport of phosphate through cellular. Intracellular proteolysis is an important catabolic event, which regulates the protein concentration in cells (Schimke, 1970; Goldberg and Dice, 1974). There exists a remarkable difference in the half-lives of proteins in rat liver and other tissues that may range from a few minutes to many weeks (Bohley *et al.*, 1987; Marzella and Glaumann, 1987). Despite such enormous diversities in half-lives, the total cellular protein content remains fairly constant under

normal conditions, implying that the synthesis and degradation of proteins are under tight physiological control (Bohley *et al.*, 1987).

The contribution of lysosomes to basal intracellular protein degradation is a matter still unsettled; their role in protein degradation during induced conditions is generally thought to be of paramount importance (Marzella and Glaumann, 1987).

The purity of the lysosomal fraction was judged by assaying three lysosomal enzymes; acid phosphatase (Barrett and Health, 1977), N-acetyl-b-Dglucosaminidase (Hultberg and Ockerman, 1972), and aryl sulfatase (Roy, 1971). The lysosome fraction was found to be 12 to 15 fold enriched over the homogenate. The cytosol fraction was assayed for these enzymes in order to determine lysosomal contamination. The activities of these enzymes in the cytosol were less than that in lysosomes. The protein concentration in lysosomes, cytosol, and homogenate was determined by the bicinochoninic acid (BCA) assay (Smith *et al.*, 1985) membranes. Hardonk and Koudstaal (1976) reported that this activity is facilitated by phosphatase or phosphotranferase action.

Acid Phosphatase:-

Acid phosphatase, a nonspecific monoesterase, pre-eminently regarded as the marker enzyme has been found in golgi cisternae and lysosomes.

The enhancement of Alkaline and Acid phosphatase is quite conceivable in animals under morbidity. In crustaceans, the distribution of phosphatase and their activity in the haemolymph, hepatopancrease (Digestive Gland) (Van, 1970),

Cuticle (Travis, 1957, 1960) and gastrolith walls, (Travis, 1963) have been observed by histochemical procedures.

Impact of Heavy Metals on Tissue Phosphatase Activity:-

The impact of heavy metals and other biocides on tissue phosphatases activity of organisms are well documented but studies in relation to endotoxin toxicity or infection in animals are rather meager.

The alterations in the alkaline and acid phosphatase activities in various organs of snails, those act as intermediate host for trematodes have been reported by number of workers, Cheng, (1962).

Alkaline phosphatase and acid phosphatase are responsible for transphosphorylation and have an important role in the general energetic of an organism. Lomte and Godhamgaonkar (1986) reported lysosomal enzymes and trematode infection in a snail, *Thiara tuberalata* (Muller) while Bendse and Karyakarte (1995) studied acid and alkaline phosphatase activities in hepatopancreas of trematode on toxicant treatment in *Melania tuberculata*.

Effect of heavy metals on a set of physiological parameters can make it possible to establish specific responses of that pollutant and may make it possible to identify a pollutant because of its pattern of physiological response. Higher toxic heavy metals bring the adverse effects on aquatic organisms at cellular level or molecular level and ultimately lead to disorder in biochemical composition, which is useful in determining different toxicants and protective mechanisms of

the body to resist the toxic effect of the substances.

There is considerable amount of literature developed for the study of effect of heavy metals concerning the enzyme systems of various animals (Bhamre, 1993; Dheshmukh, 1995; and Mazhar, 1995). Molluscs have exhibited ability to adopt heavy metals or toxicants up to certain level in different types of habitats. They show different digestive patterns. The studies on the enzymes of lamellibrancs were first exhibited by Yonge (1926) in *Oyster* and in *Lamellidens* species in India by Mukherjee and Kanungo (1954). Out of this investigation, little work has been done on alkaline and acid phosphatase enzyme activity of freshwater mussels.

All enzymes are proteins in nature and they control sub cellular functions. In the metabolism of protein, involvement of many enzymes, co-enzymes, intermediate proteins and amino acids are studied in many animals (Sekeri *et al.*, 1968).

Heavy metal ions have affinity towards the different groups of the enzymes like suphydryl groups where they tightly bound, while the amino, hydroxyl, carbaryl, imidazole and phosphate groups where they showed loose binding. Thus, metal ions act as potent enzyme inhibitors (Anderson and Webber, 1977). Hinton and Koening Jr. (1975), Sontakke (1992), Sultana (1996) and Chaudhari (2000) observed the inhibition of different enzymes after the exposure to heavy metals.

Mukhtar and Rao (1972) showed that salts of Cu, Zn, Co, Mn & Mg at 10^{-2} M inhibited the enzyme to varying degrees, while $CaCl_2$ at 10^{-4} M slightly activated the enzymes. Hinton and Koening

(1975) recorded the inhibition of various enzymes after methyl mercury intoxications. Sastry *et al.*, (1979) observed the effect of $HgCl_2$ on digestive enzymes of the bivalve, *Glycymeris yessoensis.* Lomte and Patil (1989) studied the enzyme activity of arginase, acid and alkaline phosphatase in the 5^{th} instar larva of the *Mythima* (*Pseudaletia*) *separata* after pesticidal stress. Chand *et al.*,(1988), Leo and Sabapathy, (1990), Tormanen (1997) studied the effect of metal ions on various enzymes. Nitrogen metabolites and related enzymatic activities in the body fluids and tissues. Mercury denatures proteins, inactivates enzymes and causes severe disruption of any tissue with which it comes into contact in sufficient concentration (WHO, 1991). Methyl mercury (Hg^{+2}), a form that shows primarily epithelial toxicity, can inhibit Na^+/K^+ - ATpase at low concentration (Wang and Horisberger, 1996).

Mercury can bind to phosphate; sulhydryl and imidazol group in proteins and disrupt the activity of a number of enzymes. It may also affect the transport of ions across cell and mitochondrial membranes (Ballantyne *et al.*, 1999). Histoenzymological analysis revealed toxic effects of $HgCl_2$ on hydrolytic enzymes of testicular tissues (Chaudhary & Vachhrjani, 1987). Catabolic effects are altered or induced by the different chemicals. The complete enzyme system become imbalanced and creates a hypersensitive condition against the toxic effect caused by the toxicant.

More recent research indicates that as compared to arsenite, trivalent methylated arsenic metabolites exert a number of unique biological effects, which are more cytotoxic, and genotoxic,

and are more potent inhibitors of the activities of some enzymes (Thomas *et al.*, 2001, Kitchin and Ahmad, 2003) because each arsenic species (e.g. As(III),As (V),As B, MMA^{V}, MMA^{III}) exhibits different toxicities. It may be important to take into account the fraction of total mercury and total arsenic present in the inorganic and organic forms while estimating the potential risk posed to human health through the consumption of mercury and arsenic contaminated fish and shellfish.

Heavy metal toxicity can cause a wide range of problems including severe injury to the body organs and the brain. To reduce the heavy metal load from the body, some chelating agents are used. Dimercaprol (BAC) is used for lead, arsenic and mercury toxicity and is given intramuscularly, Calcium disodium verasenate (CaNa2- EDTA) can be used in conjunction with BAL in lead toxicity, it never be used alone in treating lead toxicity because it chelates only extra cellular lead but lead is usually intracellular.

Caffeine being water soluble and common cheaper beverage, it will be cheapest preventive and curative medicine. In 1982, Takayama, long term study on the effect of caffeine in wistar rat, Gann has proved that caffeine belongs to a group of compound known as methylxanthine and it is non carcinogenic in animal model and antagonize the carcinogenic effects of chemicals in *vitro*. Under in *vitro* condition, caffeine has been reported to enhance or inhibit tumorigenesis induced by various carcinogenic agents. In 2001, Hosaka, *et al.,* has studied hepatocarcinogensis inhibition by caffeine in Ag1 rats treated with 2-

acetybrminoflurened and has showed that caffeine inhibited hepatocarcinogensis induced by 2-acetylaminoflure. Chung-Fung-Lung (1999) reported that caffeine when given in drinking water at a concentration identical to that found in 2% tea was able to inhibit lung tumours induced by 4-(Methylnitrosomino)-1-(3-pyridyl)-1-butonone (NNK). These agents can block the metabolic activation steps, scavenge the reactive intermediates or enhance the detoxification (Starvic, 1994). Wattenberg (1992) reported that any compound that can block the metabolic activation step, scavenge the reactive intermediate or enhance detoxification would be a potential chemo preventive agent. The other mode of activation involves peroxidative co oxygenation by either prostaglandin synthesis or lipid peroxidation via a free radical mediated non-enzymatic process (Sivaragah *et al.*, 1983).

Gandhi and Khanduja (1992) studied action of caffeine in altering the carcinogen activating and detoxifying enzymes in mice and reported the induction of xenobiotic detoxifying enzymes as an additional mechanism by which plant products may act as anticarcinogens, since, the induction of detoxifying enzymes is capable of competing with step in xenobiotic activation. Caffeine have been found to increase glutathione synthetase activity and reduced glutathione in liver and lungs of rat. Shelar *et al.*, (2002) suggested that decline in the rate or oxygen consumption after exposure to lethal concentration of heavy metal pesticides might also be due to its action on metabolic cycle of sub-cellular level.

Hence, an attempt has been made to study the effect of heavy metals on freshwater bivalve, *Lamellidens corrianus* with respect to changes in the levels of alkaline phosphatase and acid phosphatase enzyme activity and the impact of caffeine on the alteration in their activities.

MATERIALS AND METHODS

Selected experimental model animal, the freshwater bivalve, *Lamellidens corrianus* were collected from the Paithan dam at Paithan, Tal. and Dist. Aurangabad (M.S.). After collection, the bivalves were acclimatized in the laboratory at room condition for 2-3 days. Selected active acclimatized bivalves to approximately same size were selected for experiment.

Before starting the experiment, these bivalves were divided into three groups such as, A, B & C.

1. 'A' group bivalves were maintained as control.
2. 'B' group bivalves were exposed to acute dose ($LC_{50/2}$) of heavy metal salts, mercuric chloride (0.6 ppm) and sodium arsenate (0.9 ppm).
3. 'C' group bivalves were exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate with caffeine (5 mg/lit).

Bivalves from B group were divided into D and E group after 4-day acute exposure.

4. 'D' group bivalves pre-exposed to acute dose ($Lc_{50/2}$) of mercuric chloride/ sodium arsenate were allowed for self cure in normal water while
5. 'E' group bivalves pre exposed to acute dose ($Lc_{50/2}$) of mercuric chloride / sodium arsenates were exposed to caffeine (5 mg/lit)

for rapid recovery from tissue damage.

Some experimental bivalves of A, B & C groups were dissected after 24 hrs and 96 hrs and from D and E group after 2 days and 4 days during recovery and tissues such as gill, testis and digestive gland were separated. After removing these tissues from bivalves, 1% homogenate of each tissue was prepared in ice-cold buffer. The homogenate was centrifuged and supernatant was used to estimate the alkaline phosphatase and acid phosphatase activity.

Alkaline Phosphatase Assay:-

Alkaline phosphatase activity was assayed by the method of King (1951). The reaction mixture consisted of 1 ml (0.01 M) disodium phenyl phosphate, 2 ml carbonate-bicarbonate buffer of pH 10 and 0.5 ml tissue homogenate. It was incubated at 37°C for 1 hr. The reaction was terminated by the addition of 1 ml of Folin ciocaltaeu phenol reagent and centrifuged at 2000 rpm for 10 min. To the supernatant, 2 ml of 15% sodium carbonate was added. The blue colour complex developed was read at 660 nm. The blank readings were obtained without incubation. The initial reading of the reaction before incubation was subtracted from the final reading after the enzyme activity of the incubation. The calibration of standard curve was developed by using phenol as a standard. The activity of alkaline phosphatase enzyme was expressed as KA units/gm/hr at 37 ^{0}C at pH 10 (K.A. unit =King-Armstrong unit).

