Hemolymph factors responsible for defense reactions against pollutants and bacteria, *Vibrio alginolyticus*, in the Indian edible oyster, *Crassostrea madrasensis* (Preston)

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Doctor of Philosophy

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30-4-2004

Dr. K.C. George
Supervising teacher

Declaration

I hereby declare that this thesis is a bonafide record of research work done by me under the guidance of Dr. K.C. George, Principal Scientist, Central Marine Fisheries Research Institute and that no part thereof has been presented earlier for any degree, diploma or similar title of this or any other university.

30-4-2004

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DEDICATED TO THE

AMBITIONS OF MY

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1. INTRODUCTION

The study of the defense mechanisms in invertebrates started with marine animals. The first report on the cellular defense in invertebrates was the observation of phagocytosis in starfish larvae and *Daphnia* (Metchnikoff, 1884). It was suggested that phagocytes play a significant role in the defense mechanisms of all evolutionary stages of animals starting from unicellular animals to mammals. The study of hemolysins in the horse-shoe crab by Noguchi (1903) was the first study on the humoral immunity of invertebrates.

Since 1960, there was a renewed interest in the study of defense mechanisms in invertebrates, which was suggested to be because of two reasons: (1) to find out those mechanisms in invertebrates that are also discovered in vertebrates and (2) the development of intensive mariculture requires better knowledge of the factors that may lead to mortality of the crustaceans and mollusc due to failure of their defense systems. Marine invertebrates served as experimental materials for phylogenetic study of defense mechanisms in Animal Kingdom from late 19th century to the beginning of 20th century (Mori, 1990).

Among invertebrates, the defense systems of molluscs and insects are being studied extensively in order to understand the basics of invertebrate immune system. During the past three decades, there has evolved a considerable interest in how the molluscs defend themselves against the invaders. Immunity of molluscs is comprised of cell-mediated immunity and humoral mechanisms. The cell-mediated immunity is the function of hemocytes, while humoral immunity is provided by the serum factors. There are many evidences, which suggest that these two are interrelated (Cheng, 1990).

Hemolymph in invertebrates is analogous to blood in vertebrates. The blood of vertebrates consists of plasma and cells. The cells are red blood corpuscles and white blood corpuscles. The red blood

corpuscles carry out the function of carrying respiratory gases, while leucocytes have defensive functions. As in vertebrates, the hemolymph of invertebrates also contains plasma and cellular components. The cells do not have the function of carrying respiratory gases. They perform the defensive and healing functions. The study of these cells will give insight into the operation of disease resistance in these animals. The classification and characterization of these cells are not yet resolved and each species has got its own peculiarities. The sites where the hemocytes are produced in molluscs are not determined, but according to Cheng (1983) these cells arise from differentiation of the connective tissue cells, whereas, the hematopoietic tissue of *Biomphalaria glabrata* is reported to be the anterior pericardial wall (Sullivan, 1991).

The hemolymph plasma contains a number of glycoproteins and enzymes, which play an important role in pathogen destruction. The understanding of these factors in each species is an important step in studying the immune mechanisms of molluscs. Since aquatic pelecypods and gastropods have open circulatory systems, the volume of the hemolymph fluctuates significantly (Chu and Peyre, 1989). As a result, the number of hemocytes, volume of the serum and serum protein concentration also fluctuate. In these animals, the fluctuation in environmental conditions and presence of additives compromise both their cell-mediated and humoral immune mechanisms (Cheng, 1990).

The molluscs frequently encounter diseases due to microbial infections. They become vulnerable to infection when they are physiologically weak and attacking organisms acquire virulence. Hence, it is important to study the host-pathogen interactions with reference to molluscan defense system. The study of defense mechanisms in molluscs will throw light on the evolution of immune mechanisms in Animal Kingdom and will give information on adaptations of molluscs in hostile

environments. Since many molluscan species are candidates for aquaculture, it is essential to unravel the defense mechanisms of these animals in order to devise healthy culture practices suited for diverse environments. It is a necessary input for manipulating the molluscan immune system for the control of infectious diseases. It will be possible to select the best species with reference to the disease resistance for a particular area and also to modify the culture techniques with reference to the total defensive capacity of the candidate species. It will also provide useful information on the possibility of introducing new species of molluscs from other geographical areas. It will also be possible to control diseases by manipulating the immune responses, such as the production of transgenic disease resistant species, which will be a boon to aquaculture (Vasantha, 1996).

The study of immunotoxicity in invertebrates is still at its infancy. Full evaluation of immunotoxic potential of contaminants requires a wide range of assays to assess both cellular and humoral aspects of the immune function. Blood parameters have been recognized as valuable tools in assessing the conditions of organisms and their response to physicochemical changes in the environment (Jyothirmayi and Rao, 1987). Cheng (1989) suggested that the immunodeficiency caused by the pollutants would tilt the equilibrium between host internal defense system and the pathogen in favour of the invaders. There is an increased industrial activity in the coastal regions of India. The industrial run off is directly affecting the water bodies, which are used for molluscan culture. Another source of pollution is the intensive crop culture, which use large amount of pesticides and chemical fertilizers. The presence of these xenobiotic compounds is affecting the health of all animals, and oysters, being sedentary, are highly susceptible to the action of these compounds. The laboratory based assays need to be evaluated under field conditions

and a link between immune suppression and disease susceptibility is to be established.

The international mussel watch is a widely used marine monitoring program. This consists of measuring bioaccumulation, physiological tests, and histopathological analysis. Histopathological analysis helps to give us an idea about the health of the animal based on the morphological structure of the cells and tissues. According to Sunila (1988), the mussels are extremely tolerant to environmental pollution. However, the pollutants cause histopathological changes. In addition to environmental pollutants, the histological changes are caused by infectious agents and also by variations in the physical factors such as salinity, temperature, pH, dissolved oxygen etc. The first sign in a light microscopic study, indicating that the cells have passed the point of no return, (the point between reversible degenerative and irreversible necrotic changes) is pyknosis. It might then be too late to find the reason causing the destruction of cells. Before cell death, a series of cytological events occurs, e.g. waking up of defense mechanisms and transforming delimitedly from a normal cell to an injured cell and a dead cell. If we can get an idea about the causative agent based on the histopathological changes, it would be the most important tool in the study of marine pollution.

The purpose of the present study is to find out the various cellular and humoral factors responsible for the natural defense of edible oyster, *Crassostrea madrasensis,* which is one of the candidate species for aquaculture. Effort is made to classify and characterize these factors and also to quantify the effect of abiotic and biotic factors on the defense mechanisms of the species. The study will throw light on the use of indicators for assessing the immune responses of the animal. It is also hoped to develop various simple tests to assess immune mechanisms.

These data are highly essential to develop strategies for future disease control programs in molluscan culture. It may also help in identifying suitable sites for molluscan culture. It is hoped that the result of present work will strengthen the foundation of molluscan immunological studies in our country.

2. REVIEW OF LITERATURE

2.1. Characterization of hemolymph factors

Invertebrates, including bivalves lack the immunological memories organized by the B and T lymphocytes as in mammals. So the primitive defense mechanisms like recognition and phagocytosis of non-self materials by the hemocytes play an important role. The humoral factors are primarily hemocytic in origin. The different types of cells in the invertebrate hemolymph represent ontogenic stages in the lifespan of one type of hemolymph cell. Therefore a significant reduction in the total hemocyte count will affect the efficiency of the immunity in molluscs (Foley and Cheng, 1972; Moore and Eble, 1977; Rasmussen *et al.*, 1985). In molluscs, in contrast to the crustaceans and insects, there appears to be no difference between free and fixed hemocytes, especially phagocytes (Balquet and Poder, 1985).

2.1.1. Cellular factors

The total number of circulating hemocytes in *Crassostrea virginica* is influenced by amount of turbulence produced by the cardiac action and also by feeding and excretion cycles (Feng, 1965a).

There are two sizes of hemocyte population in the American oyster *C. virginica*, (Feng *et al.*, 1971; Foley and Cheng, 1972). The larger ones are divided into two groups, the granulocytes with a mixture of acidophilic, basophilic and refractile granules, which are electron lucid and fibrocytes with either a lobate or ovoid nuclei. The smaller cells are hyalinocytes which are either, agranular or mildly granular.

In *Mercenaria mercenaria,* the hemocytes can be broadly divided into granular hemocytes and hyaline or agranular hemocytes. The granulocytes are further classified as small granulocytes and large granulocytes. The agranulocytes constitute 2%, small granulocytes, 61% and large granulocytes constitute 37% of the total hemocytes. These hemocytes are suggested to represent different stages of

maturity, the hyalinocytes, being the immature ones, mature into semigranulocytes and finally to granulocytes. There are three types of granules in both granulocytes. They are blunt granules, identified as mitochondria, dot like granules, identified as lysosomes and refractile granules that store lipids (Foley and Cheng, 1975; Moore and Eble, 1977).

The hemocytes in *Mytilus edulis* can be divided into lymphocytes, macrophages and eosinophilic granulocytes (Moore and Lowe, 1977).

Three types of hemocytes are identified in *Mytilus californianus* in Geimsa stained preparations (Bayne *et al.*, 1979). They are small and large agranular basophils and granular acidophils. The acidophilic granules are stained positively for phospholipid with luxol fast blue. The basophils are reactive for acid phosphatase, β-glucuronidase, and N-Acetyl-B hexosaminidase, while acidophils give negative reactions. Reaction product is localized in the cytoplasmic granules presumed to be lysosomes in the basophils.

In *Lymnaea stagnalis, B. glabrata* and *Bulinus truncates* (gastropods), blood cells constitute a series of cells, which include the dark round cells (small pseudopodia, few golgi bodies and lysosomes, low enzyme contents and low phagocytic capacity) and the readily spreading cells (long pseudopodia, many golgi bodies and lysosomes, high enzyme contents and high phagocytic capacity) (Sminia and Barensen, 1980). In this concept, the phagocytosing blood cells would mature from the round cells that have the characteristics of young cells (e.g. relatively large nucleus, many free ribosomes). The hyalinocytes are suggested to be degenerative cells because of the presence of swollen nucleus, cytoplasm and disrupted cell organelles.

Cheng (1981) has reported that in *C. virginica,* the hyalinocytes is about 40%, granulocytes around 30% and fibrocytes, which

are believed to be degranulated stage of granulocytes after the digestion of antigen, around 30%.

Two classes of hemocytes exist in *Mytilus campechiensis* - agranular and granular hemocytes. In addition, a third class occurs in the blood ark, *Anadara ovalis i.e.* erythrocytes (Rodrich and Ulrich, 1984).

Rasmussen *et al.* (1985), in their study on the hemocytes of *M. edulis,* have concluded that there are only two types of cells, granular and agranular leucocytes, and that the macrophages described by the earlier workers are eosinophilic granulocytes with phagocytosed material. According to them, the granulocytes constitute much of the cell population and hyalinocytes are very less in number in circulation, and also the cells that are found in the tissues are entirely granulocytes. The hyalinocytes are generally small with a lobate nucleus, free ribosomes, rough endoplasmic reticulum and mitochondria, and sometimes a golgi complex visible in the cytoplasm. The granulocytes are of two types, the smaller one with lobate nucleus and small granules only in the paranuclear cytoplasm and surrounded by an electron lucent halo. The cytoplasm contains free ribosomes, rough endoplasmic reticulum, mitochondria and golgi complex with granules seen in the inner part. The larger granulocytes are with unlobate nucleus and cytoplasm completely filled with granules, short strands of rough endoplasmic reticulum and very few mitochondria. The granules are bigger without electron lucent halo. Cells with numerous vesicles, few granules and lytic bodies are also seen infrequently (macrophages). The granules are filled with homogeneous, moderately electron dense materials. The membrane of some of the granules containing the homogenous materials, appeared indistinct, and fusion of membranes between adjacent granules could be observed. According to them, all hemocytes have the same ontogeny.

Seiler and Morse (1988) have studied the normal and pollution related ultrastructural morphologies of the hemocytes of *Mya arenaria*. There are two types of hemocytes in this mussel. They are agranulocytes characterized by almost a lack of intracellular granules, and are roughly round with some extended pseudopodia, and have an average diameter of 7 μm. The nucleus is large with clumped chromatin and has a double walled nuclear membrane. Much of the cytoplasm contains electron lucent vesicles and patches of glycogen. Mitochondria, rough endoplasmic reticulum, and a golgi complex are prominent. The second one is the granulocyte and has numerous electron opaque granules ranging from 0.08 to 0.5 μm. The cell is roughly round with some extended pseudopodia and has an average diameter of 7 μm. The nucleus is lobate with clumped chromatin and double walled nuclear membrane. It lacks mitochondria, golgi apparatus and has very few small clear vesicles, some apparently smooth endoplasmic reticulum and little or no glycogen.

In the fresh water snail, *Viviparous ater*, there is only one type of hemocyte -the spreading hemocyte, representing the ancestral cell responsible for defense mechanisms. The cell is similar to the spreading type cells of other gastropods (Ottaviani, 1989).

The scanning electron micrographs of hemocytes of *Oncomelania hupensis* reveal that the unspread hemocytes are spherical. As they commence to spread on the glass substrate, the initial pseudopodia are lobose. Later, the granulocyte is observed to posses branched filopodia. In a fully flattened hemocyte, the filopodia have terminal swelling (Morona and Mingye, 1989).

Frachini and Ottaviani (1990) have found that the spreading hemocytes of *V.ater* are having thin pseudopodia, polymorphic nucleus and α-glycogen granules. The granules with different

morphologies are identified suggesting different stages of granule maturation. The granules show varying degrees of acid phosphatase activity.

Suresh and Mohandas (1990a) have studied the number and types of hemocytes in *Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis*. They have compared the total hemocyte number in four different size groups of both the clams and found that the reproductive cycle might cause a decrease in total cells in circulation because of migration of the cells to the gonad. If the clams are in their early growing stage or late growing stage, there are no significant differences either in total or differential counts. According to them, there are only two cell types in both the clams, namely, granular and agranular hemocytes. About one third of the total is comprised of agranulocytes.

The first report on the use of monoclonal antibodies for the antigenic characterization of the hemocytes in the mollusc is by Noël *et al.* (1994). According to them, there are three types of hemocytes in *M. edulis*. The hyalinocytes, which do not combine with antibody, basophilic granulocytes, which are granulocytes containing small granules and eosinophilic granulocytes, which are granulocytes with large granules. Hyalinocytes and the two granulocytes represent two separate cell lines.

Oubella *et al.* (1996) have reported five types of hemocytes in Manila clam, *Ruditapes philippinarum*. They are hyalinocytes (55%), neutrophilic granulocytes (39%), small basophilic hemocytes, multinucleated cells and particle loaded cells, which constituted a very less percentage. The hyalinocytes are characterized by the lack of cytoplasmic granules and a large ovoid or kidney shaped nucleus with chromatin and one or two nucleoli. The basophilic hemocytes are generally spherical with a narrow ring of dark blue cytoplasm around a

large rounded nucleus. The neutrophilic granulocytes are characterized by pink cytoplasmic granules and an eccentric hyperchromatic nucleus. The multinucleated cells are larger and exhibited two or more nuclei. The particle-loaded cells are characterized by several inclusion bodies in the cytoplasm, possibly reflecting their phagocytic ability.

Three types of hemocytes are recognized in the Giant clam, *Tridacna crocea.* They are granular cells, agranular cells and morula like cells. The granular hemocytes contain granules, which are eosinophilic and acid phosphatase positive. The hyaline cells are morphologically characterized by a relatively large nucleus, surrounded by a small volume of cytoplasm and the absence of granules (Nakayama *et al.*, 1997).

2.1.2. Humoral factors

2.1.2.1. Protein profile

The serum of molluscs is composed of proteins, enzymes, lectins, etc., which together contribute to humoral immunity.

The serum protein shows significant changes depending on the infection by a pathogen (Lee and Cheng, 1972), size of the individual and food availability (Michelson and DuBois, 1975).

The electrophoretic separation of the plasma proteins in *M. californianus* reveals about 16 proteins, which are surprisingly consistent in controls, in heavily bled ones and starved ones. However, this result cannot be interpreted as evidence against a role for humoral factors, since it is not possible to know how many proteins are present in each electrophoretic band (Bayne *et al.*, 1979).

Granath *et al.* (1987) have reported that hemoglobin and hemoglobin subunits of 160 kDa contribute to majority of the serum proteins, where as, there are a number of fairly stained polypeptide bands of 10 to 116 kDa in hemoglobin depleted serum in *B. glabrata.* The serum is analyzed by SDS-PAGE, HPLC and immunoblotting techniques.

The hemolymph proteins of Acorn barnacle, *Megabalanus rosa* is studied by two-dimensional gel electrophoresis (Muramoto *et al.*, 1996). The study reveals more than 50 proteins with molecular weights, 18-120 kDa. Several major proteins are identified to be multiple lectins and they form 10-66% of the total proteins. According to them, the PAGE maps make it possible to trace or compare the proteins of interests in animals under different physiological conditions.

2.1.2.2. Enzyme profile

The humoral immunity is provided mainly by the lysosomal enzymes. The lysosomal activity of hemolymph and body fluid is an indication of the physiological condition and the vitality of the defense system of animals.

Enzymes like lysozyme, acid and alkaline phosphatases, β-glucuronidase, lipase, glutamate-oxalacetate transaminase, and glutamate pyruvate transaminase are present in the whole hemolymph and 4000*g* pellets and supernatants in *M. arenaria* and are believed to be of hemocytic origin (Rodrick, 1979).

The granules of bivalve granulocytes have been identified as lysosomes by isolating lysosomal and hydrolytic enzymes from cell samples. The granulocytes isolated from *M. mercenaria* have different combinations of five hydrolytic enzymes. Under normal conditions, the lysosomal enzymes of the granulocytes are restricted to the lipoprotein lysosomal membrane *i.e.* being in the latent phase. However, if granulocytes are challenged by a variety of abiotic and biotic factors, the lysosomes become unmasked or destabilized *i.e.* the enzymes are released from lysosomes into the surrounding cytoplasm (Moore and Gelder, 1985). The lysosomal enzymes are released into the serum by degranulation (Cheng and Mohandas, 1985).

Takahashi *et al.* (1986) have studied the lysozymes in *Crassostrea gigas* and *M. edulis*. In both cases, the highest lysozyme activity is observed in the extracts of the digestive diverticula. Lower activities are detectable in the extracts of gills, mantle, crystalline style and in the hemolymph, but no activity in the adductor muscle. The enzyme activity is enhanced by calcium and sodium, but is inhibited by iron and iodine.

The lysosomal enzymes are synthesized in the granulocytes and released into serum where they have anti-microbial and anti-parasitic functions (Cheng, 1989; 1990).

2.1.2.3. Other humoral factors

There is evidence of an alpha-macroglobulin in *B. glabrata*. This methylamine-sensitive inhibitor decreases trypsin activity against a protein substrate (Bender *et al.*, 1992).

Thoegersen *et al.* (1992) have purified a protein inhibitor homologous to human alpha sub (2)-macroglobulin, which is a glycoprotein that is composed of two identical 180 kDa disulphide linked sub-units. It inhibits metalloproteinase thermolysin, and also serine proteinases.

Cytokine like molecule, immunoreactive TNF (Tumour necrosis factor) alpha is detected in the hemocytes and serum of *B. glabrata* by western blot analysis (Ouwe-Missi-oukem-boyer, 1994).

Asunción *et al.* (2003) have reported that the hemolymph of molluscs contains an unknown cell growth factor similar to that present in the foetal calf serum and that lipopolysaccharide induced an increase in number of α-subunits of interleukin-2 receptor in the cell membrane of hemocytes of *Mytilus galloprovincialis*.

2.2. Functions of hemolymph factors

2.2.1. Hemocytes

Hemocytes of bivalve molluscs are involved in various homoeostatic functions such as wound healing, transport of

calcium, protein regeneration, regeneration of shell, intracellular digestion, exclusion of non-self materials in the internal defense and toxification and detoxification processes of different xenobiotics (Cheng, 1981).

2.2.1.1. Phagocytosis and encapsulation

The phagocytosis and encapsulation of foreign objects are important functions of hemocytes and among these, phagocytosis is more common. Feng (1965b) has studied the uptake of rhodamine labelled gamma globulin by oyster hemocytes at different temperatures and reported increased phagocytosis at a higher temperature.

Frankboner (1971) has reported the digestion of zooxanthellae by giant clams belonging to Tridacnidae. According to him, the older zooxanthellae are selectively culled from algal population of mantle by hemocytes and are digested by lysosomes in the circulatory system as well as in interdiverticular spaces of digestive gland. These older zooxanthellae are believed to be recognized as nonself by the hemocytes.

Foley and Cheng (1975) have exposed the hemocytes of *C. virginica* and *M. mercenaria* to *Bacillus megaterium, Escherichia coli* and *Staphylococcus aureus* and have found that low temperature decreased the extent of association of the bacterial cells with the hemocytes. According to Cheng and Yoshino (1976), there is increased lipase activity in the phagocytosing hemocytes in *M. arenaria*. In molluscs, these reactions occur anaerobically (Cheng, 1976).

In *M. californianus, in vivo* studies by injecting colloidal carbon into the adductor muscle have resulted in phagocytosis of carbon particles by large basophils within 1 hour. *In vitro* phagocytosis using yeast cells and red blood cells indicates that phagocytosis occurred more selectively towards yeast within 15 minutes. The difference is

suggested to be because of the difference in surface antigenicity of the test particles. The phagocytic activity is not affected by the presence or absence of humoral agglutinins (Bayne *et al.*, 1979).

In *Bonamia ostreae* infection, there is evidence that the granular hemocytes transport the pathogen throughout the body (Balquet and Poder, 1985).

Fisher and Newell (1986) have reported that acute increase in salinity decreases hemocyte locomotion of the eastern oyster and acute decrease in salinity increases it. Fisher *et al.* (1987) have reported that acute decrease in salinity or increase in temperature reduces the ability of the hemocytes to adhere to the latex beads in the edible oyster, *Ostrea edulis*.

Cheng and Jourdane (1987) who tried implantation of allografts in *B. glabrata* have reported that encapsulation occurs in two phases. During the initial phase, granulocytes adhere to the graft, become hypertrophied and elongate. This results in the chemical alteration of the graft's surface. In the second phase, altered grafts emit a signal, which attracts additional molluscan granulocytes, which result in the formation of a granuloma.

A phospholipase-C like activity is present in the phagocytic cells in the Asian clam, *Corbicula fluminea*. This enzyme may interact not only directly with foreign intruders as a potent lytic agent, but also indirectly, by modifying the surface macromolecular composition of target cells or organisms (Yoshino, 1988).

The phagocytic process of the hemocytes of molluscs seems to differ in a number of ways from the mammalian macrophage. With a view to understand their primitive cellular immune system, Alvarez *et al.* (1989) and Alvarez and Friedl (1990) have studied the factors affecting *in vitro* phagocytosis by the oyster hemocytes (*C. virginica*).

Hemocytes withdrawn from the pericardial cavity of the American oyster were settled onto monolayers of fluorescent polystyrene microspheres. Phagocytosis was quantified by the fluorescent microscopy and flow cytometry. Phagocytosis was evident within 15 minutes after addition of the cells to the monolayer. Multiple particle phagocytosis occurred rapidly after contact between cells and particles. The number of particles taken up was proportional to the number of initial contacts between hemocytes and beads and was a function of the cell surface area. Maximum phagocytic activity was noted in a wide range of temperature (10°C-37°C) and inhibition occurred below 8°C. Inhibition of cell attachment to monolayer was suggested to be the reason for decreased phagocytosis. Actin polymerization by cytochalasin B reduced phagocytosis. Phagocytosis was not affected in the atmosphere of nitrogen or helium, which suggests that phagocytosis could occur in completely anaerobic conditions and continue for 1-2 hours. The phagocytic process was driven by an anaerobic metabolism.

The encapsulation represents aborted attempts of phagocytosis, when the foreign body is too large to be internalized. Since the granulocytes are more actively phagocytic, a reduction in the percentage of granulocytes in the hemolymph affects the immunity (Cheng, 1990).

The study on the ultrastructure of phagocytosis by hemocytes of the American oyster *C. virginica* shows that the electron dense particles within cytoplasm are extruded from the cells. The internalized latex beads are seen in the vesicles, which are phagosomes. These phagosomes appear to be able to fuse together, since more than one bead is seen within a single membrane. Degranulation is noted within the hemocytes. It is also believed that the membranes of these granules fuse to form the membrane of the phagosome. Since polystyrene beads are used,

it is assumed that a chemotactic attraction is absent. It is also reported that a direct contact of the cell with the foreign body is also unnecessary (Hinsch and Hunte, 1990).

According to Pipe and Coles (1995), the predominant methods of internal defense include the phagocytosis by hemocytes, which take place in a number of stages including recognition, chemotaxis, attachment, incorporation and destruction. Prior to phagocytosis, there will be an increase in the number of circulating hemocytes contributed by the migration of the cells from the tissues, rather than the proliferation of the cells. Other methods to deal with larger pathogen and parasites include encapsulation and nacrezation. The phagocytosis is accompanied by the release of degradative enzymes and the generation of reactive oxygen intermediates with antioxidant enzymes. Other soluble components that are released by the hemocytes include agglutinins, lysins, etc.

NADPH-oxidase activity similar to that seen in the mammalian phagocytes is seen in hemocytes of *C. gigas* (Takahashi and Mori, 2000).

2.2.1.2. Other activities of hemocytes

The inflammatory reactions of granulocytes associated with environmental or physiological changes are described by Do and Lubet (1965). These reactions increase during gonadal resorption in the end of sexual maturation stage.

Foley and Cheng (1972) have noted granulocytes as concentric layers around foreign bodies in *C. virginica.*

The acid phosphatase activity is not present in all the granulocytes, which are believed to be lysosomes (Yoshino and Cheng, 1976).

Yoshino and Davis (1983) have demonstrated that individual hemocytes of *B. glabrata* may have different populations of hemolymph-like antigens, and the interaction between some membrane components and appropriate ligands (e.g.; carbohydrate binding lectins) can result in a modulation in the expression of other groups of surface antigens.

When oysters are exposed to oil pollution, granulocytic infiltration occurs within gonadal tubules simultaneously with oocyte lysis. Such reactions occur in the connective tissue, mainly around the stomach and digestive tubules after exposure to pollutants (Calabrese *et al.*, 1984).

Wittke and Renwrantz (1984) have tried to quantify the hemocytes, which produce cytotoxic substances among the total hemocytes and found that the number of cytotoxic hemocytes and the total number of hemocytes varied greatly between different individuals. It is suggested that the transformation of normal hemocytes to cytotoxic hemocytes require triggering by nonself materials.