Acid Phosphatase Assay:-

Acid phosphatase activity was measured by the method of Gutman and Gutman (1940). The

activity was carried out in reaction mixture comprising of 1 ml (0.1 M) disodium phenyl phosphate, 2 ml citric acid buffer pH 4.9 and 0.5 ml tissue homogenate. The mixture was incubated at 37 ^{0}C to 1 hr. The reaction was inhibited by the addition of 1 ml of Folin Ciocataeu phenol reagent and mixture was centrifuged at 2000 rpm for 10 minutes. To the supernatant 2 ml of 15% sodium carbonate was added. The blue colour complex developed was read at 660 nm. The blank readings were taken without incubation.

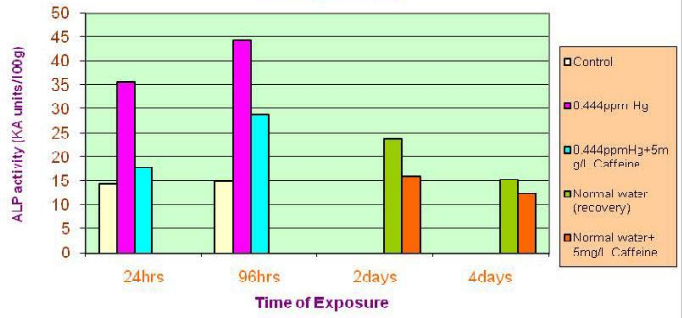
The calibration of standard curve was developed by using phenol as a standard. The activity was expressed as KA units/gm/hr at 37°C at pH 4.9. Standard deviation and student 't' test of significance are calculated and expressed in respective tables.

Table No. 3.1.1: Changes in the Alkaline phosphatase activity in selected tissues of *Lamellidens corrianus* after acute exposure to Hg^{++} without and with caffeine and during recovery. (Values represent % in dry weight)

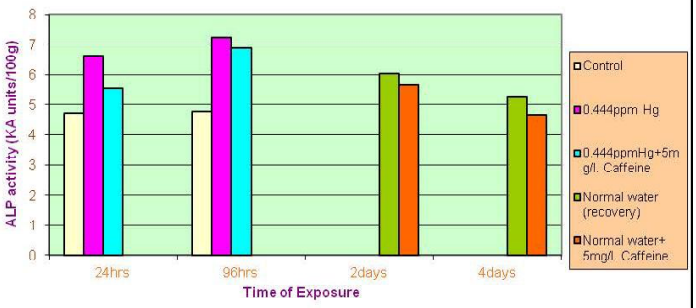
Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	14.375 ±1.273	14.870 ±1.119		
		Testis	4.730 ±0.921	4.782 ±0.897		
		Digestive Glands	5.585 ±0.978	5.503 ±0.990		
0.444 ppm Hg^{++} (B)		Gill	35.500±1.709❖❖❖ (-146.956)	7.212 ±0.779❖ (-50.815)		
		Testis	6.626 ±0.882❖ (-40.084)	7.212 ±0.779❖ (-50.815)		
		Digestive Glands	8.851 ±0.998❖ (-58.478)	8.376 ±0.775❖ (-415.646)		
0.444 ppm Hg^{++} + 5mg/lit. Caffeine (C)		Gill	17.740 ±1.500❖ (-23.408)	21.376 ±1.891❖ (-93.678)		
		Testis	5.550 ±0.773NS (-17.336)	6.890 ±0.809❖ (-44.081)		
		Digestive Glands	7.100 ±0.881NS (-27.126)	9.460 ±0.887❖❖❖ (-71.906)		
After 96hrs Exposure to 0.444 ppm Hg^{++}	Normal Water (D)	Gill			23.860 ±1.700■ (+46.230)	15.310 ±1.322■■■ (+65.498)
		Testis			6.032 ±0.883NS (+16.361)	5.244 ±0.558■ (+27.287)
		Digestive Glands			18.955±0.618■■■ (+32.200)	14.200 ±0.819■■■ (+49.957)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			16.070 ±1.403■■■ (+53.785)	12.420 ±1.222■■■ (+72.011)
		Testis			5.682 ±0.604■ (+21.214)	4.662 ±0.774■■ (+35.357)
		Digestive Glands			13.842 ±1.333■■■ (+51.219)	8.437 ±0.590■■■ (+70.267)

Values in the ( ) brackets indicate percent change over control, N.S.- Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

Fig.3.1.1 Alkaline phosphatase activity in gill of *L.corrianus* after acute exposure to Hg++ with and without caffeine and during recovery.



Alkaline phosphatase activity in testis of *L. corrianus* after acute exposure to Hg++ with and without caffeine and during recovery.



Alkaline phosphatase activity in digestive glands of *L. corrianus* after acute exposure to Hg++ with and without caffeine and during recovery.

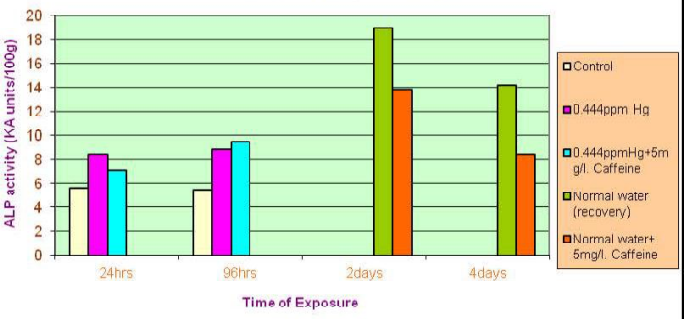
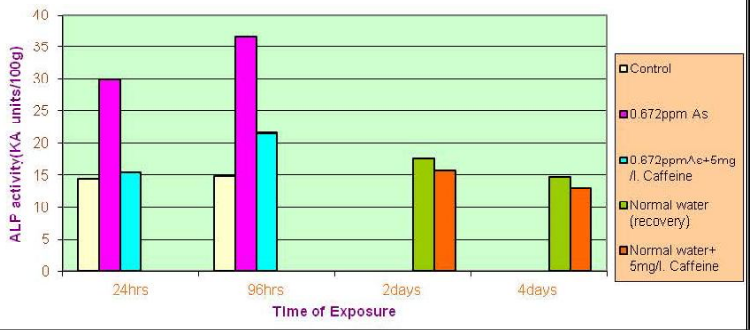


Table No. 3.1.2: Changes in the Alkaline phosphatase activity in selected tissues of *Lamellidens corrianus* after acute exposure to As^{+++} without and with caffeine and during recovery. (Values represent percentage in dry weight)

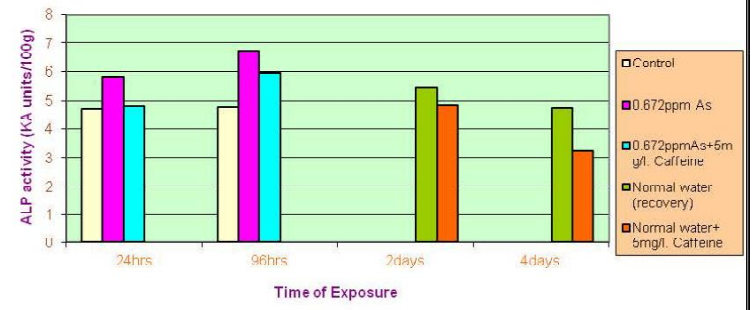
Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	14.375 ± 1.273	14.870 ± 1.119		
		Testis	4.730 ± 0.921	4.782 ± 0.897		
		Digestive Glands	5.585 ± 0.978	5.503 ± 0.990		
0.672 ppm As^{+++} (B)		Gill	29.896±1.117❖❖❖ (-107.972)	36.753±1.005❖❖❖ (-147.162)		
		Testis	5.825 ± 0.721❖ (-23.150)	6.701 ± 0.688❖ (-40.129)		
		Digestive Glands	7.637 ± 0.818❖❖❖ (-36.741)	19.862±0.782❖❖❖ (-260.930)		
0.672 ppm As^{+++} + 5mg/lit. Caffeine (C)		Gill	15.374 ±1.463NS (-6.949)	21.695 ±1.867❖❖ (-45.897)		
		Testis	4.807 ± 0.762NS (-1.627)	5.937 ± 0.798NS (-24.153)		
		Digestive Glands	6.187 ± 0.874❖❖ (-10.778)	9.244 ±0.895❖❖❖ (-27.126)		
After 96hrs Exposure to 0.672 ppm As^{+++}	Normal Water (D)	Gill			17.760±1.373■■■ (+51.677)	14.745± 1.112■■■ (+59.888)
		Testis			5.437 ± 0.987NS (+18.862)	4.750 ± 0.998NS (+29.115)
		Digestive Glands			16.987 ± 1.0400■ (+14.474)	12.550 ± 1.300■■ (+36.814)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			15.650 ± 1.689■■■ (+57.418)	13.126 ± 1.772■■■ (+64.285)
		Testis			4.842 ± 0.872■ (+27.742)	3.239 ± 0.891■■■ (+51.663)
		Digestive Glands			11.255±0.1223■■■ (+43.334)	5.832 ± 1.789■■■ (+70.637)

Values in the ( ) brackets indicate percent change over control, N.S.-Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

Fig.3.1.2 Alkaline phosphatase activity in gill of *L.corrianus* after acute exposure to As+++ with and without caffeine and during recovery.



Alkaline phosphatase activity in testis of *L.corrianus* after acute exposure to As+++ with and without caffeine and during recovery.



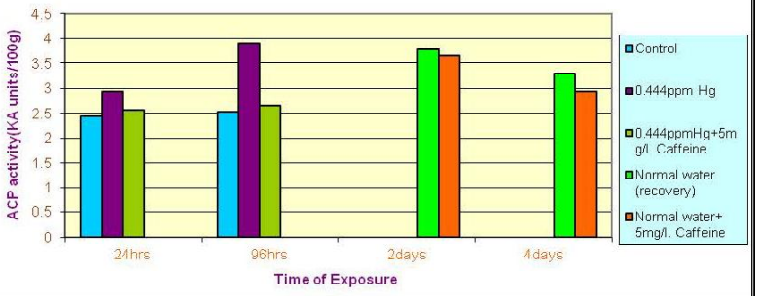
Alkaline phosphatase activity in digestive glands of *L.corrianus* after acute exposure to As+++ with and without caffeine and during recovery.

Table No. 3. 2.1: Changes in the Acid phosphatase activity in selected tissues of *Lamellidens corrianus* after acute exposure to Hg^{++} without and with caffeine and during recovery. (Values represent percentage in dry weight)

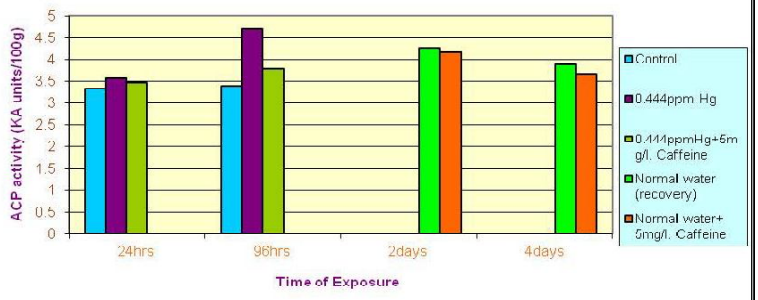
Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	2.433 ± 0.0716	2.521 ± 0.0634		
		Testis	3.324 ± 0.0762	3.389 ± 0.0587		
		Digestive Glands	3.821 ± 0.0689	3.844 ± 0.0532		
0.444 ppm Hg^{++} (B)		Gill	2.936±0.0569❖❖❖ (-20.674)	3.905±0.0487❖❖❖ (-54.898)		
		Testis	3.554 ±0.0535❖ (-6.919)	4.693±0.0452❖❖❖ (-38.477)		
		Digestive Glands	4.905±0.0467❖❖❖ (-28.369)	6.425±0.0471❖❖❖ (-67.143)		
0.444 ppm Hg^{++} + 5mg/lit. Caffeine (C)		Gill	2.561±0.0410❖❖❖ (-5.260)	2.657±0.0326❖❖❖ (-5.394)		
		Testis	3.461 ±0.0378❖ (-4.121)	3.789±0.0434❖❖❖ (-11.802)		
		Digestive Glands	3.065±0.0324❖❖❖ (-19.785)	5.231±0.0412❖❖❖ (-36.082)		
After 96hrs Exposure to 0.444 ppm Hg^{++}	Normal Water (D)	Gill			3.781± 0.0678■ (+3.175)	3.310±0.0856■■■ (+15.236)
		Testis			4.269± 0.0867■■ (+9.034)	3.873±0.0435■■■ (+17.472)
		Digestive Glands			5.709± 0.0653■■■ (+11.143)	5.134±0.0875■■■ (+20.093)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			3.650± 0.0439■■ (+6.530)	2.936±0.0367■■■ (+24.814)
		Testis			4.162±0.0397■■■ (+11.314)	3.657±0.0465■■■ (+22.075)
		Digestive Glands			5.135± 0.0401■■■ (+20.077)	4.058±0.0393■■■ (+36.840)

Values in the ( ) brackets indicate percent change over control, N.S.- Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

Fig.3.2.1 Acid phosphatase activity in gill of *L.corrianus* after acute exposure to Hg++ with and without caffeine and during recovery.



Acid phosphatase activity in testis of *L.corrianus* after acute exposure to Hg++ with and without caffeine and during recovery.



Acid phosphatase activity in digestive glands of *L.corrianus* after acute exposure to Hg++ with and without caffeine and during recovery.

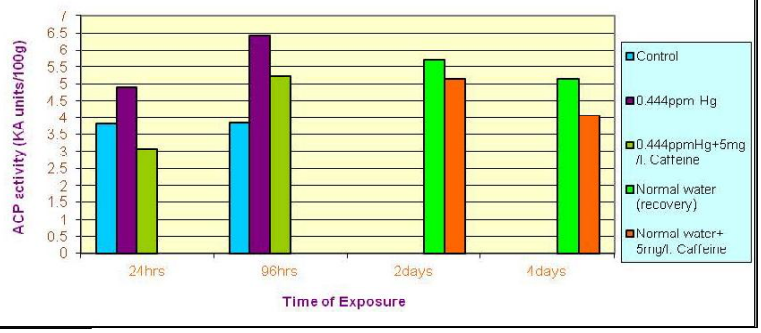
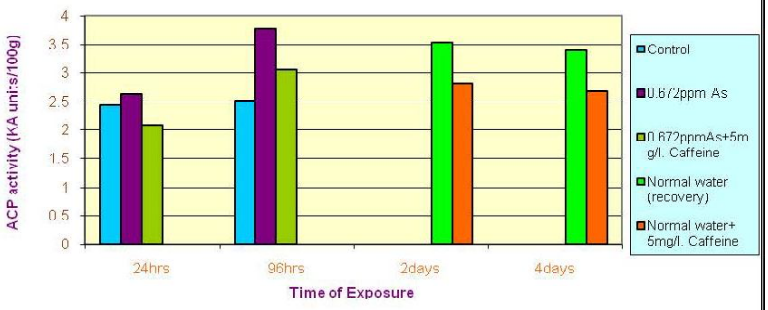


Table No. 3.2.2: Changes in the Acid phosphatase activity in selected tissues of *Lamellidens corrianus* after acute exposure to As^{+++} without and with caffeine and during recovery. (Values represent percentage in dry weight)

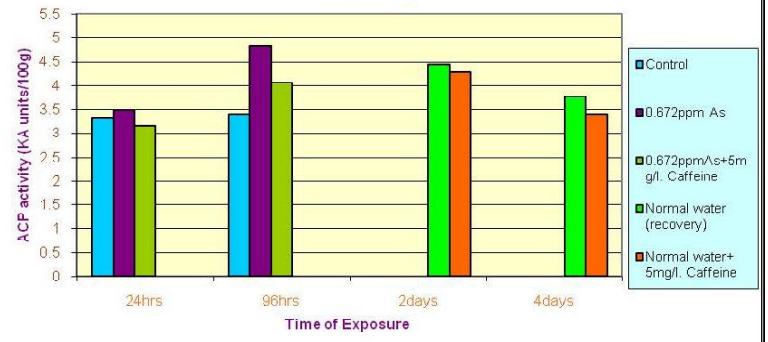
Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Gill	2.433 ± 0.0742	2.521 ± 0.0693		
		Testis	3.324 ± 0.0697	3.389 ± 0.0597		
		Digestive Glands	3.821 ± 0.0571	3.844 ± 0.0762		
0.672 ppm As^{+++} (B)		Gill	2.617± 0.0632❖ (-7.562)	3.764± 0.0451❖❖❖ (-49.305)		
		Testis	3.491± 0.0525❖❖ (-5.024)	4.821± 0.0433❖❖❖ (-42.254)		
		Digestive Glands	4.364±0.0453❖❖❖ (-14.210)	5.684± 0.0455❖❖❖ (-47.866)		
0.672 ppm As^{+++} + 5mg/lit. Caffeine (C)		Gill	2.077± 0.0401❖❖ (-14.682)	3.053± 0.0311❖❖❖ (-21.102)		
		Testis	3.156± 0.0375❖❖ (-17.403)	4.753± 0.0424❖❖❖ (-19.592)		
		Digestive Glands	3.033±0.0309❖❖❖ (-20.622)	5.423± 0.0435❖❖❖ (-41.077)		
After 96hrs Exposure to 0.672 ppm As^{+++}	Normal Water (D)	Gill			3.521± 0.0633■■ (+6.455)	3.389± 0.0866■■ (+9.962)
		Testis			4.429± 0.0871■■ (+8.831)	3.763±0.0451■■■ (+21.945)
		Digestive Glands			5.245± 0.0655■■■ (+7.723)	4.869± 0.0875■■■ (+14.338)
	Normal Water + 5mg/lit. Caffeine (E)	Gill			2.827± 0.0399■■■ (+24.893)	2.673± 0.0355■■■ (+28.985)
		Testis			4.263± 0.0401■■■ (+11.574)	3.389± 0.0481■■■ (+29.703)
		Digestive Glands			5.157± 0.0421■■■ (+9.271)	4.64 ± 0.0397■■■ (+18.121)

Values in the ( ) brackets indicate percent change over control, N.S.- Non Significant, ❖-Compared with respective (A), ■-Compared with respective 96hrs of (B),❖/■-P<0.005, ❖❖/■■-P<0.01, ❖❖❖/■■■-P < 0.001

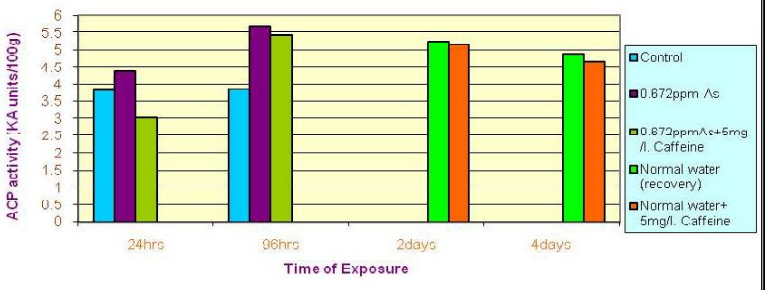
Fig.3.2.2 Acid phosphatase activity in gill of *L.corrianus* after acute exposure to As+++ with and without caffeine and during recovery.



Acid phosphatase activity in testis of *L.corrianus* after acute exposure to As+++ with and without caffeine and during recovery.



Acid phosphatase activity in digestive glands of *L.corrianus* after acute exposure to As+++ with and without caffeine and during recovery.



OBSERVATIONS AND RESULTS

Effects of acute concentration of mercury and arsenic on alkaline phosphatase activity and acid phosphatase activity of gills, testes and digestive glands of *Lamellidens corrianus* without and with caffeine and after 2 and 4 days of recovery with and without caffeine are given in table no 3.1.1 to 3.2.2. The activities of alkaline and acid phosphatase enzyme were expressed as KA unit/ gm/hr at 37°C at pH 10.0 and 4.9 respectively.

Changes in the Alkaline Phosphatase Activity after Exposure of Mercury:-

Gill:-

In the control bivalves, the KA units/100 gm wet tissue of alkaline phosphatase activity in the gills after 24 hrs was 14.375 KA units/100 gm units and after 96 hrs was 14.870 KA units/100 gm of tissue (Table no. 3.1.1). In the bivalves treated with acute concentration of mercury (0.444 ppm Hg^{++}), the alkaline phosphatase activity was 35.5 and 44.375 respectively for 24 hrs and 96 hrs of exposure periods. While in the bivalves exposed to mercury with caffeine (5 mg/lit), the alkaline phosphatase activity was 17.740 and 28.800 respectively for 24 hrs and 96 hrs of exposure periods.

During recovery from mercury intoxication, the alkaline phosphatase activity was 23.860 and 15.310 KA units/100 gm of tissue, in normal water for 2 and 4 days while in normal water and caffeine (5 mg/lit), the values for corresponding periods were

16.070 and 12.420 KA units/100 gm of tissue.

Testis:-

In the control bivalves, the alkaline phosphatase activity in the testis after 24 hrs was 4.730 and after 96 hrs was 4.782 KA units/100 gm of tissue (Table no. 3.1.1). In the bivalves treated with acute concentration of mercury (0.444 ppm Hg^{++}), the alkaline phosphatase activity was 6.626 and 7.212 respectively for 24 hrs and 96 hrs of exposure periods. While in the bivalves exposed to mercury with caffeine (5 mg/lit), the alkaline phosphatase activity was 5.550 and 6.890 KA units/100 gm of tissue respectively for 24 and 96 hrs of exposure periods.

During recovery form mercury intoxication the alkaline phosphatase activity was 6.032 and 5.244 KA units/100 gm of tissue in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for corresponding periods were 5.682 and 4.662 KA units/100 gm of tissue.

Digestive Glands:-

In the control bivalves, the alkaline phosphatase activity in the digestive glands after 24 hrs was 5.585 and after 96 hrs was 5.503 KA units/100 gm of tissue (Table no. 3.1.1). In the bivalves treated with acute concentration of mercury (0.444 ppm Hg^{++}), the alkaline phosphatase activity was 8.851 and 8.376 KA units/ 100 gm of tissue respectively for 24 and 96 hrs of exposure periods. While, in the bivalves exposed to mercury with caffeine (5 mg/lit), the alkaline phosphatase activity was 7.100 and 9.460 KA units/100 gm of tissue respectively for 24 and 96 hrs of exposure.

During recovery form, mercury intoxication

the alkaline phosphatase activity was 18.955 and 14.200 KA units/100 gm of tissue, in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for corresponding periods were 13.842 and 8.437 KA units/100 gm of tissue.

Changes in the Alkaline Phosphatase Activity after Exposure of Arsenic:-

Gill:-

In the control bivalves, the alkaline phosphatase activity in the gills after 24 hrs was 14.375 KA/100 gm units and after 96 hrs was 14.870 KA units/100 gm of tissue (Table no. 3.1.2). In the bivalves treated with acute concentration of arsenic (0.672 ppm As^{+++}), the alkaline phosphatase activity was 29.896 and 36.753 respectively for 24 and 96 hrs of exposure periods. While in the bivalves exposed to arsenic with caffeine (5 mg/lit), the alkaline phosphatase activity was 15.374 and 21.695 respectively for 24 hrs and 96 hrs of exposure periods.