In areas of focal necrosis, hemocyte infiltration is noticed in the winter disease of *Crassostrea commercialis* (Balquet and Poder, 1985). According to them, the hyalinocytes do not have any demonstrable functional and immunological correlations. But they are important as progenitor in hematopoiesis. The neoplasic conditions of the hematopoietic system are believed to arise from these cells.

Dikkeboom *et al.* (1987) have reported that the macrophage-like hemocytes of *L. stagnalis* generate reactive oxygen metabolites, when stimulated by particulate agents like latex, zymosan, *E. coli* and *Staphylococcus saprophyticus.*

Mori *et al.* (1990) have studied the H_2O_2 production by the hemocytes of scallop, *Patinopecten yessoensis* and found that the healthiest scallop produced the highest amount of H_2O_2.

Based upon the ability of the hemocytes to digest foreign materials, their motility, and their association with digestive organs, Freidl and Alvarez (1990) have suggested that they play primary roles in the bioaccumulation and depuration.

The hemocytes of *Pomacea canaliculata* require no stimulus other than calcium ions for aggregation, which is accompanied by degranulation (Shozawa and Suto, 1990).

The serous cells in *Tridacna crocea* are negative to the staining for lysosomal marker enzymes, acid phosphatase, peroxidase, etc., which clearly indicates that they do not play a role in phagocytosis, but, they may play a role in the release of anti-bacterial agents. A wound healing function of hyalinocytes is also reported (Nakayama *et al.*, 1997).

Arumugan *et al.* (2000) have found that the hemocytes of *C. gigas* and *M. galloprovincialis* use NADPH oxidase and nitric oxide synthase pathways to kill microbial pathogens as in mammals, whereas, the same activity is absent in *Ruditapes decussatus.*

2.2.2. Humoral factors

2.2.2.1. Transportation of nutrients

The hemolymph plays an important role in the transportation of protein, lipid and glycogen at the time of maturation of the gonad (Thomson, 1977).

In the bivalve, *Diplodon delodontus,* plasma proteins transport phospholipids, free fatty acids and triacylglycerols (Pollero and Heras, 1989).

2.2.2.2. Agglutination of foreign particles

The serum of *M. californianus* agglutinates *Pseudoisochrysis,* human A Rh^{+ve} erythrocytes and yeast. Agglutinins are

predominantly carbohydrate-binding proteins (lectins), which are involved in the transport functions of phagocytosis. However, there is no evidence of opsonic factors in the plasma influencing phagocytosis of yeast cells or red blood cells (Bayne *et al.*, 1979).

Fryer *et al.* (1989) have demonstrated the presence of a lectin in plasma of *B. glabrata*, which opsonize yeast for phagocytosis. Hemolymph lectins bind to the carbohydrate determinants of membrane glycoproteins or glycolipids resulting in the agglutination of invading pathogen. Agglutinins have also shown to act as opsonins, facilitating increased phagocytic activity of bivalve hemocytes (Pipe and Coles, 1995).

2.3. Environmental pollution and defense mechanisms in molluscs

The measurement of lysosomal marker enzymes like acid phosphatase in animals is an effective method to identify the stress induced by environmental pollutants (Mohandas *et al.*, 1985). If the ability of the molluscan granulocytes to synthesize lysosomal enzymes and to release them into the serum is inhibited, these cells become immunologically compromised.

The percentage of granulocytes is significantly higher (50%-60%) in *M. arenaria*, collected from polluted area compared to those from unpolluted area (30%-40%). The granules of those collected from polluted area are extremely larger. There is evidence for the hemocytes apparently moving through the kidney epithelium, which may be for excretion of particulate materials via kidney (Seiler and Morse, 1988).

Cheng (1989) has suggested that the immunodeficiency caused by the pollutants would tilt the equilibrium between host internal defense system and pathogen in favour of invaders. The detection of the molecules released in response to the specific pollutants would give an idea of the condition of animal.

Since, many cell types of marine molluscs contain lysosomes, and any cellular injury would reflect on the activity of these organelles, they are considered to be the starting point of such investigations (Suresh and Mohandas, 1990b). The lysosomes are known to detoxify the pollutants until ultimately they damaged, and then burst releasing the lysosomal hydrolases to the cytoplasm or to the extracellular environment.

It has been reported that environmental pollution causes diseases in bivalves like mantle recession, granulocytomas and proliferative blood cell disorders (Sindermann, 1993). The susceptibility towards infectious disease has shown to increase, as a result of exposure towards pollutants. The response of an organism to any change in the environment is first manifested at the cellular level.

2.3.1. Effect of organic compounds on the defense system

2.3.1.1. Changes in hemolymph factors

2.3.1.1.1. Changes in hemocyte number

Crude oil contamination has resulted in an increase in total hemocyte count in *M. edulis* (Dyrynda *et al.*, 1997). Pipe *et al.* (1995) have recorded a significant lower number of hemocytes in *M. galloprovincialis* taken from areas polluted with chlorinated hydrocarbons like lindane, DDT and PCB's. According to Fisher *et al.* (2000), the pesticides α- hexachlorohexane and mirex reduced the hemocyte number in *C. virginica*. Domoic acid is a neurotoxic amino acid secreted by a diatom in the marine environment, but it does not cause any change in the hemocyte number of *M. edulis* (Dizer *et al.*, 2001). A significant reduction in the hemocyte number in *M. arenaria* and *Mactromeris polynyma*, which are exposed to PAH and PCB is reported by Fournier *et al.* (2002).

2.3.1.1.2. Changes in the hemocyte profile

Seiler and Morse (1988) have reported that there is an increase in the number of granulocytes of *M. arenaria* collected from

polluted sites compared to those collected from non-polluted sites. They have suggested the role of granulocytes in the removal of pollutants.

Sami *et al.* (1992) have reported a reduction in the number of large hemocytes and a concomitant increase in the number of small hemocytes in *C. virginica* exposed to polycyclic aromatic hydrocarbon polluted environment.

2.3.1.1.3. Changes in hemocyte activity

Fries and Tripp (1980) have reported a decrease in the phagocytic activity of hemocytes of *M. mercenaria* exposed to phenol. The *in vitro* phagocytosis of the yeast by hemocytes decreased as a result of exposure to PCB in *M. edulis* (Cheng, 1989) and in *M. arenaria* (Beckmann *et al.*, 1992). A decrease in resistance of *C. virginica* to *Perkinsus marinus* at sub-lethal concentrations of marine antifouling agent-tributyl tin oxide is reported by Fisher *et al.* (1995). A decrease in super oxide generation and phagocytosis in *M. edulis* as a result of pollution by crude oil is reported (Dyrynda *et al.*, 1997). In *C. virginica*, the hemocyte activities increase at lower concentrations and decrease at higher concentrations, when exposed to tributyl tin oxide (Fisher *et al.*, 1999), whereas, the pesticides α-hexachlorohexane and mirex reduce the hemocyte mobility and super oxide production (Fisher *et al.*, 2000). The viability and phagocytic activity are greatly increased within 7 days of exposure to domoic acid in *M. edulis* (Dizer *et al.*, 2001). A suppression in the phagocytic activity of hemocytes of *M. arenaria* and *Mactromeris polynyma* as a result of the exposure to PAH and PCB is noticed (Fournier *et al.*, 2002). It is reported that the phagocytic activity, as detected by flow cytometry, can be used as a biomarker of exposure to organic contaminants. It is also noted that the hemocyte viability is not affected in both the species exposed to the pollutant for more than 4 weeks.

2.3.1.2. Histological changes

Sunila (1988) has studied the acute effects of PCB, Clorphen A 60, DDT, and Dieldrin each at a concentration of 5 ppm at a salinity of 7 ppt, on the gills of the mussel, *M. edulis*. None of these compounds evoked any inflammatory responses. PCB did not evoke any inflammatory reaction, but the cells were shrunken and connections between neighbouring cells became loosened. In the frontal cells, the proximal parts were granular; and lateral cilia were often missing. The endothelial cells were cuboidal and had empty spaces between them. When exposed to DDT, the changes were similar to that in PCB. The exposure to Dieldrin resulted in the formation of cavities under post lateral cells. A lack of hemocytic infiltration as a result of infection was noticed in *C. virginica,* when exposed to chemical carcinogen N-nitrosodiethylamine (Winstead and Couch, 1988)

2.3.1.3. Toxicity of organophosphorous pesticides

Egidius and Moster (1987) have reported that the mussel, *M. edulis* survived 1 ppm of Neguvon and Nuvan, both organophosphorous pesticides, whereas, lobsters and crabs could not survive at this concentration.

Toxicity and bio-concentration of organophosphorous pesticides like Dimethoate, Methidathion, Chlorfenvinphos, Chlorpyrifos and Phosmet were tested in two bivalves namely, *M. galloprovincialis* and *Venus gallina*. A concentration up to 56 mg/L was tested and mortality and sub-lethal effects were reported for *M. galloprovincialis,* but in *V. gallina* there were no effects for methidathion, chlorfenvinphos and chlorpyrifos. Dimethoate and phosmet had no effects in both the species tested. Toxic effects were moderate to nil. Both the pesticides were concentrated in soft tissues of the molluscs (Serrano *et al.,* 1995).

According to Dauberschmidt *et al.* (1996), organophosphates are toxic to the mollusc only at a concentration above 2

mg /l (96 hours). Also the tissue concentrations of the organophosphates are about 10 times higher than that present in the ambient water. The organophosphates are metabolized by mixed function mono oxygenase system present in the cytochrome P450, located mainly in the digestive diverticulae. Unlike vertebrates, the metabolism does not require addition of NADPH in invertebrates. The amount of P450 is about two times lower than the amount present in the mammalian liver (Dauberschmidt *et al.*, 1997a). It is also found that the esterase activity of the mollusc is not inhibited by the exposure to organophosphates like thiometon, disulfoton, demeton-S-methyl, whereas, the esterases of mammalian tissue show 95-100% inhibition by the same. This is suggested as one of the reasons for the high resistance towards organophosphate pesticides in molluscs. All these studies are conducted in zebra mussel, *Dreissena polymorpha.* Dauberschmidt *et al.* (1997b) have stated that in the same species, the microsomes could biotransform the organophosphates, Thiometon and Disulfoton and that the process does not require the addition of reduction equivalents, unlike the vertebrates.

2.3.2. Effect of heavy metals on the defense system

Bivalves have a capacity to accumulate potentially toxic heavy metals in their tissues far in excess of the environmental levels, which indicates that they have evolved tolerance to these metals at cellular or sub cellular levels. But the heavy metals are known to exert inhibitory effects on several physiological processes (Cheng and Sullivan, 1984; Suresh and Mohandas, 1987; 1990b; c).

2.3.2.1. Metal detoxification

George *et al.* (1976) have studied the mechanism of iron uptake and excretion by light and electron microscopy in *M. edulis.* Ferric hydroxide, which occur as particulate matter, are engulfed by pinocytosis. The engulfment by the cell membrane is followed by the pinching off to form membrane-limited vesicles. These vesicles fuse with

the lysosomes to form larger secondary lysosomes or phagolysosomes. These are transported to the basal region of cell and are voided to extra cellular spaces to get engulfed by the amoebocytes in hemolymph for excretion through byssal threads. George *et al.* (1978) have reported that copper and zinc are detoxified by hemocytes by compartmentation into membrane bound vesicles and immobilized by combining copper with sulphur and zinc with phosphorus. The amount of copper and zinc in the hemolymph is very low, compared to that in the tissues. The metals are seen mostly in amoebocytes. The concentrations of metals are the highest in gills and mantle. Granular acidophils are responsible for detoxification of copper, whereas, granular basophils are responsible for detoxification of zinc.

Roesjadi (1980) has found that copper usually binds to protein of molecular weight 14 kDa and zinc to 10.5 kDa protein. These proteins are suggested to be metallothioneins. The amount of these low-molecular weight metal binding proteins, in the gills of *Protothaca staminea* increases several fold on exposure to mercury, cadmium or copper but not by zinc, when animals are exposed to the respective metals for 28 days.

Three species of oysters namely *O. edulis, Ostrea angasi* and *C. gigas* have hemocytes, which contain copper and zinc and have dual functions - defense and metal detoxification (Pirie *et al.*, 1984). *O edulis* has two other types of granulocytes with either copper or zinc. They are believed to have a function in metal detoxification.

Viarengo *et al.* (1987) have demonstrated the significance of lysosomes and thioneins in storage and detoxification of metal ions. An increase in the metallothionein mRNA accumulation during sub-lethal cadmium challenge is reported by Unger and Roesijadi (1996) in *C. virginica.*

2.3.2.2. Changes in the cellular factors

2.3.2.2.1. Changes in the hemocyte number

The most common change due to exposure to pollutant is increase in hemocyte count, however decrease in count and no changes in count, are also been reported.

George *et al.* (1983) have observed a decrease in the number of hemocytes in *O. edulis* exposed to cadmium.

Cheng (1988a) has reported that in *C. virginica,* the exposure to copper does not cause any change in the hemocyte number. According to him, copper does not stimulate hemopoiesis or suppress it. Cadmium has stimulatory effect on the hemopoiesis, since the percentage of hyalinocyte in the cadmium exposed oysters are significantly higher than that in the control. Copper is more lethal to hemocytes than cadmium.

Suresh and Mohandas (1990b) have exposed *S. scripta* to 1, 3, and 5 ppm of copper (Cu) at a salinity of 30 ppt and *V. cyprinoides* var. *cochinensis* to 0.15, 0.30 and 0.45 ppm Cu at a salinity of 15 ppt. The total hemocyte count was taken every 24 hours for 5 days. The result indicates that in *S. scripta,* the hemocyte count does not vary significantly at any concentrations. It is suggested to be because, the concentration range of Cu is far below the LC_{50} (greater than 10 ppm) or because of the low uptake of copper at high salinity, Cu ions might not have entered into the system in sufficient quantity to cause cell mortality, and/or to induce the involvement of hemocytes in the transportation of Cu ions to the sites of storage and excretion. In *V. cyprinoides,* the total hemocyte count decreases at all concentrations from 48 hours onwards. It is suggested to be because; the concentration of Cu is close to LC_{50} value of the species. It may be also because of the high uptake at low salinity. The Cu ions may have entered the system in sufficient quantities to cause cell mortality and/or to induce the involvement of the hemocytes in the transportation

of Cu ions to sites of storage and excretion, thereby, effecting significant fluctuations in total hemocyte count. It is presumed by the authors that under metal stress, the total hemocyte count at any given time period is resulting from a balance between recruitment and loss of the hemocytes into and out of the circulatory system.

According to Pipe and Coles (1995), increase in hemocyte count after exposure to pollutants may be either because of the proliferation of cells or due to the migration of cells from tissues to circulation. In the same way, the decrease in the hemocyte count may be because of lysis, diapedesis, less recruitment or movement of the hemocytes from circulation to the tissues. They have found an increase in number of circulating hemocytes in cadmium-exposed molluscs challenged with *Vibrio tubiashi*. Pipe *et al.* (1995) have found a significantly higher number of hemocytes in the *M. galloprovincialis* taken from areas polluted with heavy metals like manganese, chromium, nickel and mercury. Pipe *et al.* (1999) have reported that copper at a lower concentration could stimulate the defense mechanisms in *M. edulis*, which is depressed at higher concentrations. Fisher *et al.* (2000) have collected *C. virginica* from 16 sites polluted with different contaminants and analysed the defense mechanisms. According to them, trace amounts of heavy metals like copper, zinc, and tin significantly increase hemocyte density. This increased defense may be for the sequestration and detoxification of these metals from the system.

2.3.2.2.2. Changes in hemocyte profile

Apart from hemocyte number, the hemocyte profile also shows difference in response to heavy metals. In most cases, there is a reduction in the number of granulocytes; though a decrease in percentage of hyalinocytes is also reported.

Pickwell and Steinert (1984) have reported increase in the granulocyte count in copper exposed mussels.

Cheng (1988a) has found that, *in vivo* exposure of *C. virginica* to 1 ppm copper reduces the percentage of hyalinocytes and, exposure to 1 ppm cadmium increases the number of hyalinocytes. According to him, since granulocytes are more involved in phagocytosis, the increase in the hyalinocytes reduces phagocytosis.

According to Suresh and Mohandas (1990b), the molluscs exposed to pollutants will need increased number of granulocytes to remove overload of pollutants and pollutant laden particulate materials, since they play a significant role in the granuloma formation and phagocytosis.

Copper exposure has resulted in a significant reduction in the number of eosinophils compared to basophils (Pipe and Coles, 1995).

2.3.2.2.3. Changes in the hemocyte activities

2.3.2.2.3.1. Phagocytosis

Cheng and Sullivan (1984) have studied the effects of *in vitro* exposure of 10 heavy metal cations on the phagocytosis of polystyrene latex beads by hemocytes of *C. virginica*. They have observed that the phagocytosing ability varies with the different metals and also with the concentration of the metal ions. Exposure to 1 and 5 mg Cd^{2+}/L, 5 mg Co^{2+}/L, 1 mg Cr^{3+}/L, 1 mg Cu^{2+}/L, 0.5 mg Fe^{3+}/L, 0.5 mg Hg^{2+}/L, 1 and 5 mg Mn^{2+}/L, 1 and 5 mg Pb^{2+}/L, 1 mg Sn^{2+}/L and 1 and 5 mg Zn^{2+}/L caused no changes in the phagocytosing ability of the hemocytes. Exposure to 1 mg Co^{2+}/L, 5 mg Cr^{3+}/L, 5 mg Cu^{2+}/L, 1 and 5 mg Fe^{3+}/L, 0.1 mg Hg^{2+}/L and 5 mg Sn^{2+}/L resulted in significantly enhanced uptake of polystyrene spheres. Finally, *in vitro* exposure to 0.5, 1 and 5 mg Hg^{2+}/L caused inhibition of phagocytosis and increased cell death.

exposure models are representative of the *in vivo* exposure. According to them, further studies are required to find out, which metabolic pathways are affected, leading to a disruption of hemocyte homeostasis that subsequently induces immuno-suppression.

2.3.2.2.3.2. Changes in other hemocyte activities

A significant reduction in the release of super oxide by the hemocytes is reported by Pipe and Coles (1995) as a result of exposure to copper. Hemocyte locomotion and super oxide production are significantly increased as a result of exposure to trace amount of copper, zinc and tin in *C. virginica* (Fisher *et al.*, 2000). According to Pruski and Dixon (2002), there is a close resemblance in the effects of cadmium on mammals and molluscs. In the studies conducted in *M. edulis,* cadmium inhibits or delays the onset of apotopsis (programmed cell death); this removes one of the main defense mechanisms responsible for protecting the organism against neoplasia.

2.3.2.3. Changes in the humoral factors

Heavy metals also modulate the activity or the release of the enzymes.

The release of acid phosphatase into the serum in copper stressed bivalves is described by Cheng and Mohandas (1985). This involves recognition sites on the surface membranes. When the concentration of copper is higher, there is no significant increase at early time periods, which is suggested to be because of an early release of enzyme due to high concentration of the metal. The hyper synthesis is also believed to be inactivated by the higher concentration within a short period of exposure. This may be the reason for the significant fall in the amount of the enzyme during 120 hours. The lysosomes are believed to include a variety of enzymes and the heterogeneity in the size and shape indicates the different functional activities of the lysosomes.

Jyothirmayi and Rao (1988) have reported that in *Lymnaea luteiola* the alkaline phosphatase activity is unaffected by ferric chloride and silver nitrate.

The study by Cheng (1989) in *C. virginica* has revealed that cadmium inhibits the release of lysozyme into serum and copper inhibits the release of acid phosphatase into serum. Copper stimulates the intracellular synthesis of β-glucuronidase in the hemocytes, but inhibits its release into serum. Serum lysozyme is significantly reduced by the cadmium exposure but copper does not cause any significant difference. Copper significantly reduces serum acid phosphatase, but cadmium does not cause any significant difference. Serum β-glucuronidase is significantly reduced by copper exposure, but cellular β-glucuronidase is significantly increased.

Suresh and Mohandas (1990c) have studied the activity of the lysosomal marker enzyme, acid phosphatase, in copper stressed *S. scripta* and *V. cyprinoides* var *cochinensis*. *S. scripta* was exposed to 1, 3, and 5 ppm and V. *cyprinoides* var *cochinensis* to 0.15, 0.30 and 0.45 ppm of Cu. These dosages were the sub-lethal doses for the respective species. The study has revealed that the activity of acid phosphatase vary from species to species. Copper ions can cause destabilization of the lysosomal membrane and the consequent release of the enzyme into the hemolymph. Copper inhibits the activity and synthesis of the enzyme. The increase in the amount of acid phosphatase within 24 to 48 hours in the serum is believed to be because of the release of the enzyme from lysosomes, due to the damage caused to lysosomal membrane by copper ion. The nonsignificant result after 72 hours is suggested to be due to the inactivation of hyper synthesis of enzyme.

According to Cheng (1990), the *in vivo* exposure to 1 ppm cadmium causes a decrease in the release of lysozymes from

granulocytes to the serum. The same concentration of copper does not change the release of lysozymes. The exposure to copper inhibited the release of acid phosphatase into serum, but cadmium does not cause any change in the release of acid phosphatase.

2.3.2.4. Histological changes

Histopathological observation in *M. edulis* exposed to 5 -10 μg/L copper shows changes in the digestive diverticulae. As compared to control, the digestive cells of digestive tubules of five mussels show loss of granules and extensive vacoulation in the cytoplasm. The tubules are dilated and contain pink staining exudate in their lumen. Some have yellow to red granules in the digestive cells. There is loss of cilia and erosion of cytoplasm of the ciliated columnar cells. The ducts are lined with non-ciliated cuboidal or squamous epithelium, instead of ciliated columnar epithelium. Areas of erosion of cytoplasm in the epithelial cells of stomach wall are noticed and instead of tall ciliated columnar epithelium, there are cuboidal to squamous cells. The muscles show varying degrees of myodegeneration and atrophy of muscle bundles. In one animal, calcified nodules are found among muscle bundles (Calabrese *et al.*, 1984.)

Sunila (1986) has reported that LC_{50} of copper for *M. edulis* at 20°C is 0.4mg/L. After 24 hrs of exposure to 0.2 to 0.8mg/L of copper, the acute histological changes in the gills are swelling of endothelial cells and the detachment of abfrontal cells. When they are exposed to cadmium (1-8mg/L), there is dilation of branchial vein. The gills are invaded by hemocytes indicating inflammatory reactions in both cases. It is suggested that lysosomes play an important role in the detoxification of these metals. In copper exposed gills, signs of intoxication are more easily observed in the abfrontal than in the frontal ends of filaments. The abfrontal cells are completely detached from

chitinous rod and lacked abfrontal cilia. There are many granulocytes inside the vein and the interfilamentar junctions are uncoupled. Interlamellar hemolymph vessels are obstructed by hemocytes. The frontal cells of two neighbouring filaments form cellular connections. There is interlamellar fusion also.

Sunila (1988) studied the acute effects of heavy metals potassium, cadmium, lead, copper, cobalt, iron and silver, each at a concentration of 5 ppm, at a salinity of 7 ppt on the gills of the mussel, *M. edulis*. The animals were exposed to the metals for 24 hours. Potassium sulfate induced an inflammatory reaction with granular hemocyte infiltration. The branchial vessels were slightly dilated and cavities were formed under post lateral cells. Cadmium chloride produced the same observation as potassium sulphate. The post lateral cells were enlarged. Apart from the above-mentioned changes, in specimens exposed to copper chloride, the abfrontal cells became detached from the chitinous rod, forming a cavity underneath. Granular hemocytes occurred under postlateral and abfrontal cells, often migrating through the epithelia. The endothelial cells were edematous and so, the epithelium appeared cuboidal. The cytoplasm of the endothelial cells was granular. With copper nitrate, the changes resembled those after copper chloride exposure, but were more severe. The endothelial cells appeared swollen and loosened from chitinous rod; however, they were still attached to it by cytoplasmic distensions. Also, frontal cells were sloughed from the chitinous rod, so that, nuclei were situated in the apical part of the cells instead of in the middle. Lead oxide produced inflammatory reaction and the branchial vessels were dilated and filled with granulocytes. Histological changes occurred in the frontal part of the gills. The lateral cells were detached from the chitinous rod and totally sloughed. Where they were still present, they did not have lateral cilia. The endothelial cells

were often sloughed; frontal cilia were unsynchronized and had mucus on their surface. No effect was produced by cobalt chloride. The post lateral cells were slightly swollen in the specimens exposed to ferric chloride. The changes caused by silver nitrate were almost similar to that for copper chloride. In all these cases, histological changes were observed in endothelial cells, thus affecting the respiration and also in ciliated cells, thus affecting the food transport.

Gill is the primary site of accumulation and toxicity of copper in the abalone *Haliotis rufescens*, followed by the foot and the adductor muscle. The reduction in the metabolic rate observed in the muscle as a result of exposure to copper may be due to mucus accumulation or cytological changes in the gills (Viant *et al.*, 2002).

2.4. Effect of infection by a pathogen/parasite on the defense system

2.4.1. Changes in the hemolymph factors

In vertebrates, the molecular signals between stimuli and host responses are either cell factors (enzymes) or bacterial factors (toxins, wall components). In *C. virginica*, chemotactic migration of the hemocytes is mediated by secreted bacterial products (Cheng and Howland, 1979).

According to Gallin (1980), the stimuli to induce defense reactions would originate at sites of host-pathogen contact or at sites of bacterial entry in tissues.

Combes and Cheng (1986) have reported that exposure of *B. glabrata* to *Aeromonas hydrophila* resulted in higher death rates, when there is crowding.

The infectious diseases in the molluscs, including parasitic diseases depend on many factors such as biology of the host, efficiency of the host defense systems, mechanisms of the agent for attachment to the host, the nutritional and other requirements of the agent, and factors which favour its multiplication (Cheng, 1989).

Based on the studies conducted on leukocyte response of *B. glabrata* to infection by trematode *Schistosoma mansoni* and *Echinostoma lie,* Mounkassa and Jourdane (1990) have suggested that the hemocytes take part in nutrient digestion and transport, wound repair and excretion.

The quality of response of the internal defense system during the period following the challenge is a major factor for the success or failure of infective process. Here, the virulence of the bio-aggressor and its interaction with the host play an important role. The toxins secreted by bacteria also play an important role in eliciting the host defense system (Oubella *et al.,* 1996).

2.4.1.1. Changes in cellular factors

2.4.1.1.1. Changes in the hemocyte number

Pauley *et al.* (1972) have conducted studies on the bacterial clearance in California sea hare, *Aplysia californica* using bacteria like *Gaffakya homari, Serratia marcescens, Micrococcus aquivivus, Pseudomonas* sp, and a gram negative rod, which are injected to pedal sinus of the host. A reduction in the number of circulating hemocytes in response to challenge by bacteria is noticed.

There is only very little leukocytic response to *S. mansoni* infection by the susceptible strain of *B. glabrata,* whereas, the resistant strain shows a lot of defense responses to infection. These responses include a considerable decrease in number of circulating hemocytes at 1 hr post exposure followed by a gradual increase to control state by 24 hr. The decrease in number is correlated to sporocyst encapsulation reactions (Granath and Yoshino, 1983a).

In *L. luteiola,* the hemocyte number does not change at the time of infection with trematode parasite, *Prosthogonimus* sp. (Jyothirmayi and Rao, 1988).

Suresh and Mohandas (1990a) studied the effect of sham injection and challenge with *Vibrio alginolyticus,* compared to the untampered control in two clam species namely, *S. scripta* and *V. cyprinoides* var. *cochinensis.* For *V. alginolyticus* challenge, 0.02 ml of bacterial suspension (1x 10^8 live cells from fresh culture, which was washed thoroughly and suspended in 0.02 ml of sterile 2% saline) was injected into the foot gonad region of clams. For sham injection, 0.02 ml of sterile saline was injected. The total hemocyte count was taken at 3, 6, 12, 24, 48, 72, 96 and 120 hours. Compared to the untampered controls, the total count was high both in the saline and *Vibrio* injected ones. The total counts in the saline controls were higher at the early time intervals. In the *Vibrio* injected ones, the count was higher both at early (6 and 12 hr) and later (48, 96 and 120 hours) periods in *V. cyprinoides* var. *cochinensis,* but only at 48 hours in *S. scripta.*

An increase in the number of circulating hemocytes, 72 hours post- challenge with pathogenic bacteria (*Vibrio* P1) in two clam species, *R. philippinarum* and *R. decussatus* is reported (Oubella *et al.,* 1993). In these species, the number of the circulating hemocytes decreases significantly, a week after starvation. It is suggested that the reversible migration of hemocytes from the tissues to the circulatory compartment or vice versa explains these variations.

Oubella *et al.* (1996) have studied the hemocytic response in the Manila clam, *R. philippinarum* after inoculation with different bacterial species of *Vibrio.* They have used live and heat killed *Vibrio* P1, the causative agent for Brown ring disease, *Vibrio anguillarum* and *Vibrio pelagius.* There was a significant increase in the total count three days after challenge with live *Vibrio* P1. But there was no response with heat-killed bacteria, *V. anguillarum* and *V. pelagius.* They found that a concentration of 10^6 bacteria/ml was required to induce host response.

There was no host response at a concentration below this. The elevated number of circulating cells in *R. philippinarum* on exposure to *V. pelagius* and *V. alginolyticus* is proposed to be the result of mobilization and consecutive migration of the resident hemocytes from the tissues towards hemolymph compartments in response to the host-pathogen interactions. It is believed to be so, because of the open circulatory system of the molluscs. It is also suggested that killing by hemocytes might have altered the pathogenecity of the bacteria and the adherence capacity of the bacteria might have lost. Concerning *V. pelagius* and *V. anguillarum*, which are pathogenic only towards larval molluscs, the epithelial borders of the pallial cavity would be efficient barriers to stop or eliminate some microorganisms.

Paillard *et al.* (1996) have reported a decrease in the total hemocyte count in *C. virginica* affected by juvenile oyster disease.

2.4.1.1.2. Changes in hemocyte profile

In *R. philippinarum*, Oubella *et al.* (1996) have reported a significant decrease in the hyalinocyte number and a concomitant nonsignificant increase of neutrophilic granulocytes, when it was challenged with *V. pelagius* and *V. alginolyticus*.

Paillard *et al.* (1996) have reported that the juvenile oyster disease in *C. virginica* is characterized by the increase in percentage of granulocytes.

2.4.1.1.3. Changes in hemocyte activities

2.4.1.1.3.1. Phagocytosis

The phagocytosis does not ensure degradation of bacteria (Rogers, 1960). Hemocytes from *A. californica*, injected with *S. marcescens* contain numerous large granules, which are assumed to be phagocytosed bacteria. The accelerated secondary clearance of bacteria in this animal is not associated with an increase in the agglutination titers. This is believed to be cell mediated. This response is noted even 1 month

after the primary challenge. Active phagocytosis is accompanied by a reduction in the number of circulating hemocytes. It is suggested that this phagocyte might migrate across epithelial borders leading to its elimination (Pauley *et al.*, 1972).

In molluscan phagocytes, there are at least three molecular sites at which signals can be recognized. One recognition site is on the cell membrane. When a chemically recognizable signal, e.g., a molecule associated with the surface of a bacterium, binds with the cell membrane recognition site, the movement of a transcytoplasmic messenger is initiated, which in turn, is recognized by a second recognition site, which is associated with the nuclear membrane of the phagocyte. Activation, i.e. molecular binding of the nuclear membrane binding site with the transcytoplasmic messenger molecule, initiate those intranuclear events that lead to the synthesis of the lysosomal enzyme on ribosomes directed by DNA-mRNA-tRNA sequence. Subsequently, the lysosomal enzyme is packed by the golgi membrane into lysosomes. Finally, a third recognition site is associated with the lysosomal membrane. The activation of this site triggers the migration of lysosomes to the cell surface where the lysosomal enzymes are released into serum by degranulation. Therefore, if the dosage of the challenging bacteria is less, there will be additional sites available for further challenging agents and so; there may be elevations of serum acid phosphatase at more time intervals. When the dosage is very high, all the recognition sites will get saturated and hence, further sites are not available for any challenging agents. So, only one peak of enzyme activity is there, which is 2 hours post challenge. If the parasite does not include the specific surface molecular configuration required to bind to the hemocytes, it cannot activate the host's hemocytic surface recognition site (Cheng and Mohandas, 1985).

Jourdane and Cheng (1987) who have studied the cellular reactions to allografts in *B. glabrata*, suggested that encapsulation of non-self, too large to be phagocytosed, occurs in two phases. (1) The foreign body emits a chemical signal which attracts an initial wave of the host hemocytes (primarily granulocytes) and (2) these hemocytes secrete lysosomal hydrolases that alter the nature of foreign body surface and emits a second signal, which attracts a second wave of host reaction cells. The recognition as nonself is followed by the formation of a granuloma composed of granulocytes from 24 hours post transplantation onwards. The capsules formed as a result of encapsulation is composed of reaction cells as well as intercellular fibrous units.

The heterotopic isograft is recognized as self by the defense system of mollusc. There is an initial wave of host reaction, which is due to the signal send by the damaged surface cells of the isografts, caused during the preparation of the isografts. These cells, however, do not release enzymes and therefore there is no second wave of host reactions. By about 72 hours, there is some regeneration of isografts. In the initial phase, larger hemocytes and elongate hemocyte are involved, but as the cells are recognized as self, these cells undergo hypertrophy (Cheng and Jourdane, 1987).

Beckmann *et al.* (1992) have compared the hemocytes of *M. arenaria* from normal and those affected by the disease, hematopoietic neoplasia, for their ability to phagocytose the yeast cells. Diseased hemocytes differ from the normal hemocytes by their high nucleo-cytoplasmic ratio. Examination by scanning electron microscope reveals that the yeast cells adhered only to the hemocytes of the normal animals and not to diseased hemocytes. The diseased hemocytes are unable to adhere and ingest yeast cells. Histochemical analysis shows

diseased hemocytes to have higher than normal acid phosphatase, nonspecific esterases and β- glucuronidase activity.

Ford *et al.* (1993) have reported the failure of the hemocytes from *C. virginica* and *C. gigas* to phagocytose *Haplosporidium* plasmodia, but it is readily phagocytosed by hemocytes of the mussel *Gaukensia demissa.*

2.4.1.1.3.2. Other hemocyte activities

Cheng and Rudo (1976) have studied the chemotactic attraction of *C. virginica* hemocytes to *Staphylococcus lactus.* Directional migration of hemocytes towards the bacteria is observed 4 hrs after incubation and, the number of migrating cells increased as the incubation temperature increased.

In *B. glabrata,* the acid phosphatase activity of hemocytes decreased by 1 hr post exposure to *S. mansoni,* followed by return to normal values by 24 hrs & 2 weeks. The peroxidase activity increased by 1 hr post exposure reached peak by 24 hrs and decreased to normal values by 2 weeks. The nonspecific esterase positive cells decreased from 0 to 12 hrs and returned to normal values by 24 hrs (Granath and Yoshino, 1983a).

Vibrio parahaemolyticus inoculated into the gastropod *Heminerita japonica* is cleared within 7 days, whereas, *E. coli* is able to survive upto 14 days (Kumazawa *et al.,* 1991).

Another method to destroy pathogen by bivalve hemocyte is by the release of various toxic oxygen molecules. This is measured by luminol dependent chemiluminescence and also by NBT (nitro blue tertrazolium) reduction. The release of reactive oxygen metabolites decreased at higher level of exposure, but increased at lower level of exposure. An antioxidant molecule produced by *S. mansoni,* which

scavenge the hemocyte derived super oxide anions was identified by Connors *et al.* (1991) in *B. glabrata.*

There is significantly higher production of reactive oxygen intermediate by individuals of *C. virginica,* which are heavily infected with *P. marinus* compared to less or moderately infected individuals. In the heavily infected individuals, there is higher number of circulating hemocytes. The resultant oxygen load may participate in the pathogenesis of the disease (Anderson *et al.,* 1992).

Cell-mediated cytotoxicity assays on poly-L-lysine treated plastic are used to compare the kinetics of the parasite (*S. mansoni*) killing, in the hemocytes of two susceptible and two resistant host strains of *B. glabrata* (Boehmler *et al.,* 1996). There are marked differences between the two resistant snail strains suggesting different mechanism of parasite recognition and killing, or both.

2.4.1.2. Changes in humoral factors

2.4.1.2.1. Changes in protein profile

Cheng (1969) have found 15 protein bands in the pond snail, *Helisoma duryi* and reported a great deal of individual variability. But there is a significant initial decrease in a rapidly migrating protein fraction in snails challenged with bacteria, which may be albumin.

Feng and Canzonier (1970) have found only four bands in the hemolymph protein of *C. virginica,* but also have reported a change in the ratio of two of the fractions that is correlated with infection by *Minchinia velsoni.*

The hemolymph protein of *B. glabrata* declines as a result of infection by *S. mansoni* (Lee and Cheng 1972).

Ford (1986a) has compared the hemolymph proteins from susceptible and resistant oysters, *C. virginica,* exposed to parasite, *H. nelsoni* (MSX). The total protein drop up to one - third of the normal level, sharply, in proportion to infection, in the animals with systemic infection.

In resistant ones, where infection is confined to the gill only, there is no change in total hemolymph protein. SDS-PAGE shows 3 rapidly migrating proteins of molecular weight between 25 kDa and 75 kDa in all the oysters. There are about 12 proteins with molecular weight upto 500 kDa. These proteins are present in much lower concentration and their frequency and clarity vary considerably, although they are infrequent in the oysters with moderate to high infections. The number of bands shows much individual variability and only a few showed all the 15 bands. The electrophoretic pattern does not show any correlation with infection or genetic pattern. The bands are less prominent in the oysters with moderate to heavy infections, when compared to oysters with light or no infections. It is suggested that, the depletion of certain metabolic substrates such as circulating protein and/or the interruption of biosynthesis pathway by the parasite is the cause of the death in infected oysters. *H. nelsoni* is a parasite against which, cellular defense is not effective, because there is no significant difference in phagocytosis. However, there are changes in the serum composition between the resistant and the susceptible strain, which indicates that non-cellular mechanisms may be critical in the defense responses against parasitism by *H. nelsoni*.

The hemolymph protein of *B.glabrata* infected with *Echinostoma paraensi* is significantly higher compared to the non-infected animal. The hemolymph contained particulate matter of about 190-200 kDa and 80-120 kDa molecular weights. There are no changes in the number of bands in the SDS-PAGE. In M-line *B. glabrata,* there are changes in soluble polypeptides, and the diffused band at around 100 kDa is more intense compared to the non-infected snail. This difference is less in 10-R2 strain of *B. glabrata.* The differences between infected and non-infected snails are quantitative rather than qualitative. As the infection further

progresses, there is a significant reduction in the hemolymph protein. The particulate materials in the hemolymph may be soluble antigen precipitated by the hemolymph, or the remnants of lysed host or parasite cells (Loker and Hertel, 1987).

In *C. virginica,* the hemolymph protein content is not affected by infection with *P. marinus* (Chu and Peyre, 1989).

The hemocytes of the gastropod, *Nerita albicilla* are able to attach and clear *V. parahaemolyticus* in the presence or absence of serum, whereas, the hemocytes of the mollusc *Clithon retropictus* are able to attach the bacteria only in the presence of serum (Kumazawa *et al.,* 1991).

According to Hubert *et al.* (1996), the plasma of *M. galloprovincialis* has cytotoxic activity against both vertebrate and protozoan cells. But it is not sensitive against *E. coli* and *V. alginolyticus.* Purification by anion exchange chromatography, followed by gel filtration, reveals a cytotoxic polymeric protein of 320 kDa, which binds to the target cell membrane. The hemolymph of the European oyster, *O. edulis* and the Pacific oyster, *C. gigas* shows anti-bacterial activity against both gram positive and gram-negative bacteria. When tested against *V. alginolyticus,* the hemocyte lysate shows more anti-bacterial activity compared to serum plasma. This anti-bacterial activity is reduced by mechanical stress and injection. The authors are of the opinion that the sensitive immuno-defense mechanisms of bivalve molluscs can greatly aid in the development of aquaculture systems by transgenics.

2.4.1.2.2. Changes in the enzyme profile.

The lysozyme levels in serum fraction of hemolymph decrease in *C. virginica* infected with *M. velsoni,* but increase in oysters infected by *Bucephalus* sp. (Feng and Canzonier, 1970).

During phagocytosis of bacteria by the hemocytes, the lysozyme is released into the serum following degranulation in hard clam, *M. mercenaria* (Cheng *et al.*, 1975).

In *B. glabrata,* the acid phosphatase values in the serum increase upto 24 hours after exposure to *S. mansoni* and then reach normal values by 2 weeks (Granath and Yoshino, 1983a).

According to Cheng and Mohandas (1985), the release of the acid phosphatase from hemocytes into serum, is at its peak at 2 hours after challenge, irrespective of challenging dose in *B. glabrata* challenged with *B. megaterium.*

In *L. luteiola,* the increase in the acid phosphatase activity of the hemolymph at the time of infection with trematode parasite, *Prosthogonimus* sp., is due to release from hemocytes rather than from digestive glands. The alkaline phosphatase activity is unaffected by the infection (Jyothirmayi and Rao, 1988).

The occurrence of red tide by *Gymnodinium* sp. and parasitism by *P. marinus* increase susceptibility of *C. virginica* to disease, but there is no correlation between lysozyme and infection by *P. marinus* (Chu and Peyre, 1989).

2.4.1.2.3. Changes in other humoral factors

A. californica is able to clear bacteria like *G. homari, M. aquivivus* and *Pseudomonas* sp. from its hemolymph within 8 hours, where as, *S. marcescens* is not cleared even after one month. The clearance occurs rapidly at high temperatures and is accelerated by previous exposure to the bacterium. This indicates a kind of primitive anamnestic response exhibited by the species. Concomitant with bacterial clearance, there is a depression in the agglutination titers. The agglutination titers return to the normal values even after 3 challenges with bacteria. Although serum does not exhibit any lytic activity, opsonic factors, which enhance phagocytosis

of chicken red blood cells, are found. A mechanism of bacterial clearance involving agglutinin acting as opsonins is hypothesized (Pauley *et al.*, 1972).

Tripp (1974) has failed to demonstrate a reaction of oyster agglutinin with a variety of bacteria.

Boswell and Bayne (1984), in a study of the hemagglutinin in the strains of *B. glabrata* resistant or susceptible to *S. mansoni,* have concluded that the agglutinin is probably not important in this particular host-parasite system. They have purified an agglutinin with a molecular weight of 89-115 kDa, which is present in the M-line *B. glabrata,* and absent in the 10-R2 strain, which could agglutinate rabbit erythrocytes.

The cytokine like molecule immunoreactive TNF alpha, which is present in hemocytes and hemolymph of *B. glabrata,* decreases at the time of *S. mansoni* infection (Ouwe-Missi-oukem-boyer, 1994).

According to Vasantha (1996), three types of serum lectins have been identified in the invertebrate hemolymph. One is homologous to the soluble C-type vertebrate lectins that bind to mannose, the second one homologous to S-type vertebrate lectins binding to galactose and the third type homologous to phase reactants such as C-reactive proteins. These serum lectins may contribute as a carbohydrate based recognition system for potentially pathogenic microorganisms.

When bacteria are at the ratio of 10:1 to the hemocytes of *M. edulis,* which are attached to a plastic surface, within 2-3 hours, rounding of the hemocytes occurs due to bacterial toxicity and for this to occur, the presence of serum is essential. The reaction does not occur in seawater. The factor present in the serum is believed to be an opsonin. Among 226 different species tested, *V. alginolyticus* and *V. anguillarum* are

found to be more toxic. The bacteria after being ingested, release toxins, or, some factors in their cell membrane, which are responsible for toxicity (Loane and Birkbeck , 1999).

Anti-*B. megaterium* and anti-*P. marinus* activity are tested in the bivalves, *C. virginica, M. edulis* and *G. demissa* plasma. It is found that, there is strong anti-bacterial activity in the plasma of *C. virginica* and *M. edulis.* But it is not detected in *G. demissa.* The anti-protistan activity is very less in *C. virginica* plasma compared to that in other two species. This is the reason for low pathogenicity of *P. marinus* in these two species. Bactericidal activity is found in hemocyte lysate from all the bivalves, suggesting the hemocytic origin of cytotoxic humoral factors. The agents other than lysozyme may be playing a significant role in these activities (Anderson and Beaven, 2001).

2.4.2. Histological changes

Experimental inoculation of the larval American oyster by *Vibrio* sp. caused detachment of the mantle cells and general atrophy (Elston and Leibovitz, 1980).

The granular hemocytes are seen as concentric layers around foreign bodies (Balquet and Poder, 1985). Such granulocytic reactions are clear in *Bonamnia ostreae* infection (protozoan disease) of *O. edulis.* The infiltration of hemocytes around the digestive tracts and gills are characteristic sign of the disease. The hemocytes transport the phagocytosed parasites through out the body. The parasites, which come out as a result of hemocyte lysis, are infectious to new cells. Thus, this disease is also termed as hemocytic parasitosis.

Elston *et al.* (1987) report an inflammatory bacteraemia in the Pacific oyster, *C. gigas* caused by systemically distributed, branching, gram-positive, acid-fast bacteria suggestive of an actinomycete. Clusters of bacteria elicit a marked inflammatory response.

Glycogenic degeneration of host vesicular cells occurs in advanced infections and results in the formation of characteristic nodules.

The histological examination of moribund *B. glabrata* infected by acid-fast bacilli by haematoxylin and eosin staining revealed large cyst-like structure surrounded by moderate, active fibrosis. Within the mass, smaller, round granulomata divided by fibrous tissue and separated by mesenchymal cells were present. Each granuloma contained numerous phagocytic amoebocytes, containing fine granular to rod shaped eosinophilic materials that were PAS positive and strongly acid fast by Zeil-Neelsen staining. The phagocytic cells appeared to migrate towards and through the overlying epithelium (Uhazy *et al.*, 1988).

In *Lymnaea truncatula* and *Lymnaea glabra,* there is a proliferative response of amoebocyte-producing organ, located between the kidney and the pericardium when infected by *Fasciola hepatica* (Rondelaud *et al.*, 1988).

Friedman (1991) has reported that summer mortality in *C. gigas* due to Pacific Oyster Nocardiosis (PON) by bacteria *Nocardia* sp. is actually caused by elevated temperature and nutrient levels during the summer months. Physiological stress caused by rapid metabolism and gonad formation is also suspected as the reason. The bacteria invoked hemocytic infiltration in the affected areas like adductor muscle, connective tissue around digestive tracts, gills and mantle. It is suggested that this bacterium is an opportunistic pathogen rather than a primary pathogen. In the winter disease of *C. commercialis* in Australia, the granulocytes are involved in the formation of focal abscesses with necrosis of connective tissue and also infiltration of hemocytes.

The infestation of *C. virginica* gonad by the metacercarieae of a trematode results in hemocytic infiltration in the gonadal area (Fisher *et al.*, 2000).

3. MATERIALS AND METHODS

3.1. Maintenance of *Crassostrea madrasensis*

The edible oysters, *C. madrasensis* (mean size 6.4 ± 1.2 cm x 4.3 ± 0.8 cm) were collected from the backwaters of Cochin around Vypeen island. They were cleaned and maintained in filtered and aerated seawater at a salinity of about 12 ppt in 2 tanks of one-ton capacity each, as stock. The animals were fed with microalgae, *Chaetoceros* sp., up to satiation. The tanks were cleaned, faecal matter and dirt were siphoned out and 50% water was exchanged daily.

The experiments were carried out in tanks of 50 liters capacity at a salinity of 12 ppt and through out the course of study, the animals were fed algae *ad libitum*. Filtered and aerated seawater and fresh water were used for water exchange and salinity adjustments. Every day the experimental tanks were cleaned and 50% of water was exchanged. Water quality parameters were checked frequently and were maintained at optimum.

3.2. Hemolymph factors of *C. madrasensis*

3.2.1. Hemolymph collection

The hemolymph was collected following the method of Foley and Cheng (1972), Chu and Peyre (1989) and Chen (1996). Hemolymph was collected from adductor muscle sinuses. A notch was filed on the dorsal aspect of the shell valve, adjacent to adductor muscle. About 0.5ml to 2ml of hemolymph was collected from the adductor muscle of each animal using a 27-gauge needle attached to a 5-ml sterile syringe and it was immediately stored at 4°C. Since the molluscan blood does not clot (Noda and Loker, 1989) anti-coagulants were not used. The hemolymph was centrifuged at 5000 rpm for 10 minutes at 4°C to remove the cells and supernatant was used for serum studies.

3.2.2. Cellular factors of the hemolymph of *C. madrasensis*

3.2.2.1 Characterization of hemocytes

3.2.2.1.1. Light microscopic studies

The characterization of hemocytes was done by Geimsa staining (Bayne *et al.*, 1979). The cells were concentrated by centrifugation at 1500 rpm for 10 minutes at 4°C and these were allowed to form monolayer on a glass slide for 45 minutes at 25°C. This was fixed using 10% methanol for 15 minutes, air-dried and stained in Geimsa (diluted 10 times with double distilled water and filtered before use) for 20 minutes, differentiated in acetone and observed under a light microscope.

3.2.2.1.2. Ultrastructural studies

3.2.2.1.2.1. Transmission electron microscopic studies

The ultrastructural studies were done using the method of Rasmussen *et al.* (1985) and Hinsch and Hunte (1990) with modification. The hemolymph withdrawn from adductor muscle was added into 3 times the volume of 3% glutraldehyde in 0.1 M sodium cacodylate buffer containing 12% glucose, (pH-7.8); mixed well and kept at a temperature of 4°C overnight. The hemocytes were then washed 3 times in cacodylate buffer and pelletised by centrifuging at 5000 rpm for 10 minutes at 4°C. The pellet was post fixed in 1% osmium tetroxide for 2 hrs, again washed in cacodylate buffer three times and pelletised. Two percent agarose prepared in cacodylate buffer of pH 7.8 at 60°C was added to the pellet by keeping the eppendorf tube in a water bath at 50°C, and mixed thoroughly and the hemocyte-dispersed-agarose was allowed to solidify. The solidified agarose cube was cut into very small pieces and dehydrated through an ascending series of acetone. The samples were further processed for transmission electron microscopy. It was infiltrated and embedded in Spurr's low viscosity plastic; ultra thin sections (60-90

nm) were cut on a LKB ultra microtome; stained with uranyl acetate and lead citrate and examined under Transmission Electron Microscope (Hitachi H-600).

3.2.2.1.2.2. Scanning electron microscopic studies

For scanning electron microscopic studies, freshly collected hemolymph was centrifuged at 1500 rpm for 10 minutes. The pellet formed was dispersed uniformly in 100µl of serum. The hemocyte suspension was poured on a clean sterile cover glass and incubated at 25°C for 45 minutes. After incubation the monolayer was rinsed with 2% seawater. The cover slip was cut into small pieces and each piece with the monolayer of hemocytes was fixed in 3% glutraldehyde in 0.1 M sodium cacodylate buffer containing 12% glucose, (pH-7.8) for 2 hours. It was washed 3 times in cacodylate buffer. The monolayers were then post fixed in 1% osmium tetroxide for 2 hrs and again washed with cacodylate buffer, three times. The cover slips were dried, coated with gold and examined under Hitachi-H- 6010 A scanning system.

3.2.2.1.3. Cytochemical studies

In the cytochemical studies, the enzymes such as acid phosphatase, prophenol oxidase and peroxidase were demonstrated in the hemocytes using the following procedures.

3.2.2.1.3.1. Acid phosphatase

As per the methods described by Sanders (1974), air-dried smears were fixed in formalin – acetone (20% formalin in 50% acetone) for 1 minute at 0^0 C and rinsed in running water. The fixed smears were incubated at 37^0 C for 1 hour in freshly prepared and filtered acid phosphatase substrate solution containing Fast Blue BBN.

3.2.2.1.3.2. Prophenol oxidase

Hemocyte smears were fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 7.4) for 1 hour at 4°C and washed 3 times (for 15 minutes each) with 0.1M phosphate buffer (pH 7.4). The slides

were then incubated in 0.1% L-dopa (dihydroxyphenylalanine) prepared in 0.1M phosphate buffer with 2% sodium chloride, for 90 minutes at 30° C, and counterstained with dilute Giemsa. Control slides were incubated in 0.1 M phosphate buffer with sodium chloride, but without L-dopa. (Smith and Söderhäll, 1983).

3.2.2.1.3.3. Peroxidase

Air dried smears were fixed in 10% alcoholic formalin for 60 seconds and rinsed in distilled water for 15 to 20 seconds. Slides were then incubated in myeloperoxidase incubation mixture for 30 seconds, washed briefly in running tap water, dried and counterstained with Giemsa. Control slides were incubated in 0.1 M phosphate buffer (Sanders, 1974).