During recovery from arsenic intoxication, the alkaline phosphatase activity was 17.760 and 14.745 in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for corresponding periods were 15.650 and 13.126 KA units/100 gm of tissue.

Testis:-

In the control bivalves, the alkaline phosphatase activity in the testes after 24 hrs was 4.730 and after 96 hrs was 4.782 KA units/100 gm of tissue (Table no. 3.1.2). In the bivalves treated with acute concentration of arsenic (0.672 ppm As^{+++}), the alkaline phosphatase activity was 5.825 and 6.701 respectively for 24 and 96 hrs of exposure

periods. While in the bivalves exposed to arsenic with caffeine (5 mg/lit), the alkaline phosphatase activity was 4.807 and 5.937 respectively for 24 and 96 hrs of exposure periods.

During recovery form, arsenic intoxication the alkaline phosphatase activity was 5.437 and 4.750 in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for corresponding periods were 4.842 and 3.239 KA units/100 gm of tissue.

Digestive Glands:-

In the control bivalves, the alkaline phosphatase activity in the digestive glands after 24 hrs was 5.585 and after 96 hrs was 5.503 KA units/100 gm of tissue (Table no. 3.1.2). In the bivalves treated with acute concentration of arsenic (0.672 ppm As^{+++}), the alkaline phosphatase activity was 7.637 and 19.862 respectively for 24 and 96 hrs of exposure periods. While in the bivalves exposed to arsenic with caffeine (5 mg/lit), the alkaline phosphatase activity was 6.187 and 9.244 respectively for 24 and 96 hrs of exposure periods.

During recovery form arsenic intoxication, the alkaline phosphatase activity was 16.987 and 12.550 in normal water and with caffeine (5 mg/lit), the values for corresponding periods were 11.255 and 5.832 KA units/100 gm of tissue.

Changes in the Acid Phosphatase Activity after Exposure of Mercury:-

Gill:-

In the control bivalves, the acid phosphatase activity in the gills after 24 hrs was 2.433 and after 96 hrs was 2.521 KA units/100 gm of tissue (Table no. 3.2.1). In the bivalves treated with acute

concentration of mercury (0.444 ppm Hg^{++}), the acid phosphatase activity was 2.936 and 3.905 respectively for 24 and 96 hrs of exposure. While in the bivalves exposed to mercury with caffeine (5 mg/lit), the acid phosphatase activity was 2.561 and 2.657 respectively for 24 and 96 hrs of exposure periods.

During recovery from mercury intoxication, the acid phosphatase activity was 3.781 and 3.310 KA units/100 gm of tissue, in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for corresponding periods were 3.650 and 2.936 KA units/100 gm of tissue.

Testis:-

In the control bivalves, the acid phosphatase activity in the testes after 24 hrs was 3.324 and after 96 hrs was 3.389 KA units/100 gm of tissue (Table no. 3.2.1). In the bivalves treated with acute concentration of mercury (0.444 ppm Hg^{++}), the acid phosphatase activity was 3.554 and 4.693 respectively for 24 and 96 hrs of exposure periods. While in the bivalves exposed to mercury with caffeine (5 mg/lit), the acid phosphatase activity was 3.461 and 3.789 KA units/100 gm of tissue respectively for 24 and 96 hrs of exposure periods.

During recovery from mercury, intoxication the acid phosphatase activity was 4.269 and 3.873 KA units/100 gm of tissue, in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for corresponding periods were 4.162 and 3.657 KA units/100 gm of tissue.

Digestive Glands:-

In the control bivalves, the acid phosphatase activity in the digestive glands after 24 hrs was

3.821 and after 96 hrs was 3.844 KA units/100 gm of tissue (Table no. 3.2.1). In the bivalves treated with acute concentration of mercury (0.444 ppm Hg^{++}), the acid phosphatase activity was 4.905 and 6.425 KA units/ 100 gm of tissue respectively for 24 and 96 hrs of exposure periods. While in the bivalves exposed to mercury with caffeine (5 mg/ lit), the acid phosphatase activity was 3.065 and 5.231 KA units/100 gm of tissue respectively for 24 and 96 hrs of exposure periods.

During recovery from mercury, intoxication the acid phosphatase activity was 5.709 and 5.134 KA units/100 gm of tissue, in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for corresponding periods were 5.135 and 4.058 KA units/100 gm of tissue.

Changes in the Acid Phosphatase Activity after Exposure of Arsenic:-

Gill:-

In the control bivalves, the acid phosphatase activity in the gills after 24 hrs was 2.433 and after 96 hrs was 2.521 KA units/100 gm of tissue (Table no. 3.2.2). In the bivalves treated with acute concentration of arsenic (0.672 ppm As^{+++}), the acid phosphatase activity was 2.617 and 3.764 respectively for 24 and 96 hrs of exposure periods. While in the bivalves exposed to arsenic with caffeine (5 mg/lit), the acid phosphatase activity was 2.077 and 3.053 respectively for 24 and 96 hrs of exposure periods.

During recovery from arsenic intoxication, the acid phosphatase activity was 3.521 and 3.389 in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for

corresponding periods were 2.827 and 2.673 KA units/100 gm of tissue.

Testis :-

In the control bivalves, the acid phosphatase activity in the testes after 24 hrs was 3.324 and after 96 hrs was 3.389 K.A. units/100 gm of tissue (Table no. 3.2.2). In the bivalves treated with acute concentration of arsenic (0.672 ppm As^{+++}), the acid phosphatase activity was 3.491 and 4.821 respectively for 24 and 96 hrs of exposure periods. While in the bivalves exposed to arsenic with caffeine (5 mg/lit), the acid phosphatase activity was 3.156 and 4.053 respectively for 24 and 96 hrs of exposure periods.

During recovery form, arsenic intoxication the acid phosphatase activity was 4.429 and 3.763 in normal water for 2 and 4 days while in normal water with caffeine (5 mg/lit), the values for corresponding periods were 4.263 and 3.389 KA units/100 gm of tissue.

Digestive Glands:-

In the control bivalves, the acid phosphatase activity in the digestive glands after 24 hrs was 3.821 and after 96 hrs was 3.844 (Table no. 3.2.2). In the bivalves treated with acute concentration of arsenic (0.672 ppm As^{+++}), the acid phosphatase activity was 4.364 and 5.684 respectively for 24 and 96 hrs of exposure periods. While in the bivalves exposed to arsenic with caffeine (5 mg/lit), the acid phosphatase activity was 3.033 and 5.423 respectively for 24 and 96 hrs of exposure periods.

During recovery form arsenic intoxication, the acid phosphatase activity was 5.245 and 4.869,

in normal water with caffeine (5 mg/lit), the values for corresponding periods were 5.157 and 4.654 KA units/100 gm of tissue.

Thus in present investigation it was observed that after acute exposure to mercuric chloride (0.444 ppm Hg^{++}) and sodium arsenate (0.672 ppm As^{+++}), there was increase in the alkaline phosphatase activity in gills, testes and digestive glands of experimental bivalves, *Lamellidens corrianus* as compared to the control bivalves. The alkaline phosphatase activity was less affected in heavy metal salt with caffeine (5 mg/lit) exposed bivalves than those exposed to only heavy metal salts. The bivalves showed faster recovery of tissues alkaline phosphatase activity in presence of caffeine than in normal water.

It was observed that after acute exposure to mercury (0.444 ppm Hg^{++}) and arsenic (0.672 ppm As^{+++}), there was increase in the acid phosphatase activity in gills, testes and digestive glands of experimental bivalves as compared to those of control bivalves.

The acid phosphatase activity was marginally affected in heavy metal with caffeine-exposed bivalves than those of only heavy metal exposed ones. The bivalves showed faster recovery of tissues acid phosphatase activity in presence of caffeine than the normal water.

DISCUSSION

The biochemical reactions during metabolic activities are taking place in presence of enzymes and the alterations accruing at cellular level due to impact of certain heavy metals, chemicals and pollutants.

The heavy metal salts enter in to the organisms either through the skin, gills or through the food and accumulate in the organisms. Alkaline phosphatase is a non-specific hydrolyses and acid phosphatase is non-specific monoesters. These enzymes are responsible for transphosphorylation and have an important role in the general energetic of an organism. The enzyme activity of alkaline phosphatase and acid phosphatase on exposure to mercury and arsenic with, without caffeine, and during recovery after toxic stress in normal water and normal water with caffeine in gills, testes and digestive glands of *Lamellidens corrianus* is given in tables 3.1.1 to 3.2.2.

Decrease or increase in the enzyme activity represents the stress on any organism that results in metabolic burden (Hanson *et al.*, 1992).

In the present investigation it was observed that after acute exposure to mercuric chloride containing 0.444 ppm mercury (Hg^{+++}) and sodium arsenate containing 0.672 ppm arsenic (As^{+++}), there was increase in the alkaline phosphatase activity in gills, testis and digestive glands of experimental bivalves, *Lamellidens corrianus* as compared to those of control bivalves.

The alkaline phosphatase activity was less in heavy metal salt with caffeine (5 mg/lit) exposed bivalves as compared to those exposed to only heavy metal salts but was higher than those of control bivalves.

The bivalves showed recovery of tissues alkaline phosphatase activity in presence of caffeine and in normal water but rate of recovery was faster in presence of caffeine. It was observed that after

acute exposure to mercury (0.444 ppm Hg^{++}), and arsenic (0.672 ppm As^{+++}), there was increase in the acid phosphatase activity in gill, testes and digestive glands of experimental bivalves, *Lamellidens Corrianus* as compared to those of control bivalves. The acid phosphatase activity was less in heavy metals with caffeine exposed bivalve's as compared to those exposed to only heavy metal salts but was higher than that of control bivalves. The bivalves showed faster rate of recovery of tissues acid phosphatase activity in presence of tissues acid phosphatase activity in presence of caffeine and normal water.

Functional significance of alkaline phosphatase was studied first by Moog (1946). It's localization in plasma membranes perhaps played an important role in the transport of phosphate ions through cellular membranes. Phosphatase is observed histochemically in different regions of digestive tract of number of insects. Pearse (1961) and Srivastava (1966) studied that high activity of alkaline phosphatase indicates increased phosphatase transfer from one alcohol to the other.

Mercury in any chemical form denatures proteins, inactivates enzymes and causes severe disruption of any tissue with which it comes into contact in sufficient concentration (WHO, 1991).

Gill *et al.*, (1990) also reported that intoxication of $HgCl_2$ (181 mg/lit. for 48 hrs.) to Rosy barb, *Puntius conchonius*, resulted in an increase in alkaline phosphatase activity in the testes. It was postulated that oral administration of $HgCl_2$ (2mg/kg/day) resulted in a highly significant increase in alkaline phosphatase activity

in the testis of rat (Ramalingam & Vimalladevi, 2002).