3.2.2.1.4. Maintenance of the hemocytes in artificial media

A study was conducted to find out the suitability of different media to support or maintain the hemocytes of *C. madrasensis*. Four types of media were used. They were 1) Hank's balanced salt solution (HBSS), 2) HBSS with 10% foetal calf serum (FCS), 3) Tissue culture media M 199 and 4) M 199 with 10% FCS. The HBSS, FCS and M 199 were obtained from HIMEDIA, Mumbai, India. The media were prepared as follows.: One vial (9.76 gm HBSS/ 9.6 gm M 199) of entire dehydrated media was added to 900 ml of double glass distilled water and stirred until it was dissolved. To the HBSS medium, 0.35 g and to the M 199 medium, 2.2 g of sodium bicarbonate was added. To each of the media, 5 units of heparin and 100 µg of streptomycin were added. The pH was adjusted to 7.4 and made up to 1000 ml using double distilled water. The media 2 and 4 were prepared by adding 10% FCS under sterile conditions. The media were sterilized by filtration through millipore 0.22µ membrane filter and stored in dark at 4°C.

The hemolymph (0.5 ml) was withdrawn from the adductor muscle sinus into a sterile hypodermic syringe with 0.5 ml of the

media. It was then transferred into sterile tubes in three replicates. The tubes were incubated at 18°C.

The viability of cells was tested using trypan blue dye exclusion technique. Trypan blue (0.1 %) was used for staining. The cells with stained nuclei were considered dead. Viable cells were counted in a hemocytometer. The total viable hemocyte count was taken at 0 hr, 24 hrs, 48 hrs and 72 hrs.

3.2.2.2. Total and differential hemocyte count:

The total and differential hemocyte counts were estimated according to the method of Nakayama *et al.* (1997). One milliliter of hemolymph was mixed with 10 µl of 0.3% of May-Gruenwald's eosin-methylene blue solution and was loaded onto a hemocytometer. It was incubated in a moist chamber for 40 minutes and observed under a phase contrast microscope. The number of cells in the four large squares was counted and the total count was expressed as mean number of cells in each of the four squares x 10^4 cells/ml of hemolymph.

For differential count, a total of 200 cells were counted and the percentage of each type of cells such as granulocytes, semigranulocytes, and hyalinocytes was calculated.

Percentage of each type of cells = Number of each type of cells x100/200

3.2.2.3. Phagocytosis

Finely ground yeast was used for phagocytic studies (Bayne *et al* 1979). The yeast cells were inactivated in 10% formaldehyde for 24 hrs. Then it was washed 3 times with filtered 2% seawater. Sufficient quantity of 2% sterile seawater was added to have a count of 4 x 10^6 cells per ml.

Hemocyte monolayers on glass slides were prepared as in the early experiment. The monolayer was rinsed with 2% seawater and incubated with yeast suspension in 2% seawater (Cheng and Rudo,

1976) for 60 minutes at 25° C. The slide was rinsed three times with 2% seawater, fixed in 10% methanol for 15 minutes, air dried and stained with Giemsa (diluted 10 times with distilled water and filtered before use) for 20 minutes, differentiated in acetone and mounted in DPX. The cells were observed under light microscope. About 200 cells were counted on each slide. The phagocytic index and endocytic index were calculated as given below.

$$\text{Phagocytic index} = \frac{\text{Number of hemocytes that showed phagocytosis}}{\text{Total number of hemocytes counted}} \times 100$$

$$\text{Endocytic index} = \frac{\text{Total number of yeast cells engulfed}}{\text{Total number of hemocytes counted.}}$$

3.2.3. Humoral factors of hemolymph of *C. madrasensis*

Cell free hemolymph was prepared according to the method of Cheng (1990). All the tests were done on the same day of collection of the hemolymph (Chu and Peyre, 1989).

3.2.3.1. Protein profile

3.2.3.1.1. Total serum protein

The total protein concentration of serum was estimated following the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The result was expressed as µg/ml of serum.

3.2.3.1.2. SDS-PAGE

Serum proteins were separated by SDS-PAGE as per the method of Laemmili (1970). Separating gel of 11.5% was used for electrophoresis. The samples mixed with equal volume of buffer were loaded. Electrophoresis was carried out at 140 V for 4 to 5 hours and the gel was stained with Coomassie brilliant blue.

3.2.3.2. Enzyme assays

Since the enzyme activities in the hemocytes are reported to be lesser compared to the serum (Chu and Peyre, 1989), assays were carried out only with the serum.

3.2.3.2.1. Serum acid phosphatase

The method followed was of Varley (1980). The acid phosphatase in the serum was allowed to react with 0.01 M disodium phenyl phosphate and citrate buffer in 1:1 proportion at a pH of 4.9. The phenol released was allowed to react with 0.6% of amino-antipyrene in 2.4% ferric cyanide solution in 0.5 N sodium hydroxide and 0.5 N sodium bicarbonate. O.D was recorded at 510 nm. The amount of phenol released per 100ml serum was determined from a standard curve constructed using known amount of phenol. The results were expressed in KA units (mg phenol released/100ml serum/hr).

3.2.3.2.2. Serum phenol oxidase

The method of Preston and Taylor (1970) was modified for the purpose. The test serum (0.3 ml) was added to 3 ml of 0.01 M L-dopa in 0.05 M tris - HCl buffer at pH 7.5. Sodium dodycel sulphate was added to the serum (mg/ml). It converted prophenol oxidase, if present, to phenol oxidase. The increase in O.D of the sample in the next 1-minute was noted at 420 nm. The phenol oxidase activity was calculated as increase in O.D/min/mg of serum protein (Δ O.D/min/mg of serum protein).

3.2.3.2.3. Serum lysozyme

The method of Parry *et al.* (1965) was modified. Dried *Micrococcus lysodeikticus,* 0.2 mg was suspended per ml of 0.05 M sodium phosphate buffer pH 6.2 to give an O.D of 0.6 at 530 nm. Fifty µl of serum was added to the above suspension to give a final volume of 5 ml. Same amount of 0.05 M buffer added to the culture suspension, served as control. The decrease in the absorbance of the solution between 0.5 minutes and 4.5 minutes was read at 530 nm. The unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001 per minute (Lysozyme unit).

3.2.4. Experimental design

3.2.4.1. Exposure to Nuvan

The effect of organophosphate pesticide on immune response was estimated using Nuvan. Experiments conducted to establish the LC_{50} values of Nuvan did not reveal any mortality even at a concentration of 10 ppm. Three concentrations of Nuvan, viz. 0.05 ppm, 0.1 ppm and 0.2 ppm were chosen along with the control in triplicates and each treatment contained 15 animals. The animals were maintained in the respective concentrations for 4 weeks before the collection of hemolymph.

3.2.4.2. Exposure to copper

The lethal concentration of copper (LC_{50}) was found to be 5 ppm at a salinity of 12 ppt for *C. madrasensis*. Thus for the experiment three sub-lethal doses viz., 0.1 ppm, 0.5 ppm and 1.0 ppm were selected along with the control. Appropriate amounts of $CuSO_4.5H_2O$ was dissolved in water of 12 ppt salinity to get the respective concentrations of copper ion. Each treatment had three replicates of 15 animals each. The animals were exposed to copper at different concentrations for four weeks before taking the hemolymph for studies.

3.2.4.3. Exposure to *Vibrio alginolyticus*

A pathogenic strain of *Vibrio alginolyticus* was used. Sixty animals each were maintained in three replications in tanks of 1 tone capacity for control and test. Live bacteria from fresh grown culture at a concentration of 10^8 cells per 0.02 ml of sterile 2% saline were injected to the test animals under sterile conditions. Sterile 2% saline (0.02 ml) was injected to the control animals. Hemolymph was collected at 2 hrs, 24 hrs, 72 hrs, 120 hrs, 1 week and 2 weeks after injection for studying the different parameters. Ten animals each were bled from both the control and the test at each time interval. In order to avoid repeated bleeding, the animals once bled were discarded.

3.3. Histological studies

Adductor muscle, gills and mantle tissues of animals exposed to various concentrations of Nuvan and copper were fixed in Bouin's fixative for 24 hours. Tissues from control group of animals were also collected and fixed. The tissues were washed in tap water and dehydrated in ascending grades of alcohol, embedded in paraffin wax and 5 µm sections were prepared in a microtome and stained by hematoxylin and eosin (Sanders, 1974).

3.4. Inflammatory responses

3.4.1. Light microscopic studies

3.4.1.1. Exposure to Nuvan

Freund's complete adjuvant (FCA) was used to induce the inflammatory responses. Three groups of animals were maintained at 12 ppt salinity at concentrations of 0 ppm, 0.1 ppm and 0.2 ppm Nuvan for two weeks. Each group contained 10 animals. All the animals were injected with 0.02 ml of FCA using a sterile hypodermic syringe at the adductor muscle under sterile conditions. The adductor muscle was cut out and fixed in Bouin's fixative on day 1, day 3, day 7 and day 14 after the injection. The tissues were washed and dehydrated, 24 hours after the fixation. It was embedded in paraffin wax and 5 µm sections were cut and stained with hematoxylin and eosin (Sanders, 1974).

3.4.1.2. Exposure to copper

The inflammatory responses were studied by injecting FCA into the adductor muscle of 3 groups of animals maintained in 0 ppm, 0.5 ppm and 1 ppm copper for two weeks. The adductor muscle was fixed and processed as already described above in 3.4.1.1.

3.4.1.3. Exposure to *V. alginolyticus*

The inflammatory responses caused by the sham (0.02 ml of sterile 2% saline) and the test (*V. alginolyticus*, 10^8cells per 0.02 ml of sterile 2% saline) injections in the adductor muscle of the *C. madrasensis*

were studied by fixing and processing the adductor muscle of the respective animals at 2 hrs, 24 hrs, 72 hrs, 120 hrs, 1 week and 2 weeks.

3.4.2. Ultrastructural studies

The adductor muscles injected with *V. alginolyticus* were fixed at 2 hrs, 24 hrs, 72 hrs, 120 hrs, 1 week and 2 weeks with 3% glutraldehyde in 0.1 M sodium cacodylate buffer (pH-7.8) at a temperature of 4°C and kept overnight. The tissues were then washed 3 times in cacodylate buffer (half an hour for each wash) and kept overnight at 4°C. It was post fixed in 1% osmium tetroxide for 2 hrs and again washed in cacodylate buffer, three times. The tissue was cut into very small pieces and dehydrated through an ascending series of acetone. The remaining procedure was the same as followed for the hemocytes.

3.5. Statistical methods

The results were analyzed by Analysis of Variance (ANOVA) technique at 5% level of significance and the means were compared by Least Significant Difference (LSD) technique (Snedecor and Cohran, 1968).

4. RESULTS

4.1. Hemolymph factors of apparently healthy *C. madrasensis*

In the present study, hemocytes, which are responsible for phagocytosis and digestion of non-self material and also the release of various enzymes, were characterized to understand the cellular factors. The humoral factors present in the serum, which include total serum protein and enzymes such as acid phosphatase, phenol oxidase and lysozyme, were also estimated. In addition, SDS-PAGE of the serum was also done. These studies were done on apparently healthy animals.

4.1.1. Cellular factors

4.1.1.1 Characterization of hemocytes

Light microscopic studies revealed two types of cells. One type is with round to oval nucleus and a small amount of cytoplasm around the nucleus, with little or no granules. These cells were designated as hyalinocytes (Plate 1). The other cells contained eccentric, oval to round nucleus with large amount of cytoplasm containing granules. The granules containing cells could be divided into two types. One with abundant granules of eosinophilic and basophilic nature and these were classified as granulocytes (Plate 2). The other type of cells contained only a few numbers of basophilic granules. These were called semigranulocytes (Plate 3).

Ultrastructural studies revealed cells containing large nucleus with a few heterochromatin clumps. Sometimes nucleoli were also visible. The cytoplasm contained rough endoplasmic reticulum and a few vesicular structures. In this type of cells, the granules were either nil or scanty (Plate 4). A number of cells contained membrane bound granules containing electron lucent materials. These granules were scanty in some cells with abundant endoplasmic reticulum (Plate 5), where as, in others; these granules occupied a major portion of cytoplasm (Plate 6). Mitochondria were visible in all these types of cells.

In scanning micrographs, the unspread hemocytes were spherical (Plate 7). The initial pseudopodia were lobose, as they began to

Plate 1: Light microscopic photograph of hyalinolocyte (100X)

Plate 2: Light microscopic photograph of granulocytes (100X)

Plate 3: Light microscopic photograph of semigranulocyte (100X)

Plate 4: Transmission electron microscopic photograph of a hyalinolocyte (12000X)

Plate 5: Transmission electron microscopic photograph of a granulocyte (12000X).

Plate 6: Transmission electron microscopic photograph of a semigranulocyte (120000X).

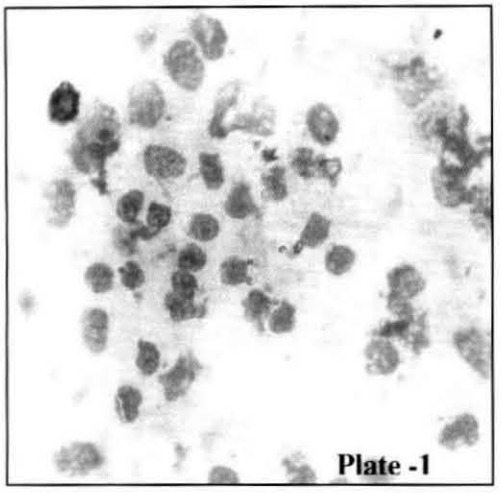


Plate -1

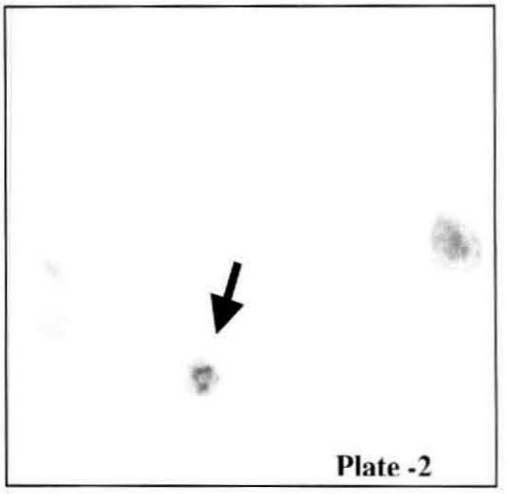


Plate -2

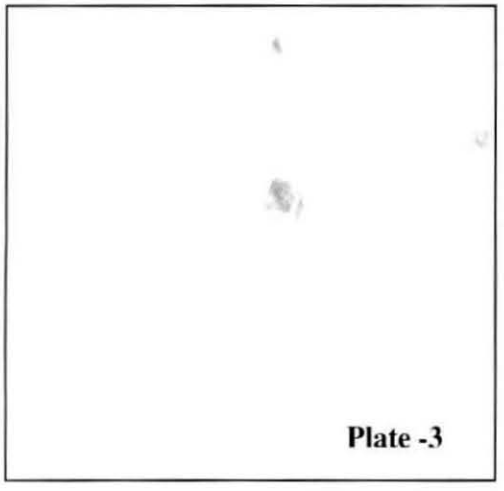


Plate -3

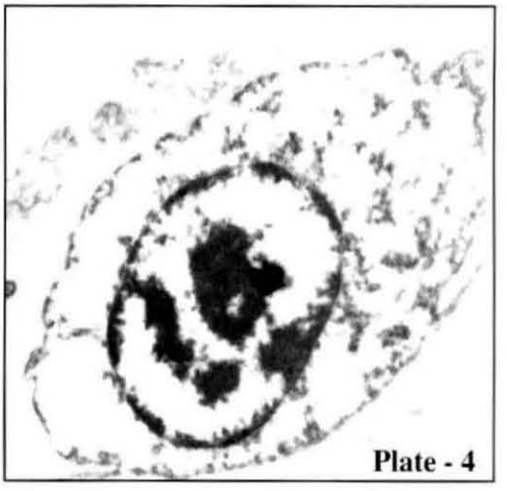


Plate - 4

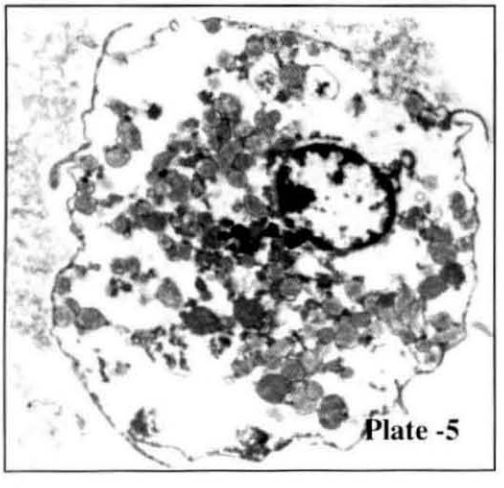


Plate -5

Plate - 6

Plate 7: Scanning electron microscopic photograph of an unspread hemocyte.

Plate 8: Scanning electron microscopic photograph of a spreading hemocyte with initial pseudopodia.

Plate 9: Scanning electron microscopic photograph of a fully spread hemocyte.

Plate 10: Hemocytes of *C. madrasensis* showing acid phosphatase activity (100X).

Plate 11: Hemocytes of *C. madrasensis* showing prophenol oxidase activity (100X).

Plate 12: Hemocytes of *C. madrasensis* showing peroxidase activity (100X).

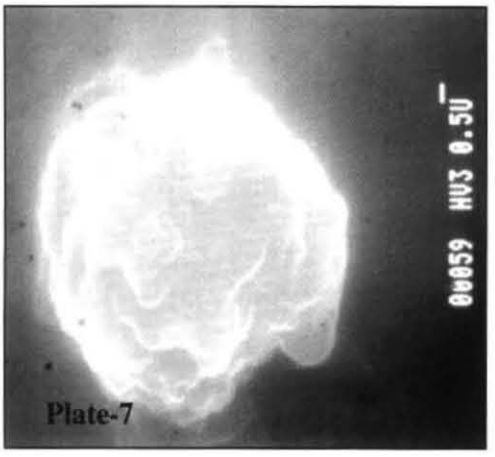


Plate-7

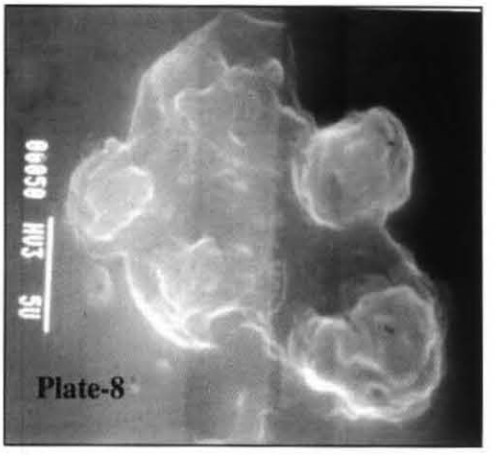
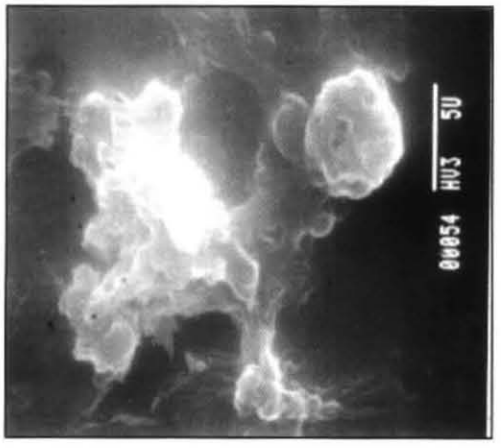


Plate-8



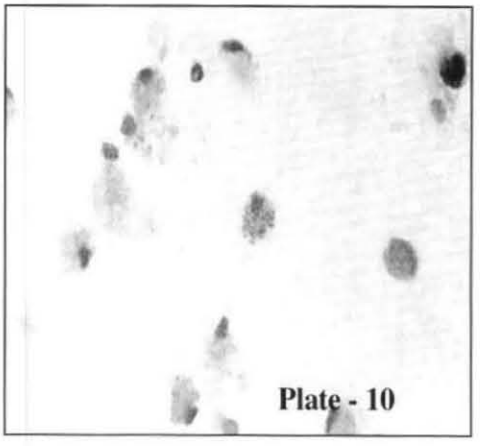


Plate - 10

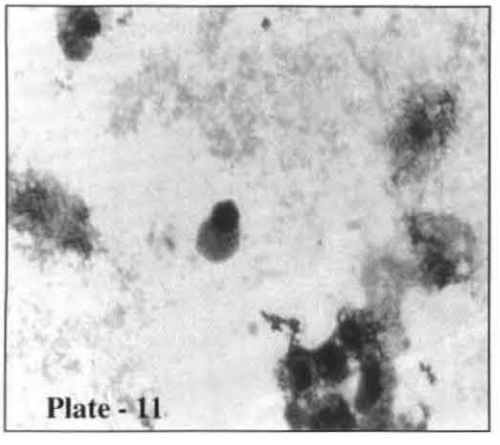


Plate - 11

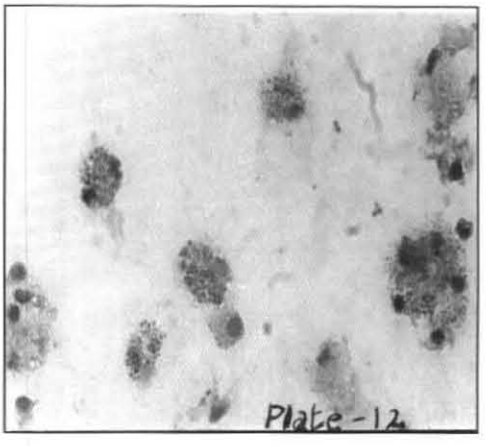


Plate - 12

spread on to the glass cover slip (Plate 8). Fully spread hemocytes with a large number of branched filopodia with terminal swelling were also seen (Plate 9).

The enzymes like acid phosphatase, prophenol oxidase and peroxidase were demonstrated in Plate 10, 11 and 12 respectively. Acid phosphatase activity was observed as scanty bluish black dot like granules in some of the granulocytes. All the granulocytes did not show acid phosphatase activity. Acid phosphatase activity was absent in hyalinocytes, however, it was observed in the semigranulocytes. Prophenol oxidase was observed as diffused bluish black pigment in cytoplasm of many cells and also in the granules. Peroxidase activity was observed as discrete dark blue granules, which were seen abundantly in all the granulocytes and semigranulocytes.

4.1.1.2. Maintenance of hemocytes in artificial media

Hemocyte counts in the different media at different time intervals are given in Table 1. In HBSS, hemocytes were maintained in viable condition for 72 hours. There was no significant ($p>0.05$) difference in the hemocyte count at the different time intervals.

HBSS with 10% FCS also, although maintained the cells for 72 hrs, the results at different time intervals were not significantly different ($p>0.05$).

In tissue culture media M 199 and in M 199 with 10% FCS, the number of cells almost doubled at 48 hrs. The increased number of cells was mainly constituted by hyalinocytes. The results at the different time intervals were significantly ($p>0.05$) different.

4.1.1.3. Total hemocyte count

The total hemocyte count of apparently healthy animals varied from 165 to 280.75 x 10^4 cells/ml of hemolymph, with a mean of 211.21± 44.40 x 10^4 cells/ml of hemolymph. The three different types of hemocytes namely, granulocytes, semigranulocytes and hyalinocytes

Table 1: Total viable hemocyte count (x 10^4 cells per ml of the respective media) of *C. madrasensis* in different media tested

Time	Repl.	HBSS	HBSS with FCS	M 199	M 199 with FCS
0 hr	1	64	63	85	71
	2	63	65	67	73
	3	64	64	60	64
	Mean	63.666	64	70.6667	69.333
	±SD	0.5773	1	12.897	4.726
24 hr	1	64	63	69	116
	2	69	63	100	102
	3	86	59	53	98
	Mean	73	61.6667	74	105.3333
	±SD	11.532	2.309	23.8956	9.45163
48 hr	1	66	61	132	158
	2	72	52	158	140
	3	68	63	137	115
	Mean	68.666	58.667	142.333	137.667
	±SD	3.055	5.8594	13.796	21.5947
72 hr	1	70	49	64	120
	2	50	67	91	90
	3	76	63	123	80
	Mean	65.333	59.667	92.6667	96.6667
	±SD	13.6137	9.4516	29.535	20.816

Table 2: Cellular factors of apparently healthy *C. madrasensis*

Replications	THC	D H C (%)			PI	EI
		G	SG	H		
1	172.75	54.41	29.96	15.63	40	0.72
2	245.5	24.13	64.16	11.71	36	0.64
3	165	24.39	53.64	21.97	42	0.59
4	196.75	82.34	9.27	9.27	63	0.65
5	206.5	80.39	11.99	7.62	48	0.55
6	280.75	86.29	8.46	5.25	58	0.93
Mean	211.2083	58.658	29.58	11.908	47.83	0.68
± SD	44.40141	28.918	24.26	6.0797	10.67	0.135

THC = Total hemocyte count (x 10^4 cells per ml of hemolymph)

DHC = Differential hemocyte count as percentage of different cells

G = Granulocyte, SG=Semigranulocyte, H=Hyalinocyte

PI = Phagocytic index as percentage of phagocytosing hemocytes

EI = Endocytic index as average number of yeast cells per hemocyte

stained by May-Gruenwald's eosin-methylene blue solution in the hemocytometer are given in Plate 13 (Table 2).

4.1.1.4. Differential hemocyte count

The percentage of granulocytes varied from 24.13 to 86.29, with a mean of 58.66 ± 28.92, the percentage semigranulocytes varied from 8.46 to 64.16, with a mean of 29.58 ± 24.26 and the percentage hyalinocytes varied from 5.25 to 21.97, with a mean of 11.91 ± 6.08 in apparently healthy animals (Table 2).

4.1.1.5. Phagocytosis

The ability of the hemocytes to phagocytose yeast cells was studied by assessing the phagocytic index and endocytic index. For apparently healthy animals, the phagocytic index varied from 36 to 63 with a mean of 47.83 ± 10.67, where as, the endocytic index varied from 0.55 to 0.93 with a mean of 0.68 ± 0.135 (Table 2). The different stages of phagocytosis are shown in Plates 14, 15, 16 and 17.

4.1.2. Humoral factors.

4.1.2.1. Protein profile

4.1.2.1.1. Total serum protein

The total serum protein concentration of hemolymph varied from 392.02 to 551.51 µg/ml of serum, with a mean of 470.8 ± 61.54 for apparently healthy animals, in the present set of experiments (Table 3).

4.1.2.1.2. SDS - PAGE

The SDS-PAGE of the hemolymph of apparently healthy animals had 4 prominent bands of 86, 74, 26 and 31 kDa. The 26 and 31 kDa bands were so intense that they appeared as a single broad zone. Apart from these, there were 6 to 8 minor bands of molecular weights varying from 52 to above 100 kDa. The standardization of the molecular weights of the different bands was done in the same lab in another set of experiment to find out the optimum salinity of the species (George *et al.*, 2001) (Plate 18).

Plate 13: The hemocytes stained with May-Gruenwald's eosin-methylene blue solution in the hemocytometer (G-Granulocyte, SG-Semigranulocyte, H-Hyalinocyte) (100X)

Plate 14: Hemocyte adhering to yeast cells (100X)

Plate 15: Hemocyte with fully engulfed yeast cells (100X)

Plate 16: Hemocyte with partially digested yeast cell (100X)

Plate17: Hemocyte with empty vacuole after the digestion of the yeast cell (100X).

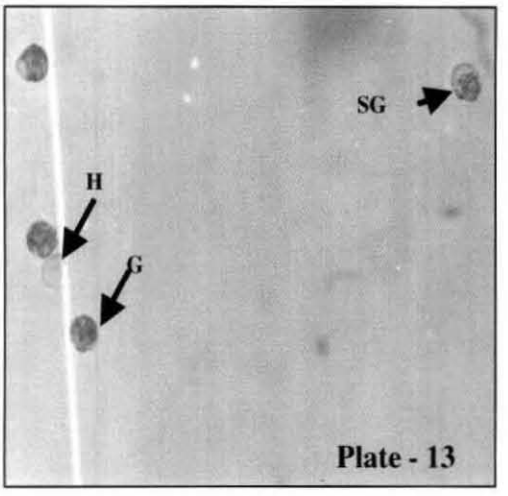


Plate - 13

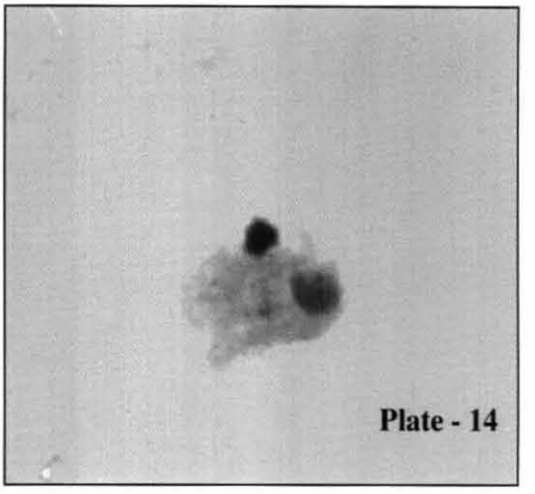


Plate - 14

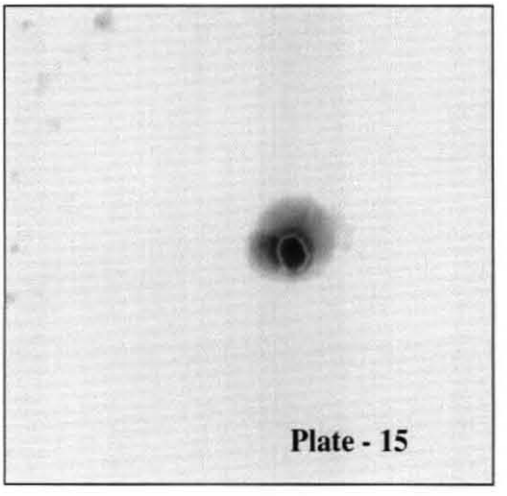
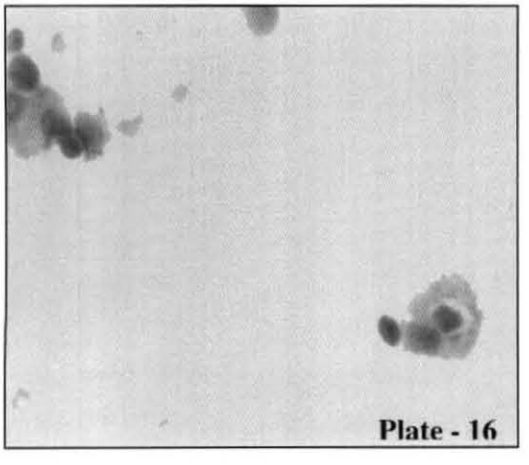


Plate - 15



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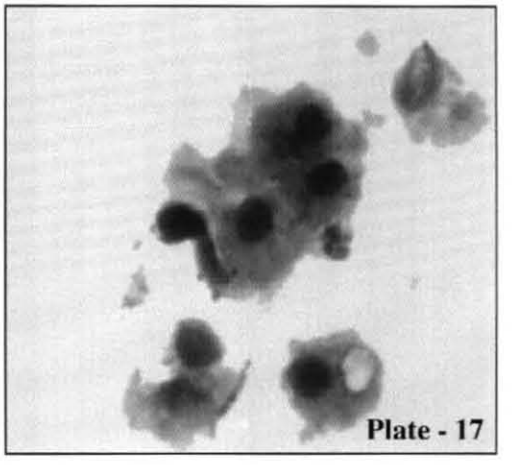


Plate - 17

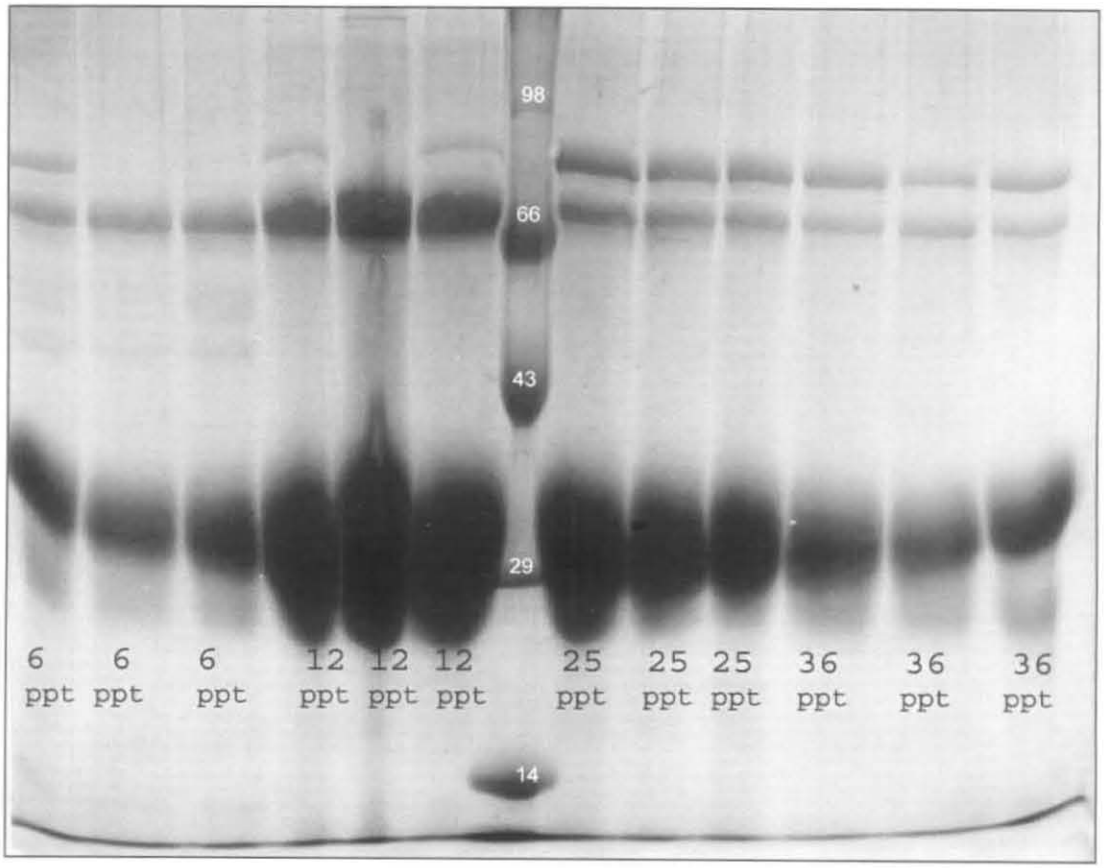


Plate 18: SDS-PAGE of apparently healthy C. madrasensis maintained at different salinities to standardize the molecular weight of the different protein bands with standard protein marker

Table 3: Serum factors of apparently healthy *C. madrasensis*

Replications	TSP	SAP	SPO	SL
1	410.8	0.417	0.01	40
2	392	0.436	0.0077	40
3	458.7	0.417	0.0073	20
4	551.5	0.465	0.0157	40
5	504.6	0.37	0.019	20
6	507.3	0.402	0.0143	30
Mean	**470.8**	**0.41783**	**0.01233**	**31.666**
±SD	**61.54**	**0.03192**	**0.00473**	**9.8319**

TSP = Total serum protein as µg/ml of serum

SAP = Serum acid phosphatase as KA units

SPO = Serum phenol oxidase as Δ OD/mg serum protein/ minute

SL = Serum lysozyme as lysozyme units per ml of serum

Table 4: Total hemocyte count (x 10^4 cells per ml of hemolymph) of *C. madrasensis* exposed to Nuvan

Replications	0 ppm	0.05 ppm	0.1 ppm	0.2 ppm
1	172.75	87.5	124	69.75
2	245.5	72.5	123.75	94.25
3	165	113.75	112	38.75
Mean	**194.4167**	**91.25**	**119.9167**	**67.58333**
±SD	**44.40885**	**20.87912**	**6.857174**	**27.81337**

4.1.2.2. Enzyme assays

4.1.2.2.1. Serum acid phosphatase

The amount of acid phosphatase (KA units) in the serum of the *C. madrasensis* varied from 0.37 to 0.465 for apparently healthy animals with a mean of 0.42 ± 0.03 (Table 3).

4.1.2.2.2. Serum phenol oxidase

The result of the phenol oxidase was expressed (ΔOD/mg serum protein/minute). Apparently healthy animals had 0.007 - 0.019 ΔOD/mg serum protein/minute with a mean of 0.012 ± 0.005 (Table 3).

4.1.2.2.3. Serum lysozyme

The serum lysozyme values for the apparently healthy animals as lysozyme units per ml of serum ranged from 20 to 40 units with a mean of 31.67 ± 9.83 (Table 3).

4.2. Effect of exposure to Nuvan on the hemolymph factors

4.2.1. Cellular factors

4.2.1.1. Total hemocyte count

The mean hemocyte count (Table 4), which was (194.42 ± 44.41 x 10^4 cells/ ml of hemolymph) at 0 ppm, decreased significantly, when the animals were exposed to Nuvan. The lowest value (67.58 ± 27.81 x 10^4 cells/ml of hemolymph) was obtained for the highest concentration of Nuvan at 0.2 ppm (Fig. 1). Analysis of variance techniques showed significant difference ($p < 0.05$) between different treatments. According to least significant difference (LSD) method, the value obtained at 0 ppm was significantly higher than all the Nuvan exposed groups.

4.2.1.2. Differential hemocyte count

The differential counts obtained for *C. madrasensis* exposed to different concentrations of Nuvan are presented in Table 5. Analysis of variance of data showed that the mean percentage of granulocytes did not significantly change with different concentrations of Nuvan (Fig 2). But the mean percentage of semigranulocytes and

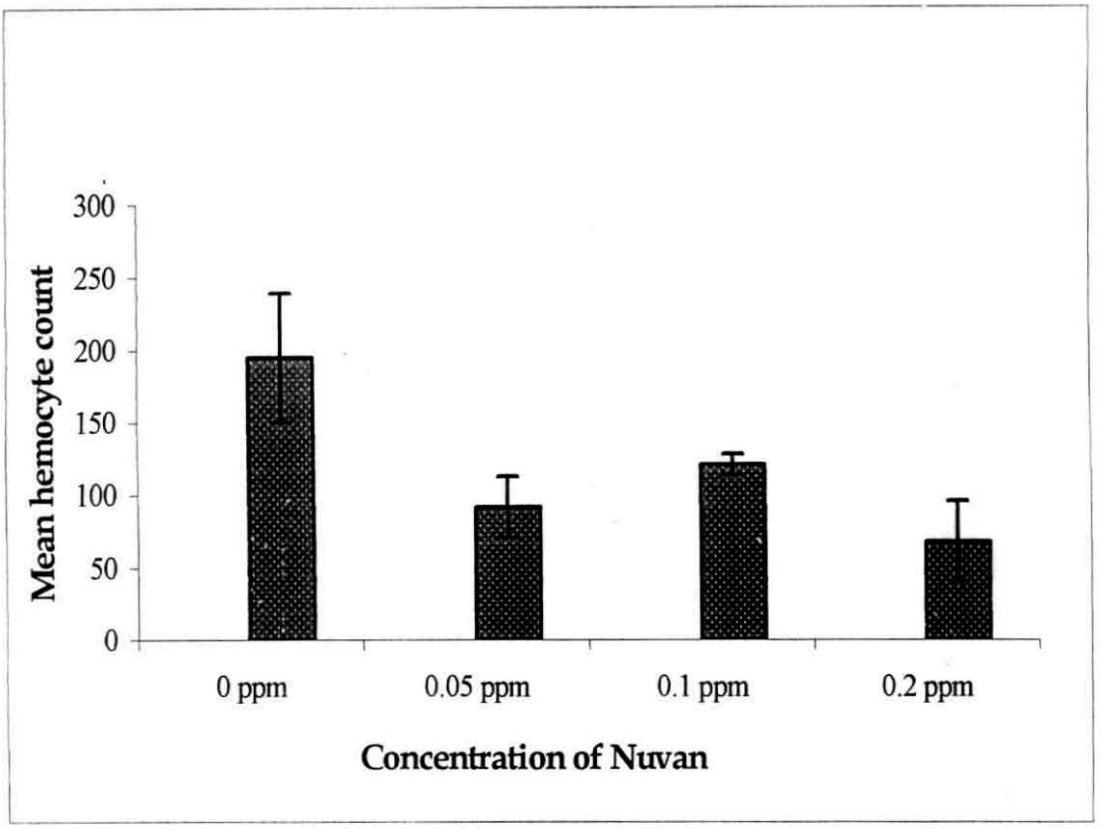


Figure 1: Mean hemocyte count (x 10^4 cells per ml of hemolymph) of *C. madrasensis* exposed to Nuvan

hyalinocytes showed significant change ($p<0.05$). The semigranulocytes showed a decrease at 0.5 ppm concentration, then increased at 0.1 ppm concentration and again decreased very much at 0.2 ppm concentration of Nuvan. The mean percentage of hyalinocytes increased significantly at 0.05 ppm and then decreased at 0.1 ppm. The value further increased significantly at 0.2 ppm.

4.2.1.3. Phagocytosis

4.2.1.3.1. Phagocytic index

The mean percentage of phagocytosing cells in the total population of hemocytes at different concentrations of Nuvan is given in Table 6. The phagocytic index reached the lowest mean value (21.33 ± 2.31) at 0.2 ppm. This value was significantly low ($p< 0.05$) compared to all other treatments. The phagocytic index showed a decreasing trend as the concentration of Nuvan increased (Fig. 3).

4.2.1.3.2. Endocytic index

Endocytic index estimated for different concentrations of Nuvan is given in Table 7. These values did not show any significant difference at 5% level. Compared to the result obtained at 0 ppm (0.65 ± 0.07), the value obtained at 0.1 ppm (0.38 ± 0.12) and 0.2 ppm (0.39 ± 0.06) were low (Fig. 4).

4.2.2. Humoral factors

4.2.2.1. Protein profile

4.2.2.1.1. Total serum protein

Table 8 shows the mean total serum protein values of *C. madrasensis*, when exposed to different concentrations of Nuvan. At 0 and 0.05 ppm, the protein concentration in the serum was not different significantly, but at 0.1 ppm and 0.2 ppm, the value significantly ($p< 0.05$) increased (Fig. 5).

Table 5: Percentage of different cells in the hemolymph of *C. madrasensis* exposed to different concentrations of Nuvan

Hemocytes	Replications	0 ppm	0.05 ppm	0.1 ppm	0.2 ppm
% Granulocytes	1	54.41	38.57	15.12	3.23
	2	24.13	38.97	7.68	31.03
	3	24.39	33.63	28.18	32.9
	Mean	**34.31**	**37.05667**	**16.99333**	**22.38667**
	±SD	**17.4076**	**2.974312**	**10.3776**	**16.61649**
%Semigranulocytes	1	29.96	22.57	54.44	32.97
	2	64.16	20.35	83.64	11.94
	3	53.64	20.34	68.3	26.45
	Mean	**49.25333**	**21.08667**	**68.79333**	**23.78667**
	±SD	**17.51691**	**1.284614**	**14.60625**	**10.765**
%Hyalinocytes	1	15.63	38.86	30.44	63.8
	2	11.71	40.68	8.68	57.03
	3	21.97	45.93	35.58	40.65
	Mean	**16.43667**	**41.82333**	**24.9**	**53.82667**
	±SD	**5.177348**	**3.671053**	**14.2801**	**11.9028**

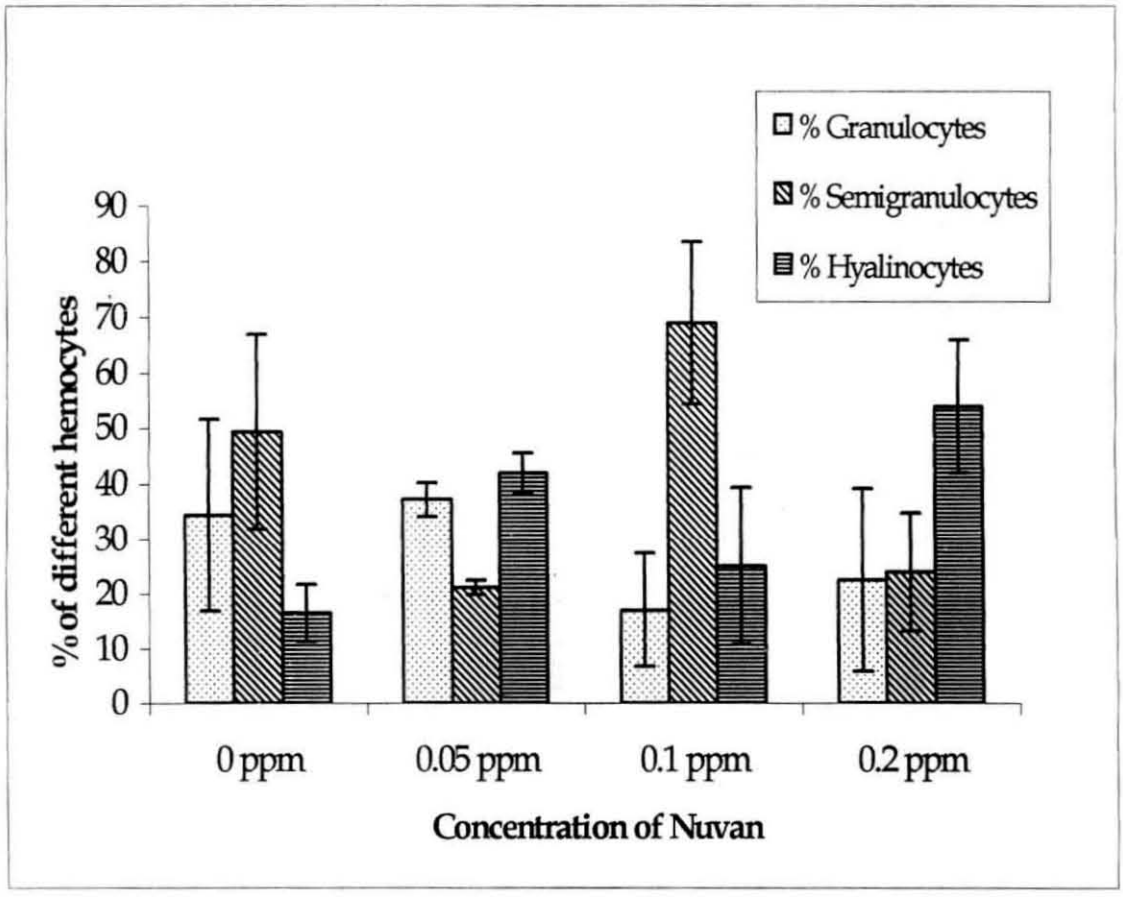


Figure 2: Mean percentage of different cells in the hemolymph of *C. madrasensis* exposed to different concentrations of Nuvan

Table 6: Phagocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of Nuvan

Replications	0 ppm	0.05 ppm	0.1 ppm	0.2 ppm
1	40	37	32	20
2	36	43	34	20
3	42	35	38	24
Mean	**39.33333**	**38.33333**	**34.66667**	**21.33333**
±SD	**3.05505**	**4.163332**	**3.05505**	**2.309401**

Table 7: Endocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of Nuvan

Replications	0 ppm	0.05 ppm	0.1 ppm	0.2 ppm
1	0.72	0.39	0.26	0.34
2	0.64	0.39	0.4	0.36
3	0.59	0.69	0.49	0.46
Mean	**0.65**	**0.49**	**0.38**	**0.39**
±SD	**0.07**	**0.17**	**0.12**	**0.06**

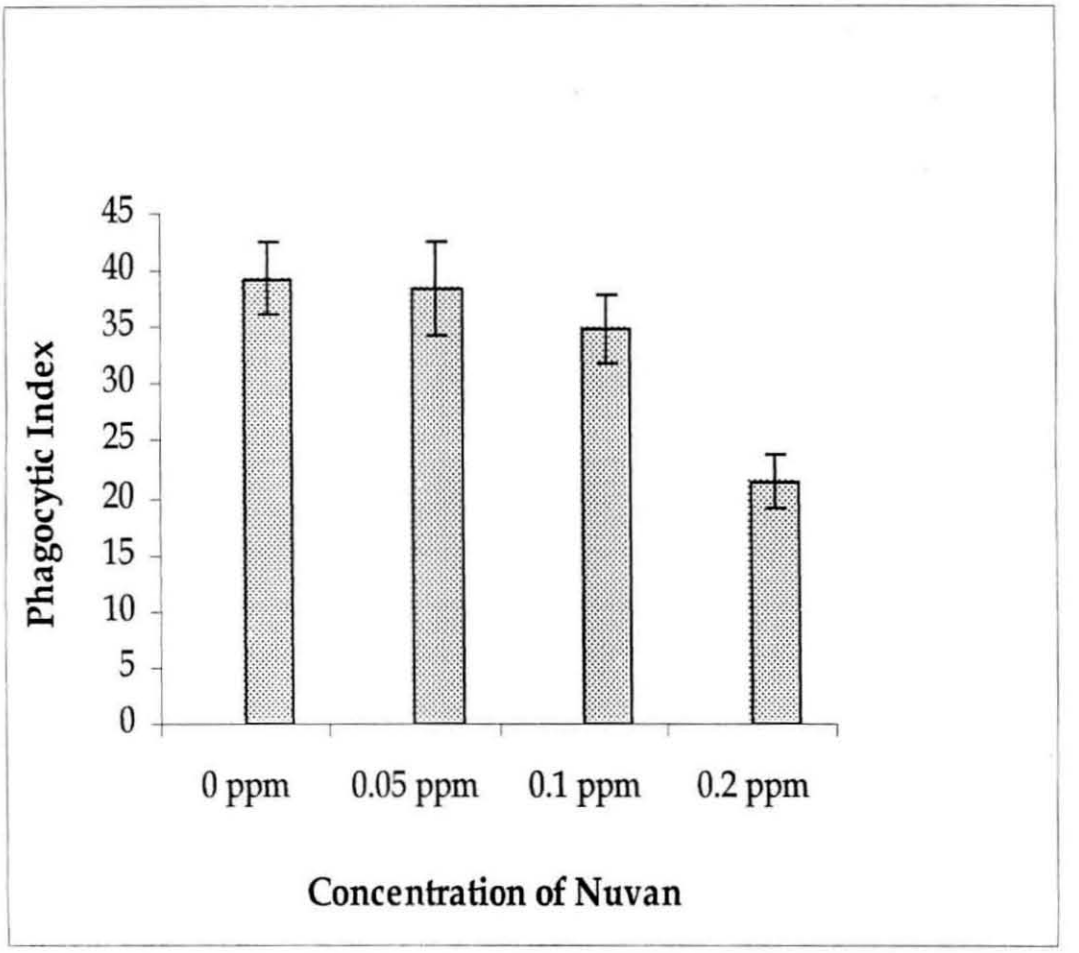


Figure 3: Mean phagocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of Nuvan

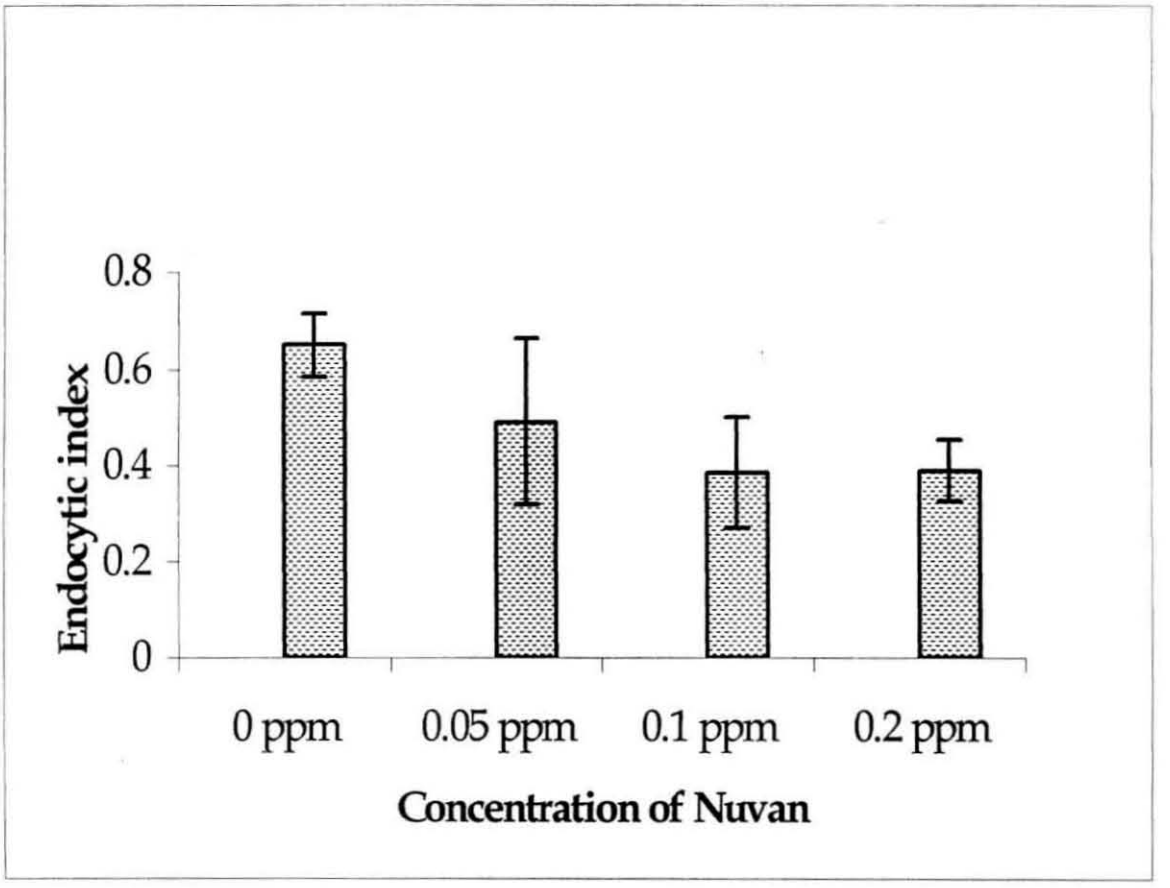


Figure 4: Mean endocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of Nuvan