Sexena and Kumar (2004) studied that the $HgCl_2$ (5mg/kg body weight) inhibited the spermatogenetic process; the unutilized alkaline phosphatase concentration was contributing to its increase in the testes of the $HgCl_2$ treated *albino* mice. Anjum and Shakoori (1994) studied that after administration of inorganic mercury to rabbits, the alkaline phosphatase activity was increased by 77% in liver. Ramalingam and Ramarani (2004) showed that in prawn *M. rosenbergii* inoculated with *Pseudomonas aeruginosa,* both acid phosphatase and alkaline phosphatase significantly increased in the haemolymph and body muscle and decreased in the hepatopancreas after 96 hrs of inoculums treatment. Similar increase in alkaline phosphatase activity in the haemolymph and body muscle of fresh water prawn (*M. lamerrei*) due to mercury intoxication was reported by Omkar and Shukla (1984). The above increase may be ascribed to the increased requirement of energy metabolites like carbohydrates and proteins. This could be possible the toxic stress in animals causes increased substrate pressure (Bostrom and Johansson, 1972; Ramalingam, 1989). Acid phosphatase, pre-eminently regarded as the marker enzyme, has been found in Golgi cisternae and lysosomes. Dutta *et al.*, (1983) concluded that both induction and inhibition of phosphatase taken place depending on the concentration of metals. Norseth, (1967) reported decrease in acid phosphatase activity due to accumulation of mercury in the lysosome and blockage in the release of enzyme. Generally, the

increased activity of acid phosphatase was attributed to the activation of the enzyme, which was kept in a latent state inside the membrane of lysososmes, due to disruption of the membrane (De Duve *et al.*, 1955). Reddy *et al.*, (1984) concluded that sensitization of cell tissues may induce proliferation of smooth endoplasmic reticulum in hepatopancreas and resulted in increased production and liberation of acid phosphatase. Batia *et al.*, (1972) were of the opinion that degradation and necrosis induced by toxicants in hepatopancreas cause release of acid phosphatase.

Ahmed *et al.*, (1997) studied the effect of copper on oxygen consumption and phosphatase in *S. serrata* and concluded that there was a decrease in acid and alkaline phosphatase activity in muscle, hepatopancreas and haemolymph. Similar observations were noted by Elumalai *et al.*, (1998) in the same crab in response to naphthalene. Bhatnagar *et al.*, (1995) studied the effect of parathyroid hormone on the fish *Clarias batrachus* and found the decreased acid and alkaline phosphatase activity in response to the toxicant.

Zinc deficiency has been shown to be correlated with a diminished activity of some enzymes. The level of a serum enzyme alkaline phosphatase decreased in zinc deficient animals and increased with zinc replenishment (Sadasivan, 1952; Van Reen, 1953). It has been postulated that the promoter region of the gene for intestinal alkaline phosphatase contains a metal responsive element, and that zinc deficiency leads to sub optional transcription of this type of enzyme (Stuart

et al., 1985, Millan, 1987).

The activity of serum alkaline phosphatase is most widely used to assess zinc status (Adeniyi and Heaton, 1980), although its response has been inconsistent in humans (Nanji and Anderson, 1983; Baer *et al.*, 1985; Weismann and Hoyer, 1985; Milne *et al.*, 1987). Alkaline phosphatase catalyses the removal of 5/phosphate from DNA, RNA, oilgonucleotides and deoxyribonucleotide triphosphates. Osteoblasts secrete alkaline phosphatase during active deposition of bone matrix. The alkaline phosphatase is believed either to increase the local concentration of inorganic phosphate or to activate the collagen fibers in such way that they cause the deposition of calcium salts. Because some alkaline phosphatase diffuses into blood, the blood level of alkaline phosphate is usually a good indicator of the rate of bone formation (Guyton, 1996).

Chatterjee and Shinde, (2002) state that, the causes of increased serum alkaline phosphatase activity are due to,

a) Necrosis of cells due to damage.
b) Increased production of enzyme in the cell.
c) Increased in the number of cells and cells mass as in cancer.
d) Patients with obstructive jaundice, and decrease alkaline phosphatase activity due to,

a) Enzyme inhibition
b) Lack of cofactor
c) Poor rate of synthesis

Alkaline phosphatase activity also increases in hepatobiliary diseases, extra hepatic cholestasius, sepsis, chronic inflammatory bowel

disease and thyrotoxicosis in serum alkaline phosphatase activity decrease.

Acid phosphatase is a lysosomal enzyme, serves as a biochemical marker for specific androgen dependent steps in spermatogenesis and is very important for tissue reorganization and tissue repair. Saxena and Kumar (2004) observed increased acid phosphatase activity in testes of $HgCl_2$ treated mice, which could be due to an increase in the leakage of the enzyme from lysosomes. Passia *et al.*, (1985) have ascribed increased acid phosphatase activity in sertoli cells with severe cell damage and in germ cells with heavy exfoliation. The exposure of $HgCl_2$ C181 mg/l for 48 hrs to Rosy barb, *Puntius onchonius* stimulated the acid phosphatase activity in its testis (Gill *et al.*, 1990).

Similar increase of acid phosphatase has been observed in organisms such as *Bubalis bubalis* exposed to malathion (Gupta and Paul, 1974). The increase of acid phosphatase activity in the haemolymph and body muscle might be attributed to increased substrate pressure consequent to endotoxin stress. Ramalingam (1990) attributed the increased acid phosphatase activity to alteration of pH in the tissue microenvironment consequent to toxicity stress mediated acid products of metabolism.

Generally, the increased activity of acid phosphatase has been attributed to the activation of the enzyme, which was stored in a latent state inside the membrane of lysosomes, due to disruption of the membrane (De Duve *et al.*, 1963). Shyamala (2001), in her study inferred that the

inflammatory changes by the inoculums of *Vibrio parahemdytcas* MTCC 451 on muscle might have caused disruption of lysosomes and enhanced the activity of acid phosphatase.

Nagpure and Zambare (2005), observed increase in alkaline and acid phosphatase activities in gill, mantle and digestive glands of *Lamellidens corrianus* and *P. cylindrica* after acute and chronic exposure to tetracycline, chloramphenical and trimethoprim. Bhatia *et al.,* (1972) and Reddy *et al.,* (1996) reported the increase in the acid phosphatase activity in the copper and zinc treated crabs since hepatopancreas was an important site of intermediary metabolism in crustaceans. Kulkarni and Nagabhushanam (1979) found higher acid phosphatase activity in the hepatopancreas. Since copper is an inhibitor of phosphatase, the bound copper suppresses the phosphatase activity in the haemolymph of *P. hydrodromous*, after the acute exposure of heavy metals. Severe damage was observed in the tissues, so the activities are increased due to the heavy metals increased enzyme activity disturbed the metabolic systems of animals leading to their death.

Chelation therapy is useful for the detoxification of heavy metals. The present work proved that, the caffeine is the useful chelator to detoxify the mercury and arsenic. Caffeine exposure increased pulse duration and showed the inactivation of the Ca^{2+} current. Oral administration of tea has been found to moderately enhance the activities of lipid peroxidase, catalase, glutathione-s-transferase which is turn protect against cancer blocking the reaction & electrophonic carcinogens

with cellular macromolecules.

El-Damerdash (2001) reported that on administration of sodium selenite in combination with mercury, partially or totally alleviated the toxic effects of mercury on ACP and ALP in rat. He concluded that selenium could be able to antagonize the toxic effects of mercury.

In present study, the main cause of increased acid and alkaline phosphatase activity can be the increase in the necrotic regions due to the stress of the mercury and arsenic. Our study indicates much distortion and damage of the tissues on exposure to the mercury and the arsenic. The caffeine binding with mercury and arsenic might have reduced the toxic effect of the heavy metals. Secondly, the free reactive oxygen generated due to the mercury and arsenic could have reacted with the caffeine as it is an antioxidant and hence has reduced the severity of the tissue damage. The improvement in the enzyme level in the presence of the caffeine indicates reduction in the tissue necrosis.

SUMMARY

- *The present investigation showed the role of caffeine in heavy metal induced alterations in the enzyme activity in an experiment model, the freshwater bivalve, Lamellidens corrianus.*
- *The enzyme activity of alkaline phosphatase and acid phosphatase in various tissues like gill, testis and digestive glands of freshwater bivalves; Lamellidens corrianus were estimated after acute exposures to mercury (0.444ppm Hg^{++}) and arsenic (0.672ppm As^{+++}) with and without caffeine.*
- *The alkaline phosphatase and acid phosphatase activity in various tissues like gill, testis and digestive glands were found to be significantly increased after acute treatment by heavy metal salts.*
- *The alkaline phosphatase and acid phosphatase activity in various tissues such as gill, testis and digestive glands were least affected when exposed to heavy metal salts with caffeine.*
- *After 96 hrs exposures to heavy metal salts, during the recovery, the bivalves showed drastic decrease in the alkaline phosphatase and acid phosphatase activity in presence of caffeine than those allowed to cure naturally.*
- *The results indicate the detoxifying effect of caffeine on heavy metal induced alterations.*

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CHAPTER : 4
BIOACCUMULATION

INTRODUCTION

Freshwater streams are seriously polluted by industrial wastes or effluents which come along with waste water of different industries such as Petrochemicals, Fertilizers, tanneries, distilleries, oil refineries, mine industries etc. Zinc, lead, mercury, copper, cadmium, chromium, etc., metals are discharged by industries into air, soil and water. They get way into human body through the food chain from the environment. When these pollutants enter into the living organisms, they produce serious effects. Man, being at higher tropic level receives large amount of these metal pollutants through food, water and even air.

The effect of heavy metals on living organisms has become a global problem. The heavy metals

have been recognized as serious pollutants of aquatic ecosystem with deleterious effects on associated organisms, which ultimately affect the ecological balance.

The bivalves are indicators of various changing environments as they show the change of the factor directly or indirectly. Because they live in close contact with water and bottom sediments, and thus are subject to pollutant exposure from both sources.

The freshwater bivalves are abundant through out the world and are commonly called as clams. They serve as excellent biological model for the study of toxicity and detoxification by different drugs as their maintenance is easy and low cost, toxicants are directly added in water, which are readily absorbed by their soft body parts, and responses are quick.

The term "Bioaccumulation" refers to the net accumulation of a chemical by aquatic organisms because of uptake from all environmental sources (e.g. water, air, food, sediment). The magnitude of accumulation can vary widely depending on the chemical and its properties. For chemical that are persistent and hydrophobic, chemical concentrations in contaminated fish and shellfish may be several orders of magnitude higher than their concentrations in water. These chemicals may also be biomagnified in aquatic food webs, a process where by chemical concentrations increase in aquatic organisms of each successive trophic level due to increasing dietary exposures (e.g. increasing concentrations from algae, to zooplankton, to forage fish to predator fish). For chemicals that are

biomagnified, consumption of contaminated fish and shellfish may pose unacceptable human health risks.

Bioaccumulation can be viewed as the result of competing rates of chemical uptake and elimination (chemical loss) by aquatic organisms. When the rates of chemical uptake and elimination achieve balance, the distribution of the chemical between the organism and its source is said to be at steady state. Under the steady-state conditions, a Bioaccumulation factor is the ratio (in L/kg) of the concentration of a chemical in the tissue of an aquatic organism to its concentration in water, in situations where both the organism and its food are exposed (USEPA - 2000).

Heavy metals are very harmful pollutants as they remain in the nature for a very long period. Uptake of heavy metals through food chain in human being may cause various physiological disorders like hypertension, sporadic fever, renal damage, liver cirrhosis etc. The bioaccumulation of heavy metal toxicants in aquatic animals depends on availability and persistence of the contaminants in water and the physico-chemical properties of toxicants.

Aluminum, arsenic, cadmium, chromium, lead and mercury can cause chronic or acute poisoning and hence should be absent in the drinking water. The role of heavy metals (the non-degradable and cumulative chemicals) as pollutants is widely recognized (Ballester, *et al.*, 1980) and some heavy metals like Pb and Cd are known to interfere with the functioning of biological systems (Villareal Trevino *et al.*, 1986). Metal pollution

hazards to living organisms are well known (Guthrie and Perry, 1980). Nayak (1999) reported that heavy metals are known toxicants, which inflict acute disorders in aquatic organisms. Mercury is very much toxic heavy metal among other heavy metals. Reviews of the pharmacology and chemistry of mercury (Hg) compounds have been presented elsewhere, (W.H.O., 1990; Suzuki *et al.* 1991). The main sources of Hg, in the diet such as fish and marine mammals, are also rich sources of selenium (Cuvin, 1991).