Table 8: Total serum protein (μg/ml of serum) of *C. madrasensis* exposed to different concentrations of Nuvan

Replication	0 ppm	0.05 ppm	0.1 ppm	0.2 ppm
1	410.83	409.12	642.74	613.68
2	392.02	419.57	609.12	664.96
3	458.69	415.39	580.06	637.04
Mean	**420.5133**	**414.6933**	**610.64**	**638.56**
±SD	**34.37364**	**5.259718**	**31.36763**	**25.67377**

Table 9: Serum acid phosphatase (KA units) of *C. madrasensis* exposed to different concentrations of Nuvan

Replications	0 ppm	0.05 ppm	0.1 ppm	0.2 ppm
1	0.417	0.473	0.511	0.455
2	0.436	0.53	0.511	0.53
3	0.417	0.492	0.549	0.511
Mean	**0.423333**	**0.498333**	**0.523667**	**0.498667**
±SD	**0.01097**	**0.029023**	**0.021939**	**0.038991**

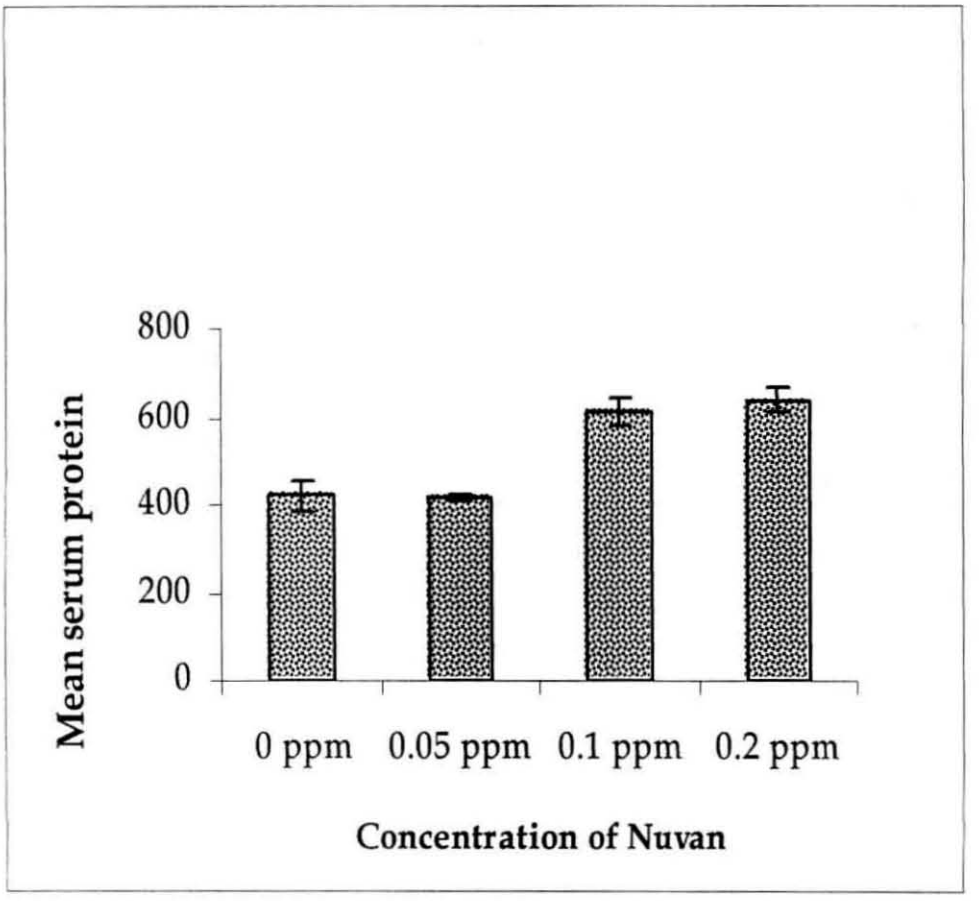


Figure 5: Mean serum protein (μg/ml of serum) of *C. madrasensis* exposed to different concentrations of Nuvan

4.2.2.1.2. SDS- PAGE

In the control, the major bands obtained were of molecular weights 86 kDa, 74 kDa, 26 kDa and 31 kDa. About 8 minor bands were also visible. Among these minor bands, 3 bands were of molecular weight above 100 kDa. Below that, there were 90 kDa, 60 kDa and 52 kDa bands.

At a Nuvan concentration of 0.05 ppm, only 1 band of above 100 kDa was detected. The 90 kDa band was also absent. The 86, 74, 26 and 31 kDa bands were more intense compared to the control. The 60 and 52 kDa bands were very feeble (Plate 19).

At a concentration of 0.1 ppm Nuvan, the minor bands of molecular weights 90 kDa and above were absent. The major bands of 86, 74, 31 and 26 kDa bands were more intense compared to the control.

At a concentration of 0.2 ppm of Nuvan, the bands of molecular weights 90 kDa and above were absent. All the other bands were less dense compared to the control.

4.2.2.2. Enzyme Assays

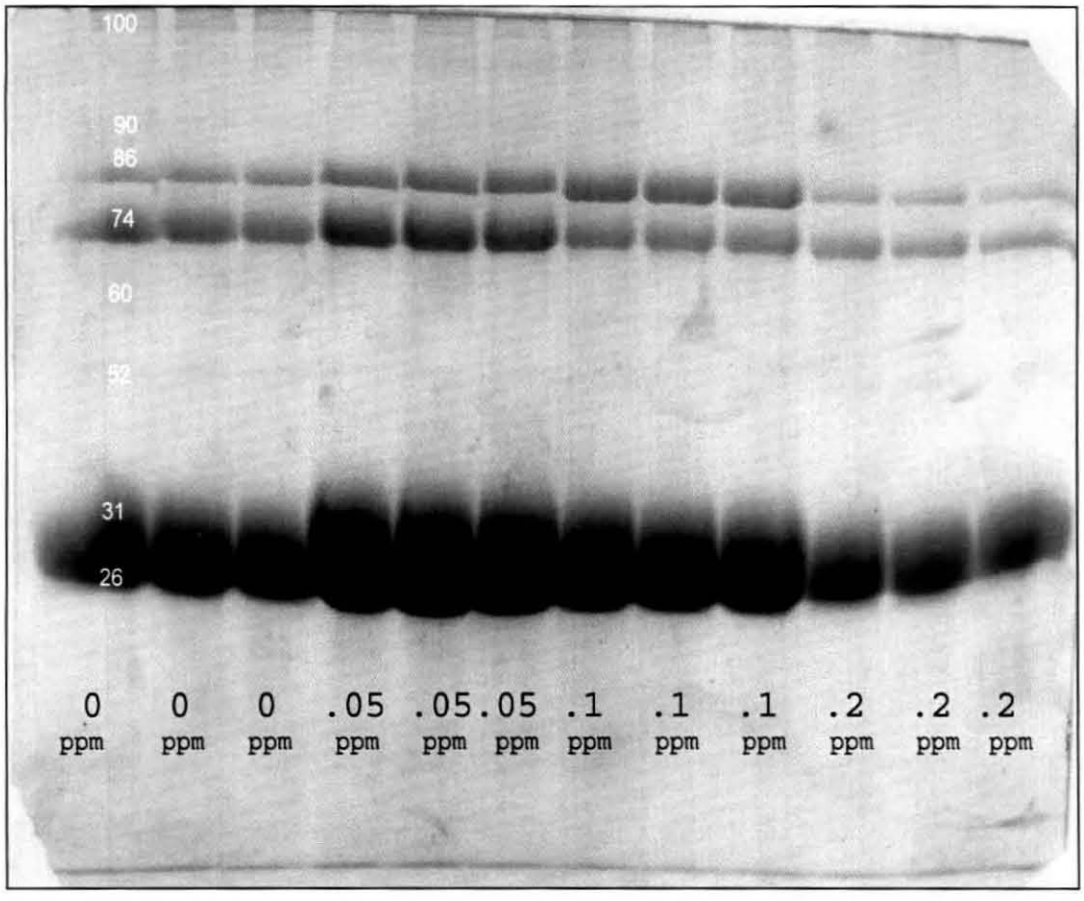
4.2.2.2.1. Serum acid phosphatase

The mean amounts of acid phosphatase in the serum of *C. madrasensis*, exposed to different concentrations of Nuvan are presented in Table 9. The mean serum acid phosphatase levels in the three treatments with Nuvan were significantly higher ($p < 0.05$) than the control. The highest amount (0.52 ± 0.02 KA units) was in the treatment with 0.1 ppm Nuvan (Fig. 6). Thus the value of acid phosphatase increased significantly in Nuvan treated animals.

4.2.2.2.2. Serum phenol oxidase

The mean amounts of serum phenol oxidase as Δ OD/mg serum protein/minute of *C. madrasensis* exposed to different concentrations of Nuvan are given in Table 10. Compared to 0 ppm (0.008 ± 0.002), the value increased significantly ($p < 0.05$) at 0.05 ppm (0.011 ± 0.004).

Plate 19: SDS - PAGE of the serum of *C. madrasensis* exposed to different concentrations of Nuvan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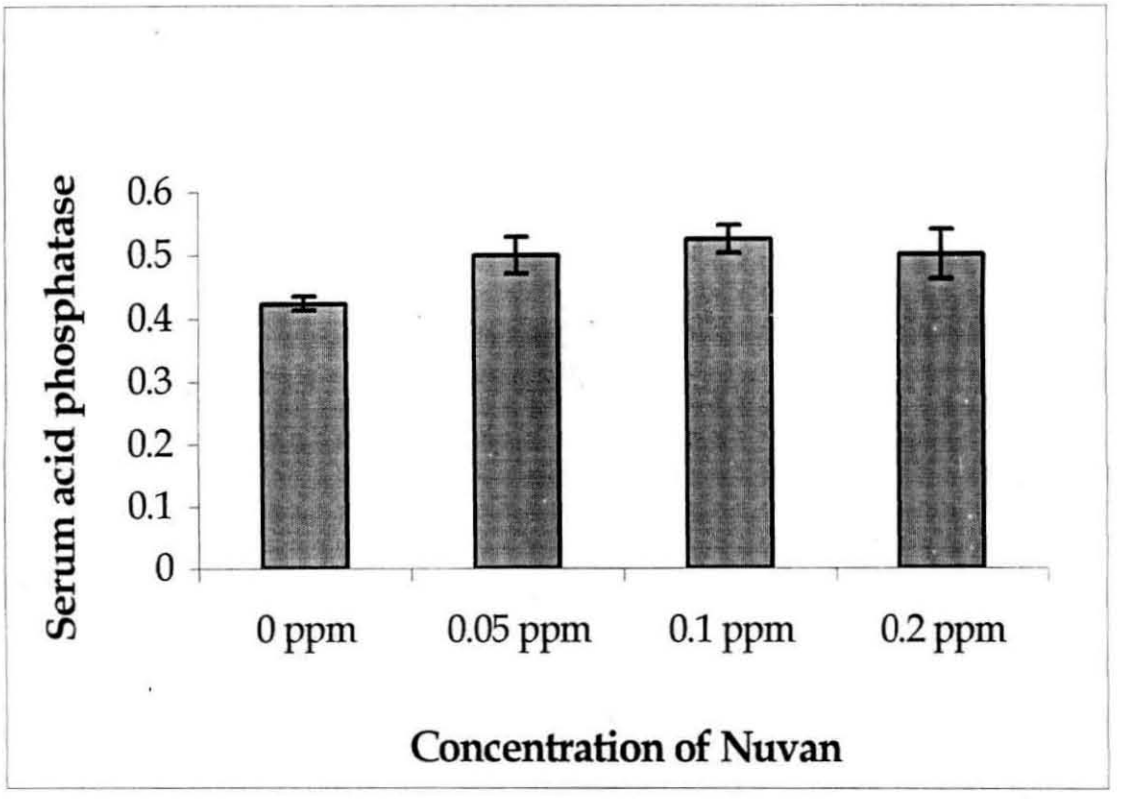


Figure 6: Serum acid phosphatase (KA units) of *C. madrasensis* exposed to different concentrations of Nuvan

Table 10: Serum phenol oxidase (Δ OD/mg serum protein/ minute) of *C. madrasensis* exposed to different concentrations of Nuvan

Replications	0 ppm	0.05 ppm	0.1 ppm	0.2 ppm
1	0.01	0.0077	0.0063	0.0023
2	0.0077	0.015	0.004	0.0047
3	0.0073	0.0087	0.002	0.002
Mean	**0.008333**	**0.010467**	**0.0041**	**0.003**
±SD	**0.001457**	**0.003958**	**0.002152**	**0.00148**

Table 11: Serum lysozyme (lysozyme units per ml of serum) of *C. madrasensis* exposed to different concentrations of Nuvan

Replications	0 ppm	0.05 ppm	0.1 ppm	0.2 ppm
1	40	120	140	320
2	40	160	120	180
3	20	160	80	140
Mean	**33.33333**	**146.6667**	**113.3333**	**213.3333**
±SD	**11.54701**	**23.09401**	**30.5505**	**94.51631**

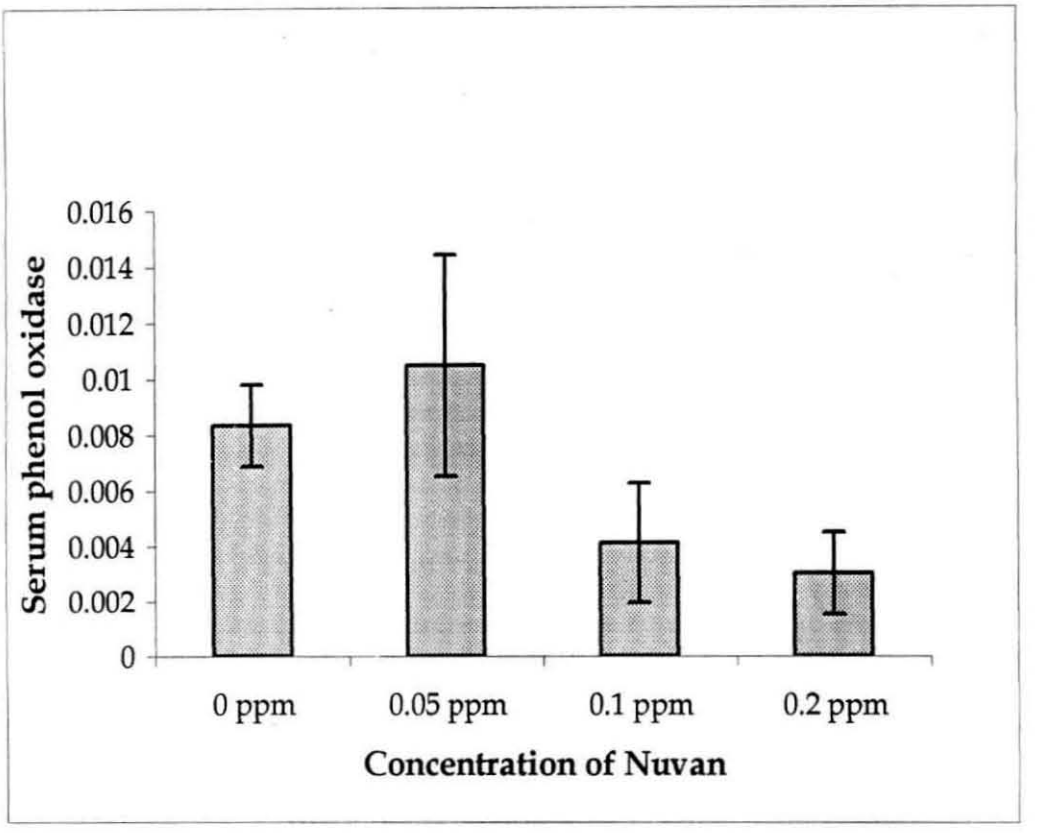


Figure 7: Mean serum phenol oxidase (Δ OD/mg serum protein/ minute) of *C. madrasensis* exposed to different concentrations of Nuvan

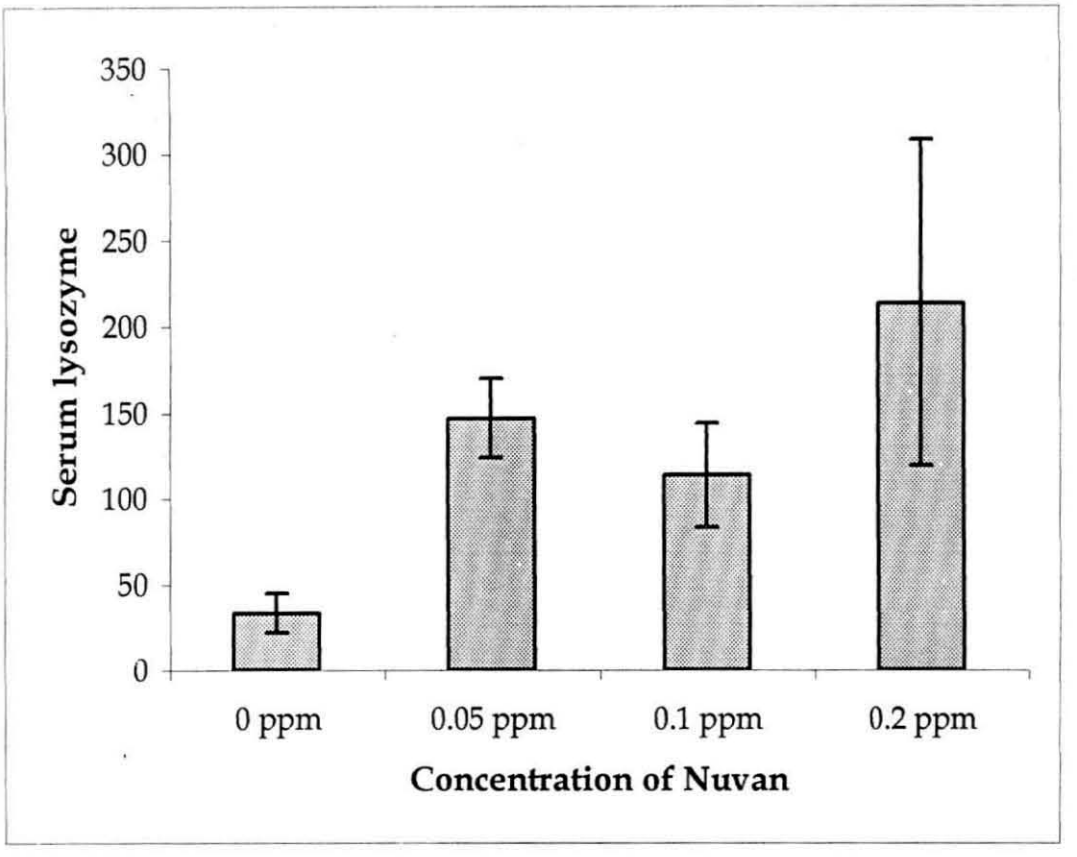


Figure 8: Mean serum lysozyme (lysozyme units per ml of serum) of *C. madrasensis* exposed to different concentrations of Nuvan

At 0.1 ppm, the value significantly reduced (0.004 ± 0.002) (Fig. 7). Thus the values showed an initial increase followed by a fall at higher concentrations of Nuvan.

4.2.2.2.3. Serum lysozyme

The mean serum lysozyme values, as lysozyme units per ml of serum, obtained when *C. madrasensis* was exposed to different concentrations of Nuvan are presented in Table 11. The amount of serum lysozyme increased significantly in all the treatment groups ($p < 0.05$) (Fig. 8). The highest value was obtained for the highest concentration of Nuvan (213.33 ± 94.52 lysozyme units/ml of serum).

4.3. Effect of exposure to copper on the hemolymph factors

4.3.1. Cellular factors

4.3.1.1. Total hemocyte count

Table 12 gives the mean hemocyte count in treatments with different concentrations of copper. The mean hemocyte count of control was significantly high compared to all other treatments. At the highest concentration of copper, the count was significantly ($p < 0.05$) low (Fig. 9).

4.3.1.2. Differential hemocyte count

The differential hemocyte counts recorded for different copper concentrations are presented in Table 13. The highest mean percentage of granulocytes (83.01 ± 3.01) was recorded for the control (0 ppm). The values obtained at all other treatments were significantly ($p<0.05$) low. The value at 0.5 ppm copper was significantly lower than that obtained at all the other treatments. Exposure to copper had resulted in a significant reduction of granulocytes in all the treatments (Fig.10). The percentage of semigranulocytes was significantly ($p < 0.05$) high in all the treatments with copper. The mean percentage of hyalinocytes at 0.5 ppm and 1 ppm were significantly high compared to the values obtained at 0 and 0.1 ppm. Thus, there was an increase in the percentage of semigranulocytes and hyalinocytes, when the animals were exposed to copper.

Table 12: Total hemocyte count (x 10^4 cells per ml of hemolymph) of *C. madrasensis* exposed to different concentrations of copper

Replications	0 ppm	0.1 ppm	0.5 ppm	1 ppm
1	196.75	116.25	170.5	69.25
2	206.5	85.25	73.25	68.25
3	280.75	83.5	137.75	75
Mean	**228**	**95**	**127.1667**	**70.83333**
±SD	**45.94222**	**18.42383**	**49.48127**	**3.642915**

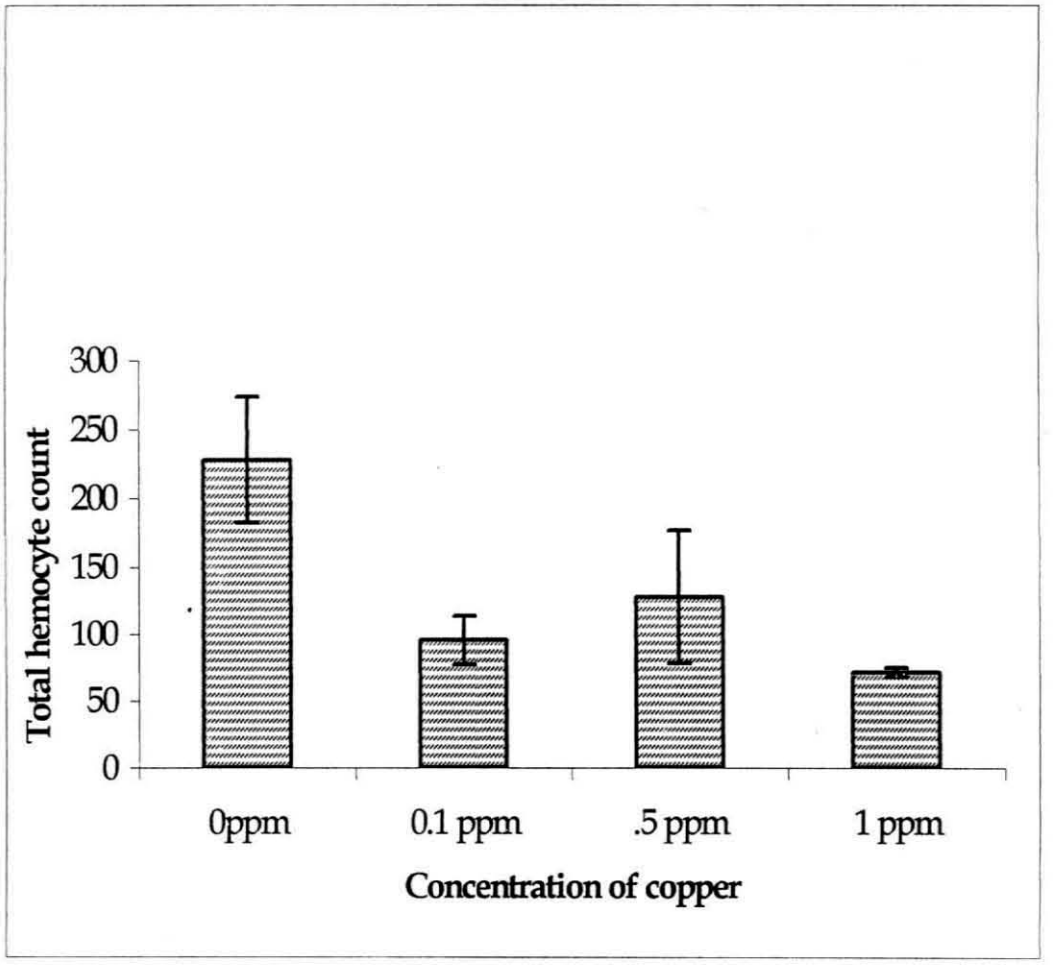


Figure 9: Mean hemocyte count (x 10^4 cells per ml of hemolymph) of *C. madrasensis* exposed to different concentrations of copper

4.3.1.3. Phagocytosis

Both phagocytic index and endocytic index showed significant difference between the treatments at 5% level.

4.3.1.3.1. Phagocytic index

The values obtained for phagocytic index are given in Table 14. A concentration of 0.1 ppm of copper had a stimulatory effect on phagocytic index (60 ± 4). But at 0.5 ppm (29 ± 8.5), and at 1 ppm of copper (19.33 ± 5.86) there was significant reduction ($p < 0.05$). The lowest value for mean phagocytic index was obtained for the highest concentration of copper (Fig. 11).

4.3.1.3.2. Endocytic index

The mean endocytic index of the hemocytes of *C. madrasensis* at different concentrations of copper are recorded in Table 15. The result showed that 0.1 ppm of copper had a boosting effect on the endocytic index (1.25 ± 0.08). The value obtained at this concentration was significantly high ($p < 0.05$) compared to that at 0 ppm copper. When the concentration increased to 0.5 ppm, the mean endocytic index (0.56± 0.09) significantly ($p < 0.05$) reduced. The lowest value (0.39 ± 0.09) was obtained for the highest concentration of 1 ppm copper (Fig. 12).

4.3.2. Humoral factors

4.3.2.1. Protein profile

4.3.2.1.1. Total serum protein

The mean total serum protein concentrations of hemolymph at different concentrations of copper are given in Table 16. The mean total serum protein values increased significantly ($p < 0.05$) at 0.1 ppm compared to the control (833.04 ± 44.44 µg/ml of serum). At 0.5 ppm, the value decreased significantly ($p < 0.05$) and reached the lowest value (402.84 ± 17.1 µg/ml of serum) at the highest concentration of copper (1 ppm) (Fig.13).

Table 13: Percentage of different cells in the hemolymph of *C. madrasensis* exposed to different concentration of copper

Hemocytes	Replications	0 ppm	0.1 ppm	.5 ppm	1 ppm
%Granulocytes	1	82.34	60	52.79	61.74
	2	80.39	68.04	40.61	58.97
	3	86.29	64.97	52.99	60.67
	Mean	**83.00667**	**64.33667**	**48.79667**	**60.46**
	SD	**3.005966**	**4.057245**	**7.090567**	**1.396889**
%Semigranulocytes	1	9.27	25.16	19.65	29.96
	2	11.99	8.5	38.57	24.18
	3	8.46	24.85	28.13	27
	Mean	**9.906667**	**19.50333**	**28.78333**	**27.04667**
	SD	**1.849117**	**9.530427**	**9.476905**	**2.890283**
%Hyalinocytes	1	9.27	14.84	27.56	24.18
	2	7.62	8.5	20.82	16.85
	3	5.25	10.18	18.88	12.33
	Mean	**7.38**	**11.17333**	**22.42**	**17.78667**
	SD	**2.020718**	**3.284651**	**4.555831**	**5.98027**

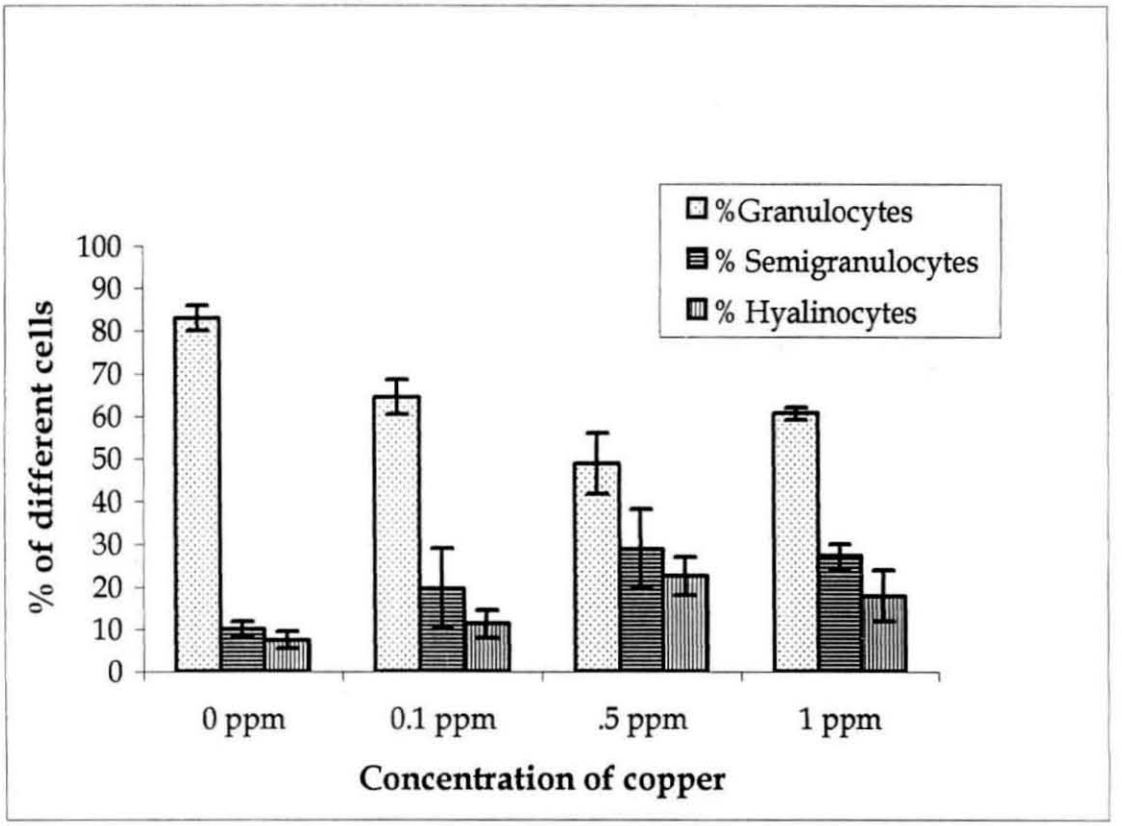


Figure 10: Mean percentage of different cells in the hemolymph of *C. madrasensis* exposed to different concentrations of copper

4.3.2.1.2. SDS - PAGE

In the control without copper, there were the usual major bands of 86, 74, 26 and 31 kDa. Apart from these, there were 2 minor bands of above 100 kDa, and the other minor bands of molecular weight, 90 kDa, 66 kDa, 60 kDa, 58 kDa and 52 kDa.

At a concentration of 0.1 ppm copper, all the minor bands were present with the same intensity as that of the control. The intensity of the major bands of 86 and 74 kDa were almost same as that of control. But the 26 and 31 kDa bands were denser than the control.

At a concentration of 0.5 ppm of copper, the three bands above 100 kDa were absent, 90 kDa band was very feeble, 86 and 74 kDa bands were more denser than the control. The 66, 60 and 52 kDa bands were feebly present, but 58 kDa band was absent. The 26 and 31 kDa bands were highly intense. Thus the major bands were denser and minor bands were either feeble or absent compared to the control.

At a concentration of 1 ppm of copper, the result was similar to 0.5 ppm, but 58 kDa band was present and 52 kDa band was absent (Plate 20).

4.3.2.2. Enzyme Assays

4.3.2.2.1. Serum acid phosphatase

The mean values of serum acid phosphatase of *C. madrasensis* exposed to different concentrations of copper are presented in Table 17. The highest mean value for serum acid phosphatase (0.41± 0.05 KA units) was obtained for the control without copper. As the copper concentration increased, the amount of serum acid phosphatase significantly ($p< 0.05$) decreased and the lowest value (0.09 ± 0.02 KA units) was obtained for the highest concentration of copper (Fig. 14).

4.3.2.2.2. Serum phenol oxidase

The mean amounts of phenol oxidase as Δ OD/mg serum protein/minute in the serum of *C. madrasensis* exposed to copper are

Table 14: Phagocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of copper

Replication	0 ppm	0.1 ppm	0.5 ppm	1 ppm
1	63	56	28	17
2	48	60	38	26
3	58	64	21	15
Mean	**56.33333**	**60**	**29**	**19.33333**
± SD	**7.637626**	**4**	**8.544004**	**5.859465**

Table 15: Endocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of copper

Replication	0 ppm	0.1 ppm	0.5 ppm	1 ppm
1	0.65	1.33	0.5	0.46
2	0.55	1.25	0.52	0.43
3	0.93	1.18	0.66	0.29
Mean	**0.71**	**1.253333**	**0.56**	**0.393333**
±SD	**0.196977**	**0.075056**	**0.087178**	**0.090738**

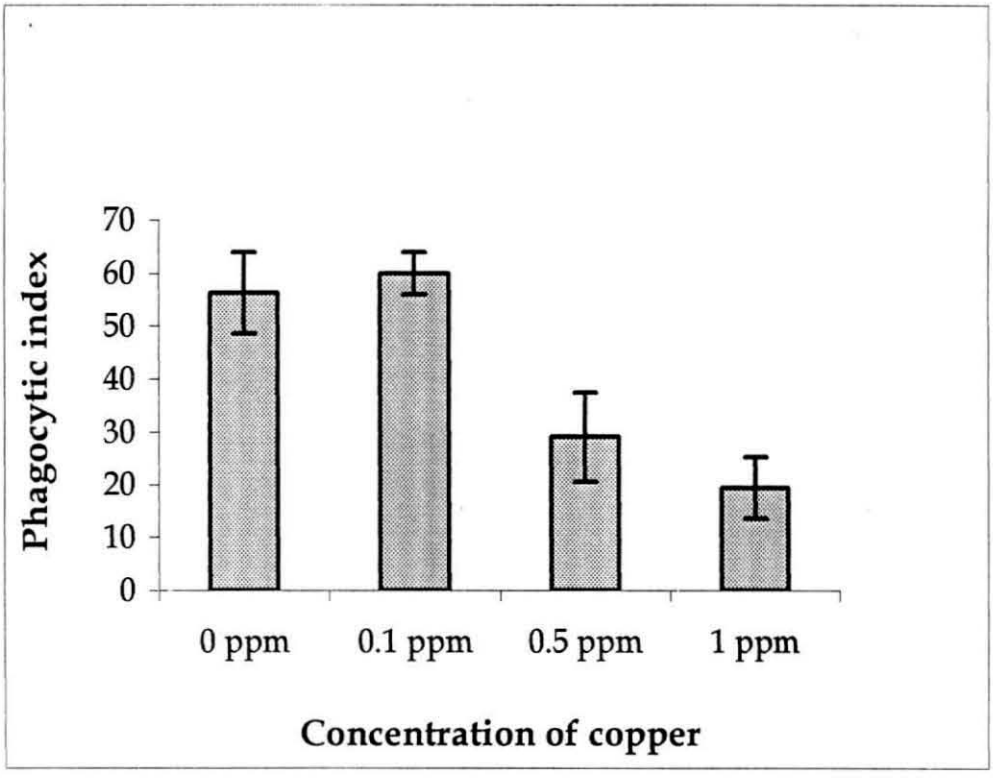


Figure 11: Mean phagocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of copper

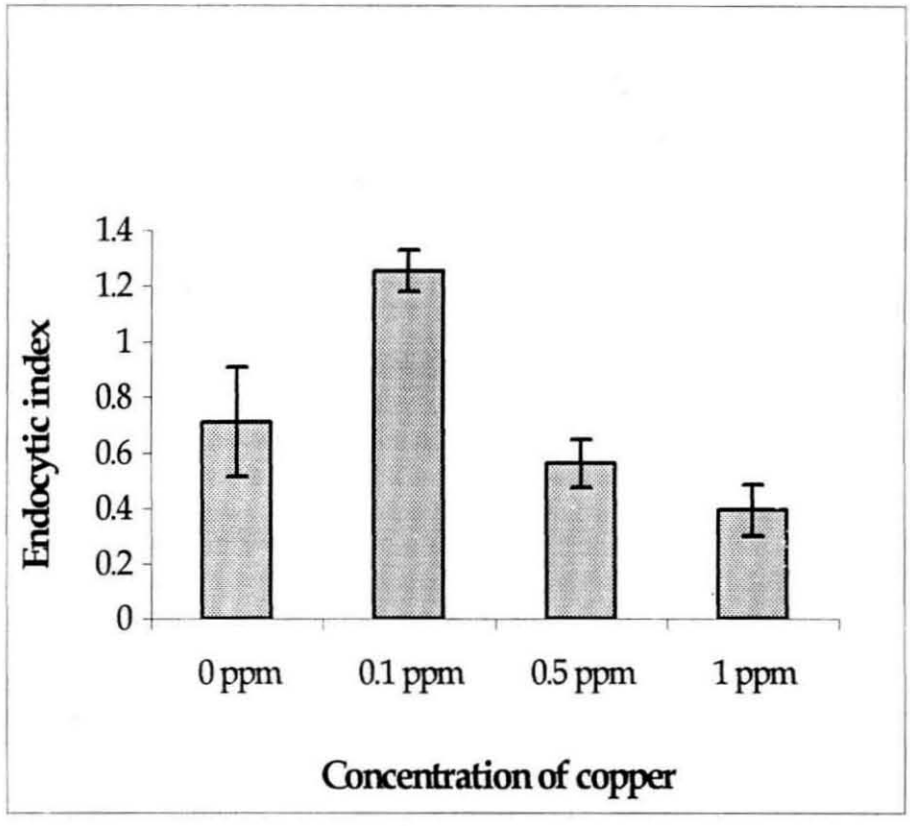


Figure 12: Mean endocytic index of hemocytes of *C. madrasensis* exposed to different concentrations of copper

Table 16: Total serum protein (μg/ml of serum) of *C. madrasensis* exposed to different concentrations of copper

Replications	0 ppm	0.1 ppm	0.5 ppm	1 ppm
1	551.51	879.75	688.99	422.56
2	504.62	828.06	711.9	393.78
3	507.28	791.3	687.92	392.18
Mean	**521.1367**	**833.0367**	**696.27**	**402.84**
± SD	**26.33768**	**44.43451**	**13.54655**	**17.09675**

Table 17: Serum acid phosphatase (KA units) of *C. madrasensis* exposed to different concentrations of copper

Replications	0 ppm	0.1 ppm	0.5 ppm	1 ppm
1	0.465	0.317	0.095	0.085
2	0.37	0.243	0.106	0.063
3	0.402	0.296	0.116	0.106
Mean	**0.412333**	**0.285333**	**0.105667**	**0.084667**
±SD	**0.048336**	**0.038136**	**0.010504**	**0.021502**

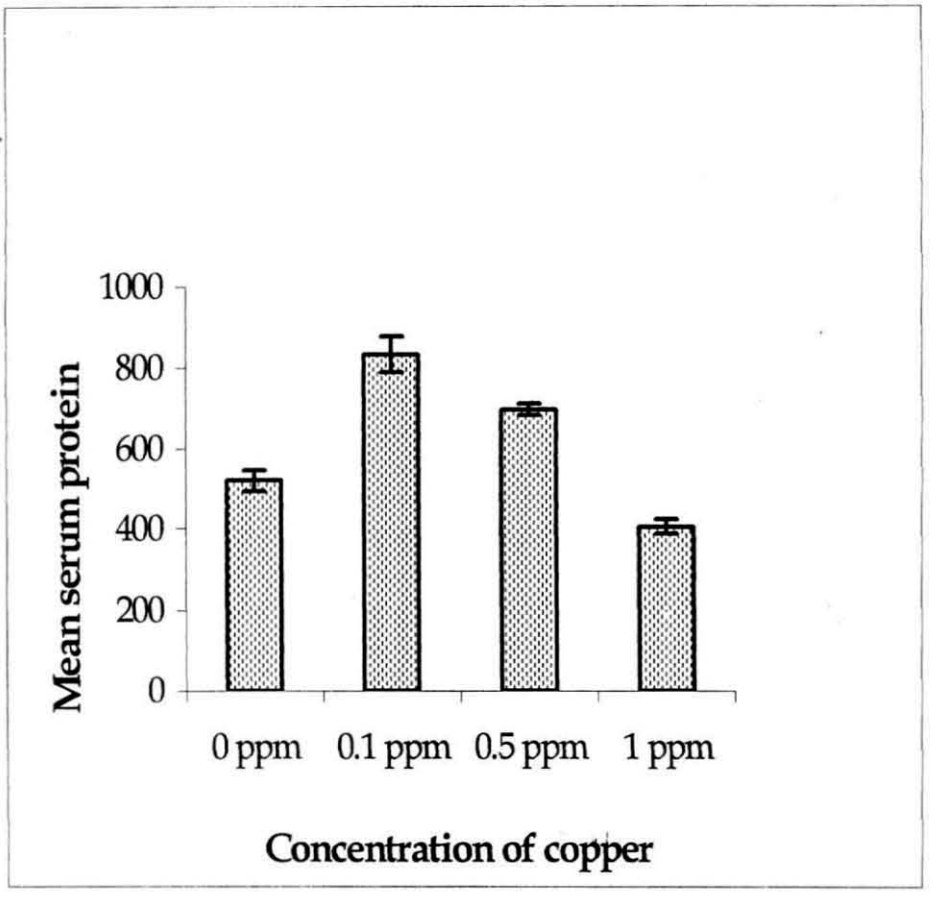
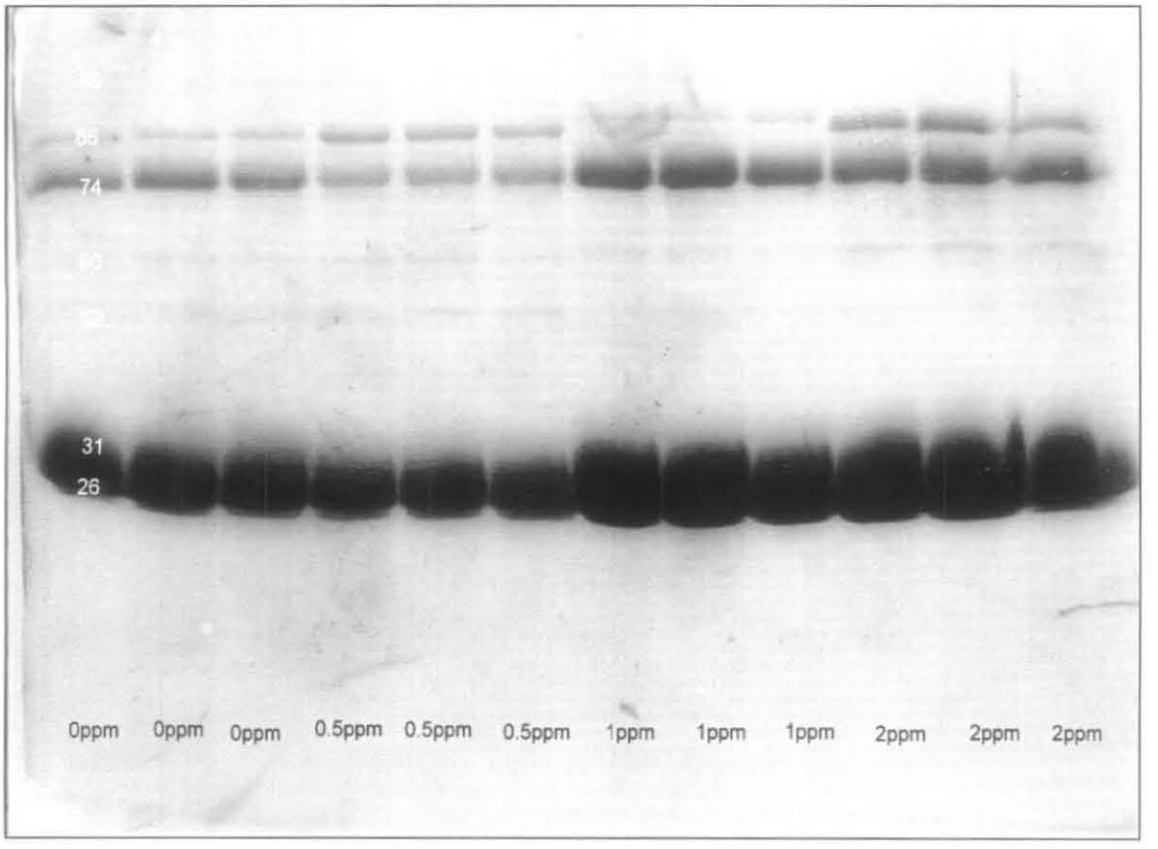


Figure 13: Mean serum protein (μg/ml of serum) of *C. madrasensis* exposed to different concentrations of copper

Plate 20: SDS- PAGE of the serum of *C. madrasensis* exposed to different concentrations of copper


74
31
26
0ppm
0ppm
0ppm
0.5ppm
0.5ppm
0.5ppm
1ppm
1ppm
1ppm
2ppm
2ppm
2ppm

recorded in Table 18. The serum phenol oxidase in all the treatments with copper was significantly ($p< 0.05$) low compared to the control without copper (Fig. 15).

4.2.2.2.3. Serum lysozyme

The mean amounts of lysozyme in the serum of *C. madrasensis*, when exposed to copper are given in Table 19. At a concentration of 0.1 ppm, the amount of lysozyme in the serum of the *C. madrasensis* showed an increase (43.33 ± 15.28 lysozyme units/ml of serum) (Fig. 16) followed by a fall in the values at 0.5 ppm and 1ppm concentrations of copper. However, the result was not statistically significant at 5% level.

4.4. Effect of exposure to *V. alginolyticus* on the hemolymph factors

4.4.1. Cellular factors

4.4.1.1. Total hemocyte count

The total hemocyte count at different time intervals such as 2 hours, 24 hours, 72 hours, 120 hours, 1 week and 2 weeks after injection with *V. alginolyticus* (test) are given in Table 20. Analysis of variance showed significant difference ($p< 0.05$) between control and test groups at different time intervals. The results obtained for the test (537.17 ± 7.75 x 10^4 cells/ml of hemolymph) was significantly high compared to the control (344.56 ± 111.17 x 10^4 cells/ml of hemolymph) at 2 hours of injection. The mean total hemocyte count dropped significantly in both control and test at 24 hours. The fall in the total hemocyte count observed at 24 hours after injection in the test was more than that in the control group. At 120 hours after injection, the value of control (212.5 ± 25.59 x 10^4 cells/ml of hemolymph) returned to the normal values. At 2 weeks, the values obtained for both control and test were similar and within the normal range (Fig. 17).

4.4.1.2. Differential hemocyte count

The mean percentages of granulocytes, semigranulocytes and hyalinocytes showed significant difference ($p< 0.05$) in

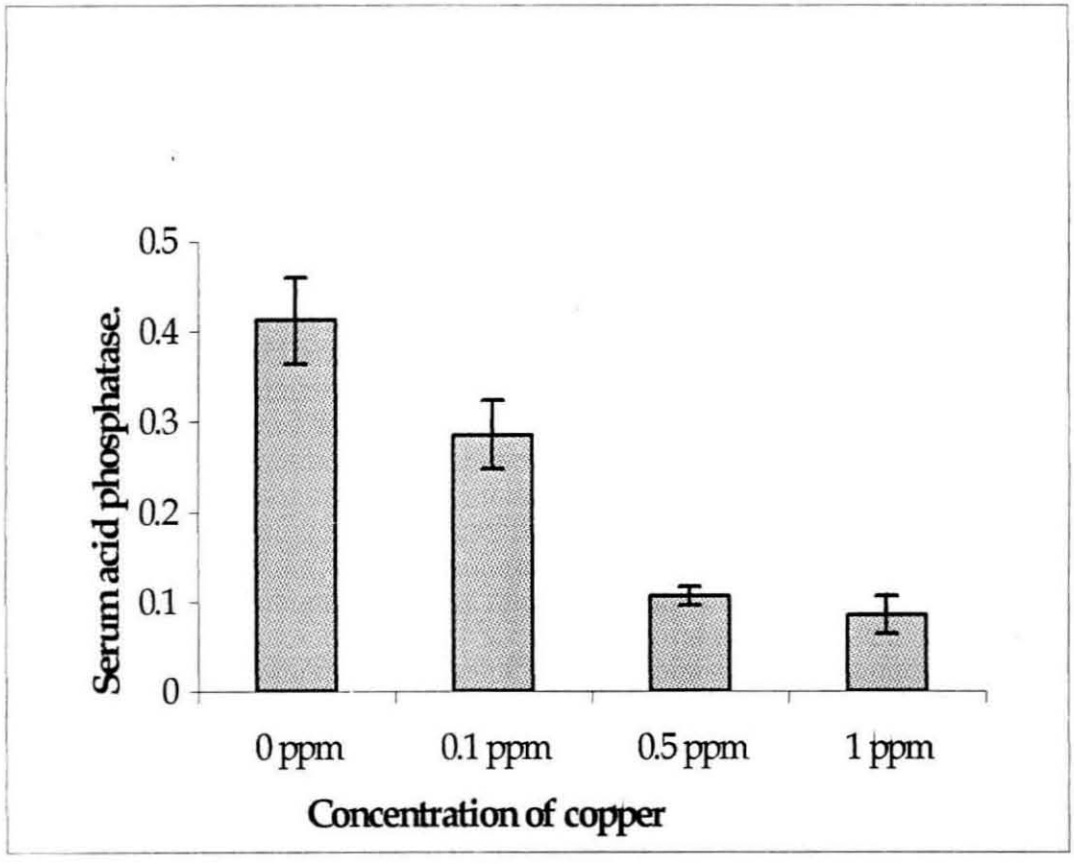


Figure 14: Mean serum acid phosphatase (KA units) of *C. madrasensis* exposed to different concentrations of copper

Table 18: Serum phenol oxidase (Δ OD/mg serum protein/ minute) of *C. madrasensis* exposed to different concentrations of copper

Replications	0 ppm	0.1 ppm	0.5 ppm	1 ppm
1	551.51	879.75	688.99	422.56
2	504.62	828.06	711.9	393.78
3	507.28	791.3	687.92	392.18
Mean	**521.1367**	**833.0367**	**696.27**	**402.84**
± SD	**26.33768**	**44.43451**	**13.54655**	**17.09675**

Table 19: Serum lysozyme (lysozyme units per ml of serum) of *C. madrasensis* exposed to different concentrations of copper

Replications	C	0.1 ppm	0.5 ppm	1 ppm
1	40	60	30	20
2	20	40	40	20
3	30	30	20	20
Mean	**30**	**43.33333**	**30**	**20**
±SD	**10**	**15.27525**	**10**	**0**