Methyl mercury (MeHg) has been one of the most dramatic and best-documented examples of the bioaccumulation of toxins in the environment, particularly in the aquatic food chain (Boudou and Ribeyre, 1997). Methyl mercury (MeHg) intoxication has been a public health problem from many decades (W.H.O., 1990).

Inorganic mercury was reported to be teratogenic in fish (Weish, 1977). The mode of action of mercurial is non-selective or non-specific inhibition of enzymes containing especially iron and sulfhydryl sites. The toxicity of mercury was studied by many workers (Akiyama, 1970; Webeser, 1975; and Dhanekar *et al.*, 1985). The freshwater mussels have relatively higher ability to accumulate mercury from the medium, than other existing fauna (Balogh *et al.*, 1988). Krishnakumar *et al.*, (1990) studied the accumulation, distribution and depuration of mercury in the green mussel, *Perna viridis*.

Arsenic is a chemical that bioaccumulates in tissues of aquatic organisms but does not biomagnify in the aquatic food chain (Woolson, 1975; Wagemann *et al.*, 1978; Spehar *et al.*, 1980,;

Maeda *et al.*, 1990; Chen and Folt 2000, Mason *et al.*, 2000). Arsenic Bioaccumulation factors for upper trophic level freshwater and estuarine fish and shellfish typically consumed by humans, generally range between 5L/kg and 5000 L/kg (Langston, 1984;Baker and King, 1994; Chen *et al.,* 2000; Chen and Folt, 2000; Mason *et al.,* 2000; Cooper and Gillespie, 2001; Giusti and Zhang, 2002). Despite the recent attention focused on arsenic uptake and accumulation in aquatic biota, much uncertainty in the mechanisms and bioaccumulation potential of the various forms of arsenic in the environment still exists. The consensus in the literature is that upwards of 85% to 90% of arsenic found in edible portions of marine fish and shellfish is organic arsenic (arsenobetaine (ASB), Arsenocholine (ASC), dimethylarsinic acid (DMA) and that appropriately 10% is inorganic arsenic (Goessler *et al.*, 1997; Ochsenkuhn - Petropulu *et al.*, 1997; De Gieter *et al.*, 2002; Johnson and Roose, 2002). Less is known about the forms of arsenic in freshwater fish, but there is evidence that organic arsenic may be prevalent (Kaise *et al.*, 1997; Field based study) or considerably less (Maeda *et al.*, 1990, 1992, 1993; Suhendrayatna *et al.*, 2001, 2002 b).

More recent research indicates that when compared to arsenite, trivalent methylated arsenic metabolites excrete a number of unique biological effects, are more cytotoxic and genotoxic, and are more potent inhibitors of the activities of some enzymes (Thomas *et al.*, 2001; Kitchin and Ahmad, 2003). Because each arsenic species (e.g. As (III), As (V), ASB, MMA^{V}, MMA^{III}) exhibits different

toxicities, it may be important to take into account the fraction of total arsenic present in the inorganic arsenic (Spehar *et al.*, 1980; USEPA, 1987) and organic arsenic forms when estimating the potential risk posed to human health through the consumption of arsenic contaminated fish and shell fish.

From the large volume of water (Stanzikowska *et al.*, 1976; Pusch *et al.*, 2001), mussels uptake and accumulate in their bodies useful, non essential (e.g. Be, Sr, Ba) and toxic metals without noxious effects (Korringa 1960; Lobel *et al.,* 1990; Metcalfe-Smith *et al.*, 1992; Adams and Shorey 1998; Byrne and Vesk 2000). The accumulation of several metals is due to the low capacity of these molluscs for discriminating among metals, which are similar in some characteristics such as ionic radius (Jeffree *et al.*, 1993; Metcalfe - Smith, 1994). Mussels also possess a variety of effective detoxification mechanisms to reduce the toxicity of the metal uptake (Mason and Jenkins, 1995; Vesk and Bytrne, 1999; Byrne, 2000; Byrne and Vesk, 2000). In addition, adult mussels are sedentary and some species (e.g. *Dreissena polymorpha, Mytilus edulis*) are geographically widespread and very abundant at all time of the year. The characteristics described, makes mussels useful indicators of the abundance and spatial distribution of metals in aquatic ecosystems (Ravera and Vuido, 1961; Gaglione and Ravera, 1964; Ferrington *et al.*, 1983; Zarnezki, 1987; Doherty *et al.*, 1993; Oertel, 1998; and Sures *et al.*, 1999).

Bivalves are effective tools for monitoring

long-term trends, especially for bioaccumulative trace organics. Bivalves are of limited use in monitoring trends for those trace elements that do not accumulate in tissues, as integrators of water contamination for mercury and arsenic, or for estimating mercury transfer to higher levels of the food web. Bivalves are but one of many tools to determine the transfer and potential magnification of contaminants to higher tropic levels. The current use of non-resident species appears sub optimal in this regard.

The mussel is regarded as suitable species because it accumulates metals, is sessile, has relatively long life span, is large enough for individual analysis, and can tolerate relatively wide range of temperature and salinity (Phillips, 1976). The ability of lamellibranches molluscs to concentrate heavy metals to levels, excess of those in the hydrophere is well documented (Waldichuk, 1974). Heavy metal uptake by endocytosis in mollusc has also been documented. Many times the insoluble particulate colloidal forms of the heavy metals exist in the water, which are endocytosed by the living organisms. Endocytosis is the engulfment of metal by epithelial cell membrane limited vesicles by the cell. The blue mussel, *Mytilus edulis* has been used widely as Surveillance organisms (Claisse, 1989). Naqvi *et al.*, (1990) reported that cadmium, lead and arsenic exposed Cray fish accumulated these metals rapidly. Goodrich *et al.*, (1991) stated that exposure to heavy metals through air, water and the food chain has induced a wide variety of toxic effects in human and animals. Ruiter (1995) reported that heavy

metals have high toxicity and worldwide distribution in the aquatic environment. They are known to accumulate in sediments. In molluscs and fishes, they are bioconcentrated even when released in minute quantities into the environment.

Metal pollution hazards to living organisms are well known (Guthrie and Perry, 1980).The toxicity and bioaccumulation of heavy metals to some marine biota from the Egyptian costal waters. Wang *et al.*,(1996) studied the kinetic determinations of trace elements, bioaccumulated in the mussel, *Mytilus edulis*. The accumulation of metal in different species is the function of their respective membrane permeability and enzyme system (Mitra *et al.*, 2000). Pani *et al.,* (2002) and Canivet *et al.*, (2001) studied toxicity and bioaccumulation of arsenic and chromium in epigen and hypogen in freshwater macro-invertebrates tolerates, and higher values of Zn, Ni, Hg, Cr and Pb were observed in comparison to water in fish. Accumulation of toxins such as mercury, cadmium, arsenic, nickel, aluminum and pesticides can damage the body in an insidious and cumulative way. The concept of internal cleansing and detoxification has been around for centuries. Detoxification of the body refers to the cleansing by the bowels, kidney, lung, the liver and blood since these are the organs involved in the detoxification of chemicals and toxins from the body.

Cleansing of the body system is done internally through fasting and externally through skin cleaning. Enemias can also be used but are normally reserved for cases where intense cleansing

is required, as is often the case in using detoxification as a way to treat cancer (Such as coffee enemia used in the Gerson therapy). The removal of toxic metal through chelating allows the body functions at an optimal level. During the chelating process, a synthetic amino acid, called EDTA is administered to the patient by a slow intravenous drip over a painless three-hour process. Once in the blood stream, EDTA works through a complex cascade of chemical reactions thus resulting in the binding of unwanted metals such as cadmium, calcium, lead and mercury. These metals are than excreted through the urine because of chelating therapy and removal of toxic metals and calcium from body (Public Health service, 1981). However EDTA has many side effects on the health. So, caffeine is found to have antioxidant activity. This activity of caffeine can protect the damage of tissues, chemicals and genetic materials of organisms from heavy metal generated free oxygen radicals. Mike McLaughlin (2000) has found that coffee has capacity to bind the heavy metals. Dissolved heavy metal ions are positively charged, caffeine contains uncharged or negatively charged molecules, the metal ions might be taken out of solution by binding to negatively charged molecules in the coffee which indicates that caffeine can have the capacity to remove the heavy metals from living organisms. According to Kolayly (2004), oxygen from second and sixth position of caffeine forms chelate with heavy metal.

In current study, it is proposed to study the efficacy of caffeine in the detoxification of mercury and arsenic with respect to their tendency of

bioaccumulation in an experimental animal model, the freshwater bivalve, *Lamellidens corrianus.*

MATERIALS AND METHODS

Selected experimental model animal, the freshwater bivalve, *Lamellidens corrianus* were collected from the Paithan dam at Paithan Tq. Paithan, Dist. Aurangabad (M.S.). After collection, bivalves were acclimatized in the laboratory condition at room temperature for 2-3 days. The healthy and active acclimatized bivalves of approximately same size were selected for experiment.

Before starting the experiment, these bivalves were divided into three groups such as A, B and C.

(1)' A' group bivalves were kept as control,

(2) 'B' group bivalves were exposed to acute dose ($LC_{50/2}$) of heavy metal salts, mercuric chloride (0.6 ppm) equivalent to 0.444 ppm Hg^{++} and sodium arsenate (0.9 ppm) equivalent to 0.672 ppm As^{+++},

(3) 'C' group bivalves were exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate with caffeine (5 mg/L.).

After 4 days, bivalves from group 'B' were divided into two groups D and E

(4) 'D' group bivalves which were pre-exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate were allowed to cure naturally in the normal water while

(5) 'E' group bivalves which were pre-exposed to acute dose ($LC_{50/2}$) of mercuric chloride and sodium arsenate were allowed to cure in the normal water with caffeine (5 mg/l.).

These experimental bivalves of A, B and C group were dissected after 24 hrs and 96 hrs and from D and E groups of recovery after 2 days and 4

days. Tissues such as testis and digestive glands, from all five groups bivalves were dried at 80 ^{0}C in an oven till constant weight was obtained. These powders obtained were stored in airtight specimen bottles by waxing the cork outside. The dried powders of these different tissues of control and experimental animals were used to estimate bioaccumulated respective heavy metals.

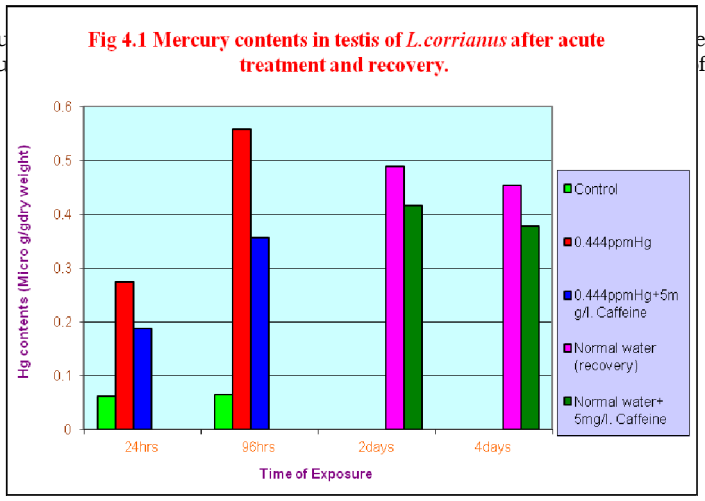
The 500 mg tissue powder was digested in 10ml of acid mixture (HNO_3: Perchloric acid) in (4:1) ratio on hot plate until dryness. The digested mixture was cooled and diluted to 50 ml by double distilled water in volumetric flask and was filtered by (Whatman grade 541) filter paper. From each sample, respective heavy metal was estimated by Atomic Absorption Spectrophotometer (Chemito.)

The concentration of mercury and arsenic accumulated in the tissue of each exposure period in each tissue was recorded and the results are given in the tables 4.1, 4.2, figure 4.a, and 4.b.

Table No. 4.1: Mercury content (mg/g dry weight) in selected tissues of *Lamellidens corrianus* after acute exposure to Hg^{++} without and with caffeine and during recovery.

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Testis	0.062	0.065		
		Digestive Glands	0.023	0.027		
0.444 ppm Hg^{++} (B)		Testis	0.274	0.358		
		Digestive Glands	0.124	0.489		
0.444 ppm Hg^{++} + 5mg/lit. Caffeine (C)		Testis	0.188 (-31.38)	0.356 (-36.20)		
		Digestive Glands	0.113 (-8.870)	0.404 (-17.38)		
After 96hrs Exposure to 0.444 ppm Hg^{++}	Normal Water (D)	Testis			0.489 [-12.365]	0.454 [-18.637]
		Digestive Glands			0.409 [-16.359]	0.325 [-33.537]
	Normal Water + 5mg/lit. Caffeine (E)	Testis			0.416 [-25.448]	0.378 [-32.258]
		Digestive Glands			0.319 [-34.764]	0.218 [-55.419]

Valu ed B
Valu f 96
hrs



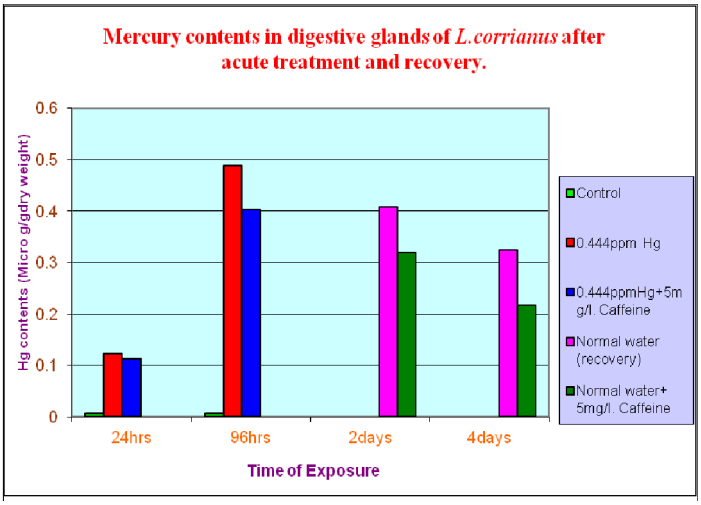


Table No. 4. 2: Arsenic content (mg/g dry weight) in selected tissues of *Lamellidens corrianus* after acute exposure to As^{+++} without and with caffeine and during recovery.

Treatment		Tissue	24hrs	96hrs	Recovery	
					2days	4days
Control (A)		Testis	0.016	0.016		
		Digestive Glands	0.011	0.011		
0.672 ppm As^{+++} (B)		Testis	1.026	1.044		
		Digestive Glands	1.089	1.119		
0.672 ppm As^{+++} + 5mg/lit. Caffeine (C)		Testis	1.023 (-0.2923)	1.036 (-0.7662)		
		Digestive Glands	0.990 (-9.090)	1.092 (-2.412)		
After 96hrs Exposure to 0.672 ppm As^{+++}	Normal Water (D)	Testis			1.010 [-3.256]	0.969 [-7.183]
		Digestive Glands			1.105 [-1.251]	1.067 [-4.647]
	Normal Water + 5mg/lit. Caffeine (E)	Testis			0.942 [-9.770]	0.917 [-12.167]
		Digestive Glands			1.073 [-4.110]	0.975 [-12.868]

Values in the ( )brackets indicate percent change over respective treated B
Values in the [ ] brackets indicate percent change over respective B of 96 hrs

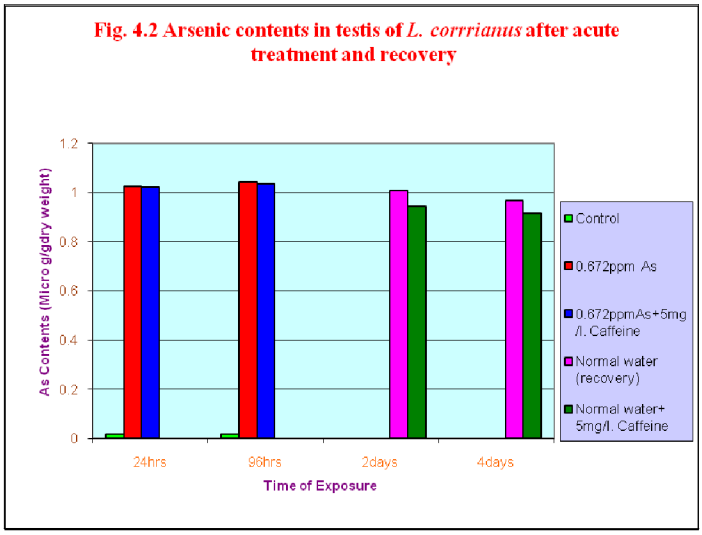
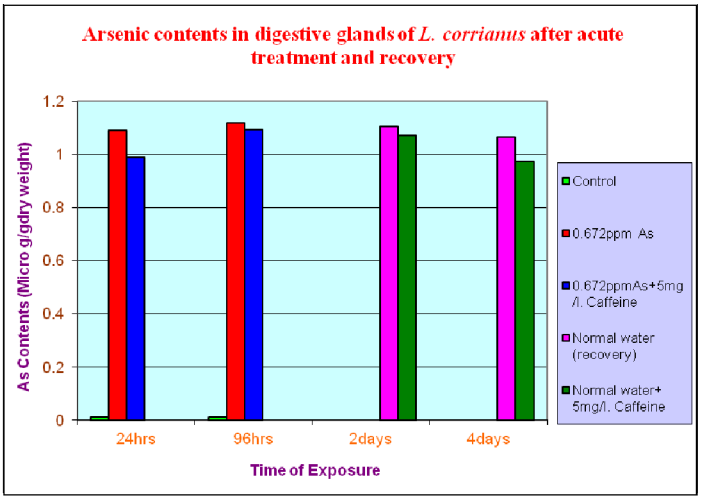


Fig. 4.2 Arsenic contents in testis of L. corrrianus after acute treatment and recovery
As Contents (Micro g/gdry weight)
1.2
1
0.8
0.6
0.4
0.2
0
24hrs
96hrs
2days
4days
Time of Exposure
Control
0.672ppm As
0.672ppmAs+5mg /l. Caffeine
Normal water (recovery)
Normal water+ 5mg/l. Caffeine


Arsenic contents in digestive glands of L. corrianus after acute treatment and recovery
As Contents (Micro g/gdry weight)
1.2
1
0.8
0.6
0.4
0.2
0
24hrs
96hrs
2days
4days
Time of Exposure
Control
0.672ppm As
0.672ppmAs+5mg /l. Caffeine
Normal water (recovery)
Normal water+ 5mg/l. Caffeine

OBSERVATIONS AND RESULTS

Bioaccumulation of mercury and arsenic in testis and digestive glands of *L. corrianus* exposed to mercury (0.444 ppm) and arsenic (0.672 ppm) with and without caffeine and during recovery has been summarized in tables 4.1 and 4.2 and figure 4.a and 4.b.

After 24 hrs and 96 hrs of acute exposure to heavy metals, it was observed that there was an increased in mercury and arsenic concentration in the tissues of *L. corrianus* with respect to time as compared to those of control bivalves.

Bioaccumulation of Mercury:-

Mercury contents are expressed in mg/g dry weight of tissue. Minute quantity of mercury was observed in control group animals as compared to the treated groups.

Testis:-

The control group of animal tissue showed 0.65 mg/g mercury in testis while the amount of accumulation of Hg in presence of $HgCl_2$ (0.444 ppm Hg^{++}) for 24 hrs was 0.274 mg/g. The amount of Hg in the testis after 96 hrs was 0.558 mg/g.

The amount of bioaccumulated mercury in $HgCl_2$ with caffeine exposed bivalves was less as compared to those exposed to only $HgCl_2$ in respective period of exposure and for 24 hrs it was

0.188 mg/g and for 96 hrs was 0.356 mg/g. The bivalves pre-exposed to $HgCl_2$ showed faster recovery with caffeine than those allowed to cure naturally. The bioaccumulated Hg as observed after 2 days was 0.416 mg/g and after 4 days was 0.378 mg/g in naturally curing bivalves while after 2 days, it was 0.489 g/g and after 4 days was 0.454 mg/g in caffeine treated recovering bivalves.

Digestive Glands:-

The control group of animals showed 0.008 mg/g mercury in digestive gland while the amount of bioaccumulated Hg in presence of $HgCl_2$ (0.444 ppm Hg^{++}) for 24 hrs was 0.124 mg/g and after 96 hrs was 0.489 mg/g. The amount of Hg^{++} accumulated was lower in $HgCl_2$ with caffeine treated bivalves at respective period of exposure, as for 24 hrs was 0.113 mg/g and for 96 hrs was 0.404 mg/g. The bivalves pre-exposed to $HgCl_2$ showed fast removal of Hg with caffeine than those allowed to cure naturally. The amount of accumulated Hg observed after 2 days was 0.319 mg/g and after 4 days was 0.218 mg/g in caffeine exposed bivalves and in those allowed to cure naturally, the amount of accumulated Hg^{++} for 2 days was 0.409 mg/g and 4 days was 0.325 mg/g.

The bioaccumulation data recorded in table No. 4.2 and Fig. 4.b indicates the amount of bioaccumulated arsenic in presence of sodium arsenate (0.672 ppm As^{+++}) that was increased with increase in exposure period.

Bioaccumulation of Arsenic:-

The control group of animals showed minute quantity of arsenic in their tissues as compared to those of experimental group animals.

Testis:-

Bivalves from control group showed 0.016 mg/g arsenic in testis while the amount of accumulated As in presence of Sodium arsenate (0.672 ppm As^{+++}) for 24 hrs was 1.026 mg/g and after 96 hrs was 1.044 mg/g. The amount of accumulated arsenic was lower in As with caffeine, exposed bivalves as compared to those exposed to only As in respective period of exposure and after 24 hrs exposure was 1.023 mg/g and after 96 hrs exposure was 1.036 mg/g. The bivalves pre-exposed to As showed fast arsenic depletion with caffeine than those allowed to cure naturally. The amount of accumulated As as observed after 2 days was 0.942 mg/g and after 4 days was 0.917 mg/g while in those bivalves allowed to cure naturally, the amount of accumulated arsenic after 2 days was 1.010 mg/g and after 4 days was 0.969 mg/g.

Digestive Glands:-

The control group of animals showed 0.011 mg/g arsenic in digestive gland. The amount of bioaccumulated As in presence of sodium arsenate (0.672 ppm As^{+++}) for 24 hrs was 1.089 mg/g and after 96 hrs was 1.119 mg/g. The amount of As^{+++} accumulated was lower in Sodium arsenate with caffeine exposed bivalves as compared to those exposed to only sodium arsenate at respective period of exposure that was for 24 hrs, 0.990 mg/g and for 96 hrs 1.092 mg/g. The bivalves pre-exposed to sodium arsenate showed fast removal of As with Caffeine than those allowed to cure naturally. The amount of accumulated As observed after 2 days was 1.073 mg/g and after 4 days was 0.975 mg/g in caffeine exposed bivalves and in

those allowed to cure naturally, the amount of accumulated Hg^{++} for 2 day was 1.105 mg/g and 4 days was 1.067 mg/g.

DISCUSSION

Heavy metals are known pollutants, which inflict disorders in aquatic ecosystem and there accumulated concentrations are significantly higher in the aquatic biosphere. Accumulation of heavy metals in tissues occurs independently of the uptake of new metal. The high level of heavy metals causes deleterious effects on organisms. Heavy metal uptake and concentration in the food chain, especially those terminating in the human beings have renewed interest largely due to several instances of human intoxication (Moore and Ramamoorthy, 1984; Muralidharan and Raja, 1997).

The ratio between bioaccumulation and exposure concentration with periods of exposure has been shown by various investigators (Pragatheeswaran, 1987; Sayer *et al.*, 1989; Barber and Sharma, 1998; Vijayaraman *et al.*, 1998; 1999; Senthilnathan *et al.*, 1998 and Senthilnathan and Balasubramaniam, 1998).

In invertebrates or other animals, bioaccumulation of heavy metals is recorded, but bivalves are one of the most important groups of animal for metal bioaccumulation as was also evident from some of the recent studies. Bivalves are very good stress indicators for many contaminants, particularly lipophilic compounds such as chlorinated hydrocarbons and PAHs, because their contaminant body burdens equilibrate with corresponding contaminants in the

surrounding environment relatively quickly (Russell and Gobas, 1989; Stephenson, 1992). However, not only all contaminants are bioaccumulated in the same way by bivalves, and bivalve species differ in their bioaccumulation characteristics.

ANOVA results also indicated relatively little temporal variation in the bioaccumulation of copper, mercury, PAHs, and PCBs. Catsiki (l986) stated that the bioaccumulation of copper and zinc in tissues of molluscs are dependent on the species and the reaction of these organisms during the period of study to environmental conditions. Harrison (1969) reported that the strong attraction between ions and organic ligands influence the deposition of metal in the body and their rate of excretion. Pb binds strongly to tissues and is slowly excreted. Consequently, with continuing intake it tends to accumulate to a high degree in the body of mollusc.

Cuvin (1994) observed that, concentration of cadmium and mercury in *Oreochromis nilotisus* increased with exposure period. Mason *et al.,* (2000) observed that, concentration of heavy metals is more in detoxifying organs.

Accumulation of heavy metals in the hepatopancreas, in various crustaceans, was reported by many workers as in *P. duorarum* in response to cadmium (Nimmo *et al.*, 1971); *Rangia cuneata* exposed to mercury (Dillonand Neff, 1978); *Potamonautes warreni* exposed to zinc and lead (Du Preeze *et al.*, 1993); *M. malcolmsonii* in response to cadmium and zinc (Vijayanaman *et al.*, 1999;); *M. malcolmsonii* exposed to nickel (Kabila *et al.*, 1999)

and *U. annulipes* in response to cadmium and mercury (Suresh, 2001). The positive correlation between the concentrations of zinc and arsenic in the fish muscle always shows. Mahajan and Zambare (2005) reported that the bioaccumulation of mercury, arsenic and lead in *Bellamya bengalensis* increased with the increases in exposure period to chronic concentrations. Paulose (1987) recorded the bioaccumulation of mercuric chloride and methyl mercuric chloride in *Lymnaea acuminata* and observed that the accumulation gradually increased up to 7.78 mg/kg body weight on the fifth week. The concentrations of the non-essential metals (lead, arsenic) in the aquatic organism depend mainly on their environmental levels.

Sontakke (1992) observed the increase in the bioaccumulation of Hg in the freshwater snail, *Thiara tuberculata* with respect to the time. Jaykumar (2002) found that in the bioaccumulation of copper and zinc in hepatopancreas of crab, *Spiralotte phusa hydrodroma* in control group was 222.75 and 233.74 mg/g wet weight of tissue. However, bioaccumulation rate increased in the crab treated with copper. The copper bioaccumulation in lower sub lethal concentration (25.46 ppm) exposed crab was 741.37 and 801.03 mg/g-wet weight of tissue after 15 and 30 days respectively. The amount of zinc in hepatopancreas of control crabs was 906.37 & 1012.97mg/g-wet weight of tissue for 15 days and 30 days. In the crabs exposed to sub lethal concentration (24.37 ppm) of zinc, the bioaccumulated zinc was 1749 and 2226.24 g/g fresh weight tissue.

Metal concentrations in tissues of the

freshwater fish, *Coapoeta barroisi* from the Sehan River observed. Amount of iron and zinc have been found to be highest in tissues in every season. In general, lead and cadmium have been found at lower levels, while the amount of copper was dependant on the availability of heavy metals in the aquatic ecosystem and its impact on flora and fauna had been reported by many investigators (Nayak, 1999 and Shrinivas and Balaparameshwararao, 1999).

The heavy metal concentration in Grass carp *Ctenoogartbgodon idella* was higher in liver as compared to other parts of the body i.e. head, abdomen and tail; mercury concentration in grass carp fish of upper lake were, less than 0.6 mg/g dry weight. The range of Hg was in between 0.14 to 0.31 mg/g in head portion, 0.034 to 0.085 g/g in abdomen portion and 0.113 to 0.869 g/g in liver.

Mahajan (2005) reported that the bioaccumulation of mercury, arsenic and lead in *Bellamya bengalensis.* On exposure to $HgCl_2$ (0.109 ppm), bioaccumulated Hg increased with increase in exposure period as compared to control animals. In whole body, bioaccumulated Hg in presence of $HgCl_2$ (0.109 ppm) for 7 days was 920.0 mg/kg, after 14 days was 1786.0 mg/kg and after 21 days was 2144.0 mg/kg. In the snails exposed to $HgCl_2$ with caffeine the amount of the Hg after 7 days was 740.0 mg/kg, after 14 days was 1056.0 mg/kg, while after 21 days was 1640.0 mg/kg. The gastropod snails pre exposed to $HgCl_2$ showed fast depletion of Hg^{++} in presence of caffeine. Finally, he indicated that mercury, arsenic and lead accumulation is less when exposed with 5 mg/l.

Caffeine in presence of respective concentrations of only heavy metals.

The main constituent a coffee is caffeine. The kidney manufacture and discharge up to 100% more urine on caffeine intake, investigation state the increased urinary excretion of calcium, magnesium, sodium and chloride after oral doses of caffeine. Increased pulse duration and showed the inactivation of Ca^{++} current. Caffeine binds divalent cations of calcium, which indicates that caffeine can bind other reactive divalent cations of heavy metals. Cadmium association with caffeine is an indicator of such binding.

Shomer and Nickelson (1994) reported that the altered caffeine sensitivity of malignant hypothermia susceptible (MHS) skeletal muscle fiber bundles is due to an altered caffeine sensitivity of the MHS calcium ion release channel protein. Sugiyama *et al.*, (2001) studied suppressive effect of caffeine on hepatitis and apoptosis induced by tumor necrosis factor L, but not the anti - F as antibody in mice and observed that caffeine had no significant effect on anti-F antibody induced hepatitis and apoptosis. These results suggest that caffeine differentially affected TNF- receptor -and F as mediated hepatitis and apoptosis. Caffeine has been found to have anti carcinogenic effect against N-nitosamines, polycyclic aromatic hydrocarbons and other carcinogens responsible for induction of tumors in animals.

Deposition of copper within the organism is affected by drugs and metabolites that associate with the metal. 27% increases in kidney copper in gerbils in response to caffeine feeding and an effect

of caffeine on copper concentration in the brain. Vitamin A transport from the liver to the blood may be linked with copper metabolism.

Metal complexing agents such as those found in coffee may affect copper uptake and mobilization. Greger and Emery (1987) reported that rats fed on coffee had elevated liver copper levels. Coffee is reported to affect iron but not copper metabolism in lactating women.

The interactions between caffeine and metal ions can be through its oxygen and nitrogen atoms, because of the blockage on N1, N3 and N7 atoms by methyl groups. Caffeine probably binds to metal ions through its oxygen atom of second and sixth position. Abbasoglu *et al.*,(2002) studied that caffeine would bind to metal ions through its second O and sixth O atoms in the gaseous phase.

Nafisi *et al.,* (2002) investigated the binding constants of caffeine and theophylline with Ca^{++} and Mg^{++} and reported that both alkaloids form a very weak complex with Ca^{++} and Mg^{++} ions, with caffeine to be 29.8 and 22.4 M- respectively. Theophylline is the metabolic intermediate of the caffeine catabolism in the body. Caffeine inhibited hepatocarcinogenesis induced by2-acetylaminoflure.

Kolayly *et al.*, (2004) published a paper in the journal of Food Science published by Elsevier giving the binding capacities of caffeine with different micronutrients. According to him, the binding strength of the caffeine is weaker than that of the EDTA and since all nitrogen groups are blocked by methylation, metals probably form complexes with second and sixth oxygen of caffeine. Though the binding strength of caffeine with metal

ions is weaker than that of EDTA, it is sufficient to drag the metal ions bound to the proteins because the metals are usually bound to –SH groups of proteins and the bond energy of the oxygen metal complex is stronger than sulphydril-metal bond. EDTA is very toxic as compared to the caffeine, and it removes essential micronutrients from the body.

The caffeine metal-chelate complex being of small molecular weight can easily pass through the membranes of the cells of the epithelia of the tubules of the kidney and can be effectively excreted out.

The humans are regularly exposed to the toxic heavy metals however the impact of the toxicant is usually bypassed and the problems such as headache are suppressed by taking the common analgesic and antipyretics. No body refers the doctor for the relief from the heavy metal toxicosis. Caffeine, commonly found in the daily beverage in the regular use can be safe and useful to protect the body against the heavy metal toxicosis and it's bioaccumulation. Caffeine is toxic at very high levels and the records shows that ten grams of caffeine taken at a time is lethal. In comparison, a strong cup of tea or coffee contains not more than eighty Mgs of the caffeine.

SUMMARY

- *The bioaccumulation of mercury and arsenic in Lamellidens corrianus was studied.*
- *The mercury and arsenic accumulation increased with the increase in exposure period of acute concentration of mercury (0.444ppm Hg^{++}) and arsenic (0.672ppm As^{+++}).*
- *The amount of mercury and arsenic accumulation is less when exposed with 5 mg/lit. Caffeine in presence of respective concentration of heavy metals as compared to those exposed to only heavy metal.*
- *The Lamellidens corrianus pre-exposed to mercury (0.444ppm Hg^{++}) and arsenic (0.672ppm As^{+++}) showed rapid removal of the heavy metal in presence of caffeine than those maintained in normal water.*

FINAL CONCLUSION:-

Finally on the basis of experimental observations and results of this investigation, It is confirmed that the Caffeine (1, 3, 7 - Trimethylxanthine) is a Modulator of Heavy Metal toxicity in the experimental Pelecypod Model, *Lamellidens corrianus*. The present investigation indicates that, Caffeine has a protective and curative role in the heavy metal induced alterations. Experimental results indicate the detoxifying effect of Caffeine induced alterations. Caffeine repairs or recovers damages due to the heavy metal intoxication. The Caffeine has Ant-Oxidant and Anti-cancer or carcinogenic activity. This study concluded that heavy metals are removed from the body in presence of Caffeine. This investigation confirmed that Caffeine (1, 3, 7 - Trimethylxanthine) is a Modulator of Heavy Metals intoxication.

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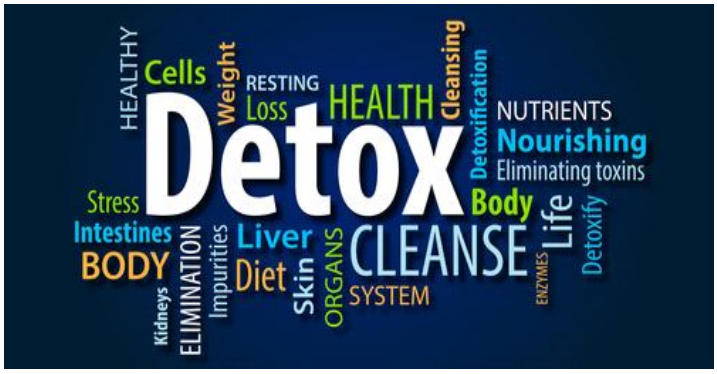

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