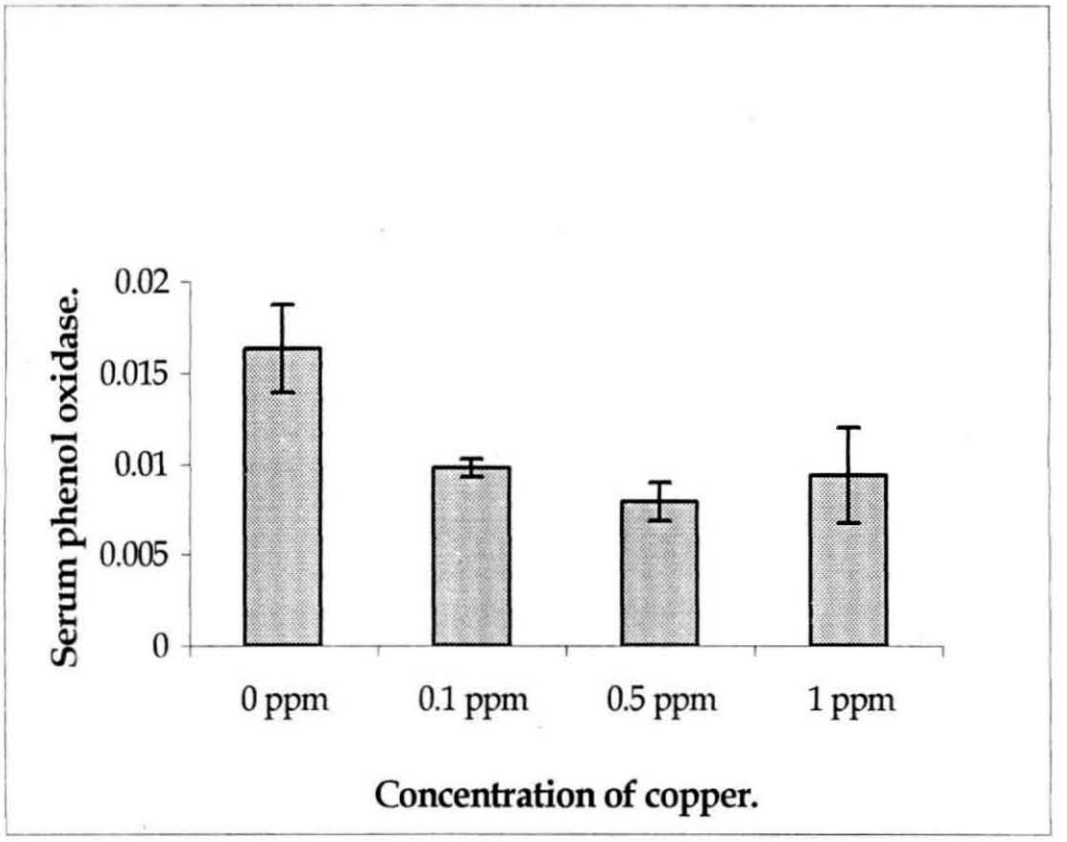


Figure 15: Mean serum phenol oxidase (Δ OD/mg serum protein/ minute) of *C. madrasensis* exposed to different concentrations of copper

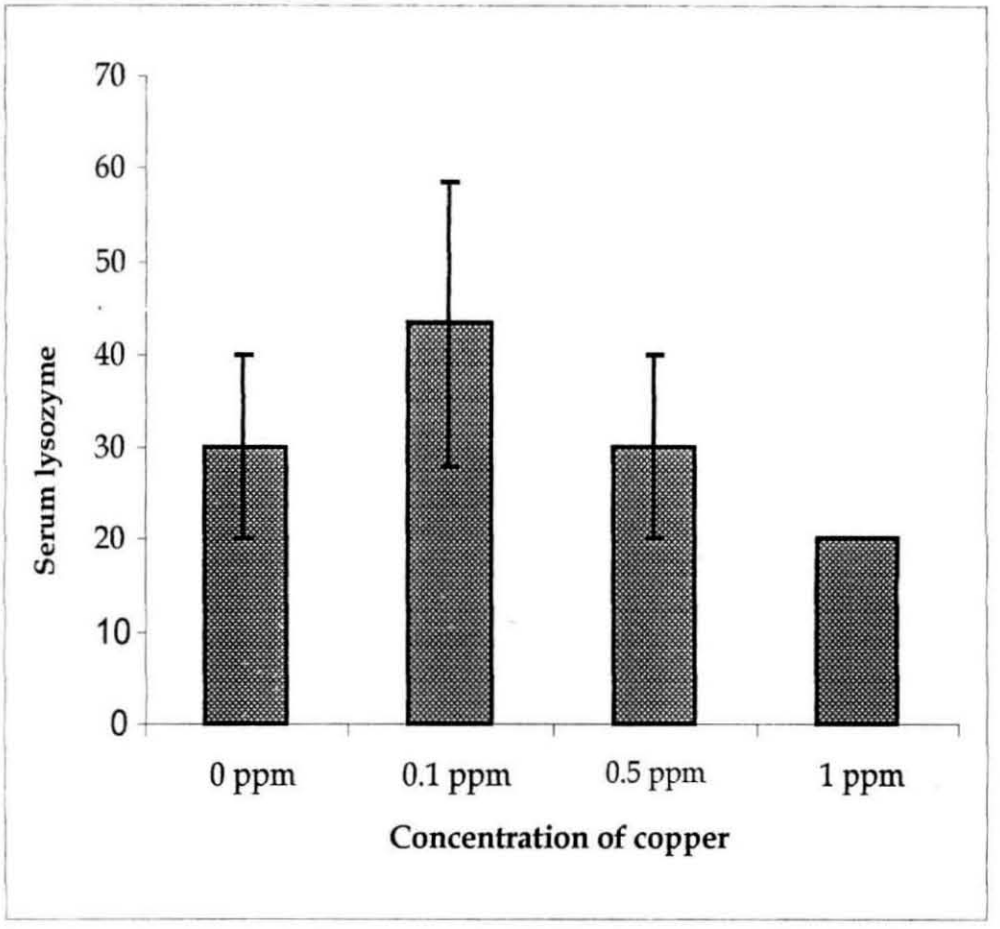


Figure 16: Mean serum lysozyme (lysozyme units per ml of serum) of *C. madrasensis* exposed to different concentrations of copper

the control and the test at different time intervals (Table 21).

The mean percentage of granulocytes at different time intervals for test were significantly ($p< 0.05$) higher than the control at all the time intervals except at 2 weeks, where the result obtained for the control and the test were not significantly different (Fig. 18).

Up to 72 hours, the mean percentage of semigranulocytes was significantly ($p< 0.05$) lower in the test animals injected with *V. alginolyticus* compared to the control animals injected with saline (Fig. 19). At 120 hours, the mean value obtained for the control dropped significantly ($p< 0.05$), but the test (26.58 ± 6.04) value was almost the same as that obtained at 72 hours. At 1 week the mean percentage of semigranulocytes in both the control and the test were very low. But, by 2 weeks there remained no difference between mean percentage of semigranulocytes for the control and the test (Fig. 19).

The mean percentage of hyalinocytes in the test was less compared to the control up to 24 hrs after injection. At 72 hours, the mean percentage of hyalinocytes in the test increased and both control and test gave almost similar values. At 120 hours, the mean percentage of hyalinocytes in the control increased significantly (91.24 ± 2.71), but the test value did not show much change. At 1 week the percentage of hyalinocyte decreased to a very low value (2.98 ± 1.7) in the test. By 2 weeks, the control and test gave almost normal and similar values (Fig. 20).

4.4.1.3. Phagocytosis

4.4.1.3.1. Phagocytic index

The mean phagocytic index of the hemocytes for the control and the test are given in Table 22. The analysis of variance showed no significant difference ($p>0.05$) between control and test at different time intervals (Fig. 21).

Table 20: Total hemocyte count (x10^4 cells/ml of hemolymph) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Time interval	2 hrs	24 hrs	72 hrs	120 hrs	1 week	2 weeks
Control	271.5	39	41	184	136.5	122
	472.5	36	27.5	233.5	155	127
	289.67	36.75	38.75	220	235	122
Mean	**344.55667**	**37.25**	**35.75**	**212.5**	**175.5**	**123.667**
±SD	**111.17401**	**1.56125**	**7.23274**	**25.5881**	**52.3522**	**2.88675**
Test	537	17.2	12.25	87	89.5	117.5
	545	15	14	83	92	122.5
	529.5	15.75	10	93	77	112.5
Mean	**537.16667**	**15.9833**	**12.0833**	**87.6667**	**86.1667**	**117.5**
±SD	**7.751344**	**1.11841**	**2.0052**	**5.03322**	**8.03638**	**5**

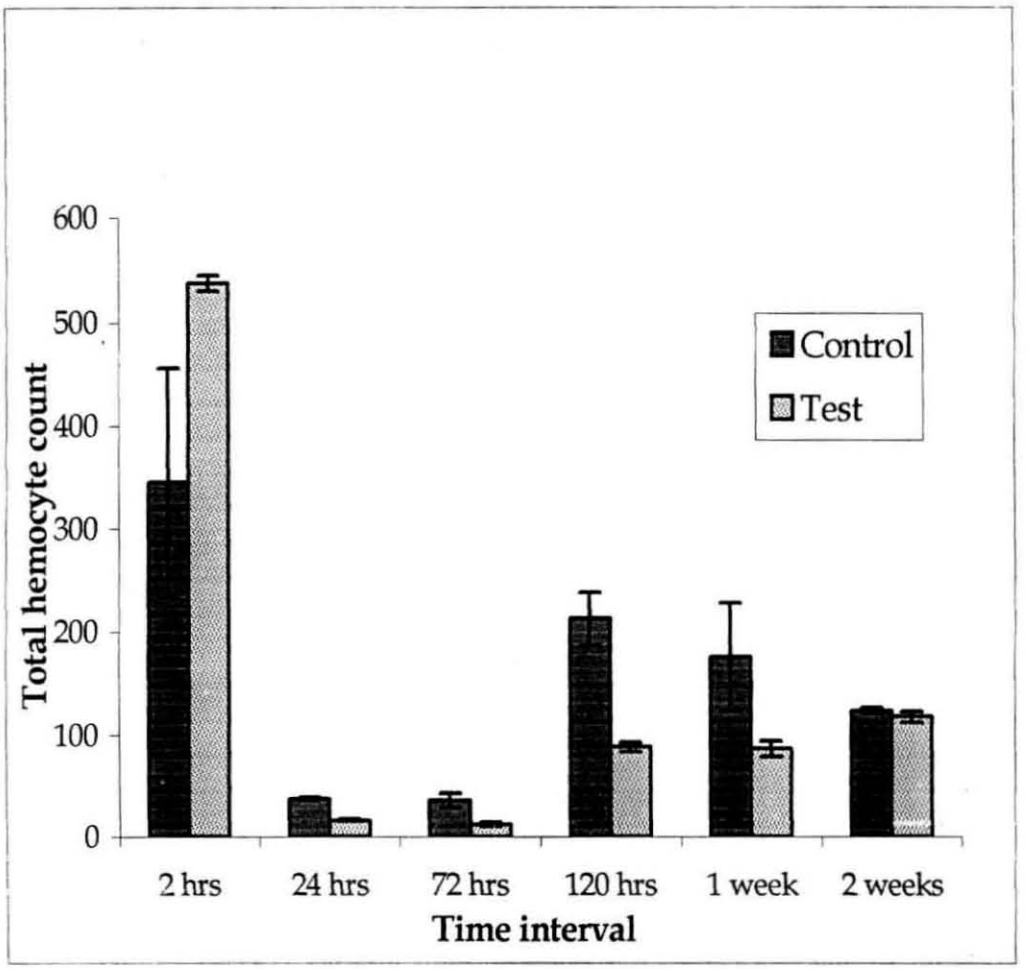


Figure 17: Mean hemocyte count (x10⁴ cells/ml of hemolymph) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Table 21: Percentage of different cells of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Percentage Cells/Time intervals		2 hrs	24 hrs	72 hrs	120 hrs	1 week	2 weeks
Control		43.65	57.05	6.12	0	21.25	38.52
	Granulocytes	24.23	46.43	11.79	0.43	18.06	35.04
		38.21	19.73	2.5	1.14	15.11	36.74
	Mean	35.36333	41.07	6.803333	0.523333	18.14	36.76667
	±SD	10.01807	19.2287	4.682546	0.575702	3.070782	1.740153
		34.07	21.15	40.82	6.53	13.55	34.02
	Semigranulocytes	40.64	25.71	64.29	11.35	15.48	39.37
		31.19	59.18	52.5	6.82	11.7	35.23
	Mean	35.3	35.34667	52.53667	8.233333	13.57667	36.20667
	±SD	4.843583	20.76582	11.73504	2.703005	1.890141	2.805536
		22.28	21.8	53.06	93.47	65.2	27.41
	Hyalinocytes	35.13	27.86	33.92	88.22	66.46	25.59
		30.6	21.09	45	92.04	73.19	28.03
	Mean	29.33667	23.58333	43.99333	91.24333	68.28333	27.01
	±SD	6.517487	3.720676	9.609627	2.714154	4.295746	1.268227
Test		86.03	75.36	27.44	44.83	79.89	35.32
	Granulocytes	88.33	88.33	33.64	31.33	82.61	37.96
		85.74	74.6	26.45	34.41	79.87	40.44
	Mean	86.7	79.43	29.17667	36.85667	80.79	37.90667
	±SD	1.419049	7.716988	3.896926	7.075	1.576198	2.560417
		8.57	7.25	24.39	33.33	18.99	40.43
	Semigranulocytes	3.3	10	30.91	21.69	14.13	35.1
		8.69	22.22	29.68	24.73	15.58	35.11
	Mean	6.853333	13.15667	28.32667	26.58333	16.23333	36.88
	±SD	3.077862	7.968603	3.46428	6.037262	2.495002	3.074394
		5.4	17.39	48.17	21.84	1.12	24.25
	Hyalinocytes	12.48	1.67	35.45	46.98	3.26	26.94
		5.57	3.18	43.87	40.86	4.55	24.45
	Mean	7.816667	7.413333	42.49667	36.56	2.976667	25.21333
	±SD	4.03946	8.672971	6.47025	13.11001	1.732465	1.498677

4.4.1.3.2. Endocytic index

The mean endocytic indexes of hemocytes at different time intervals, when exposed to bacteria are recorded in Table 23. Upto 24 hours after injection, both the control and test gave very low values for endocytic index. The value started to increase by 72 hours in the control and reached normal value by 120 hours (0.79 ± 0.13). The values at 2 hrs, 24 hrs and 72 hrs were lower than the values obtained at other time intervals. In the test, normal value was reached only by 2 weeks (0.66 ± 0.09) (Fig. 22). The values at all the other time intervals were low in the test compared to control. Analysis of variance showed no significant difference between control and test at different time intervals (LSD method), except at 24 hours, where the test value was significantly lower than the control value.

4.4.2. Humoral factors

4.4.2.1. Protein profile

4.4.2.1.1. Total serum protein

The mean total serum protein values as µg/ml of serum in the hemolymph of *C. madrasensis* at different time intervals, after injection with sterile saline (control) and saline with *V. alginolyticus* (test) are given in Table 24. The analysis of variance at 5% level showed significant difference between the control and the test at different time intervals. After 72 hrs of injection, the control gave a significantly high value (1090.22 ± 8.71 µg/ml of serum) compared to test. The test value significantly increased compared to the control after that, and reached the maximum value (1049.38 ± 205.76 µg/ml of serum) at 120 hrs. At the same time, the control value significantly decreased and reached normal values. After 1 week of injection, test values were also normal (Fig. 23).

4.4.2.1.2. SDS - PAGE

Two hours after injection, the control gave bands of molecular weights 90, 86, 74, 60, 52, 31 and 26 kDa. In the test samples, the 90

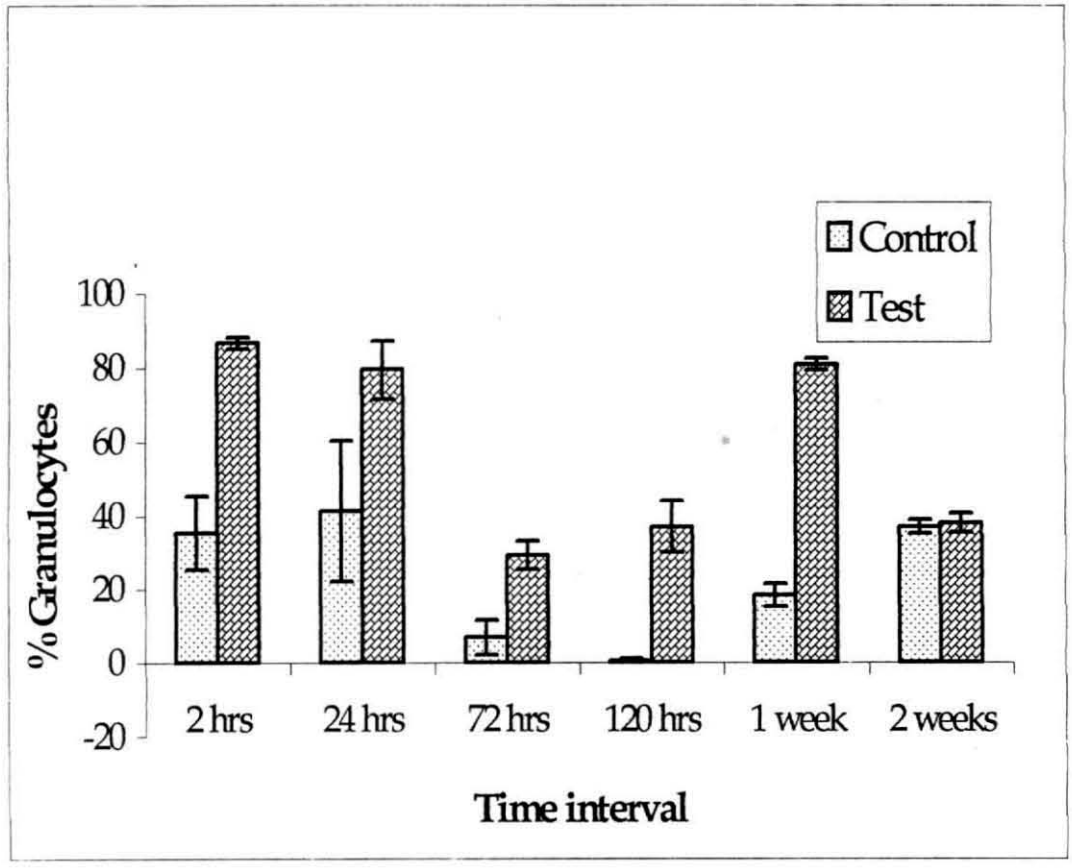


Figure 18: Mean percentage of granulocytes of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

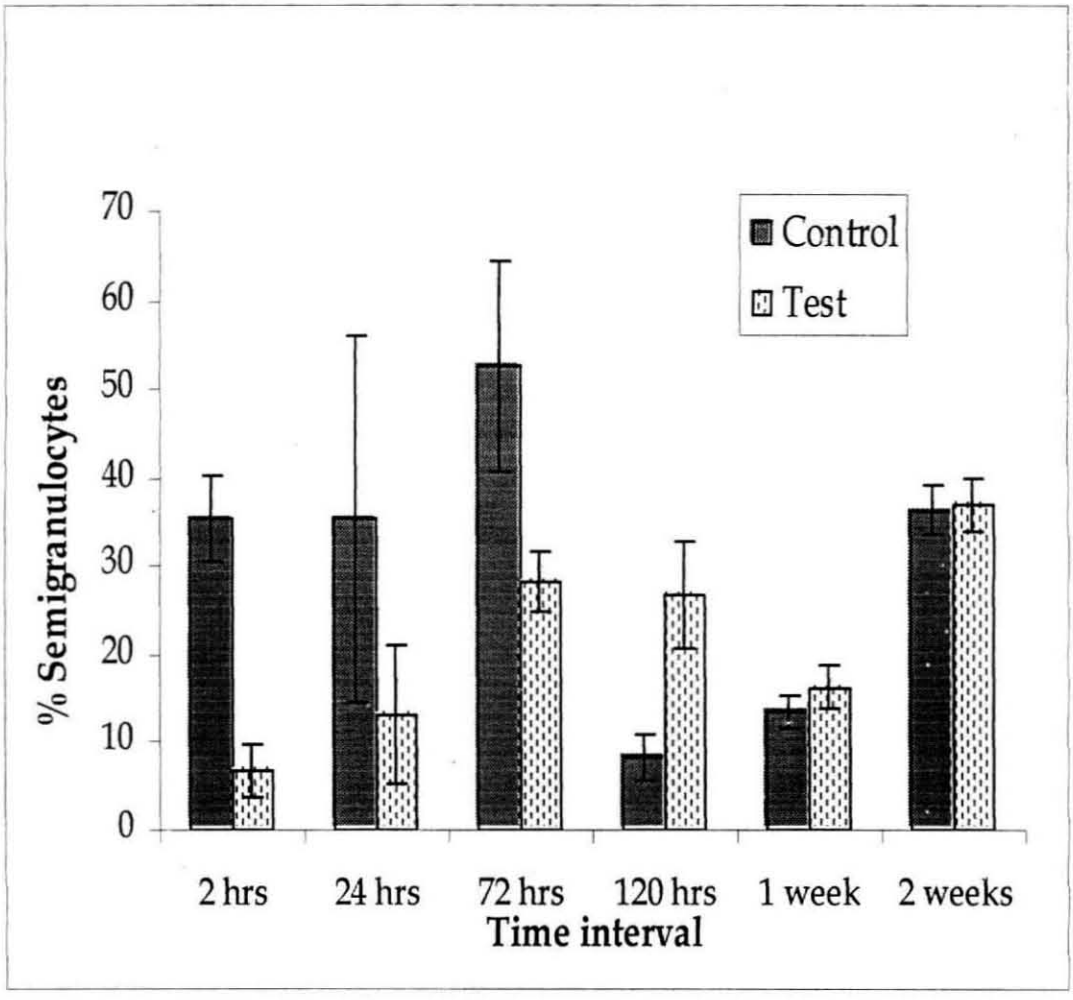


Figure 19: Mean percentage of semigranulocytes of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

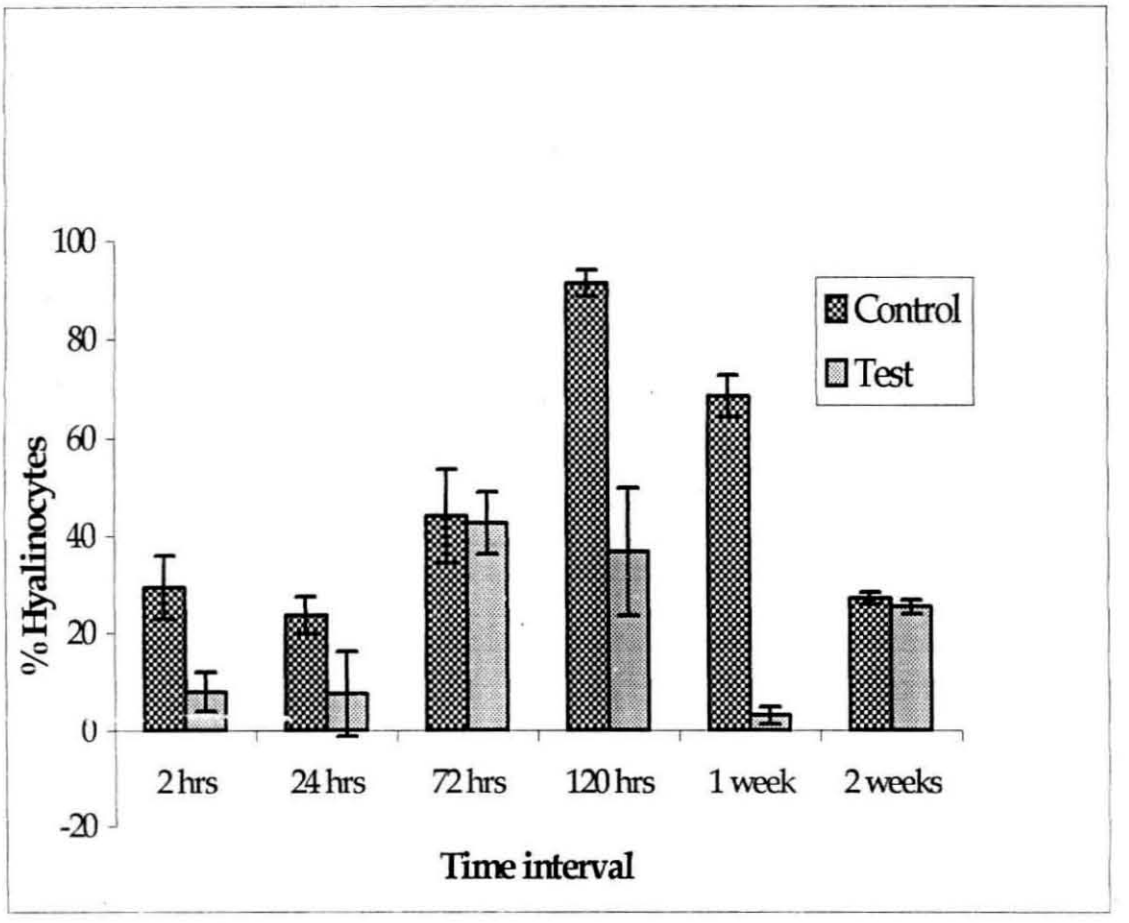


Figure 20: Mean percentage of hyalinocytes of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Table 22: Phagocytic index of hemocytes of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Treatments	Rep/Time	2 hrs	24 hrs	72 hrs	120 hrs	1 week	2 week
Control	1	29	18	44	37	46	50
	2	31	23	50	56	38	56
	3	29	32	52	32	45	48
	Mean	**29.66667**	**24.33333**	**48.66667**	**41.66667**	**43**	**51.3333**
	±SD	**1.154701**	**7.094599**	**4.163332**	**12.66228**	**4.358899**	**4.1633**
Test	1	23	16	46	19	26	40
	2	17	25	42	28	44	42
	3	30	38	31	21	25	47
	Mean	**23.33333**	**26.33333**	**39.66667**	**22.66667**	**31.66667**	**43**
	±SD	**6.506407**	**11.06044**	**7.767453**	**4.725816**	**10.69268**	**3.60553**

Table 23: Endocytic index of hemocytes of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Treatments	Replication	2 hrs	24 hrs	72 hrs	120 hrs	1 week	2 week
Control	1	0.34	0.16	0.48	0.86	0.84	0.93
	2	0.35	0.27	0.43	0.86	0.88	0.84
	3	0.39	0.54	0.52	0.64	0.65	0.75
	Mean	**0.36**	**0.323**	**0.477**	**0.787**	**0.79**	**0.84**
	±SD	**0.026**	**0.195**	**0.045**	**0.127**	**0.122**	**0.09**
Test	1	0.28	0.18	0.37	0.24	0.43	0.59
	2	0.15	0.33	0.28	0.35	0.73	0.76
	3	0.36	0.49	0.33	0.24	0.39	0.64
	Mean	**0.263**	**0.333**	**0.327**	**0.277**	**0.517**	**0.663**
	±SD	**0.106**	**0.155**	**0.045**	**0.063**	**0.186**	**0.087**

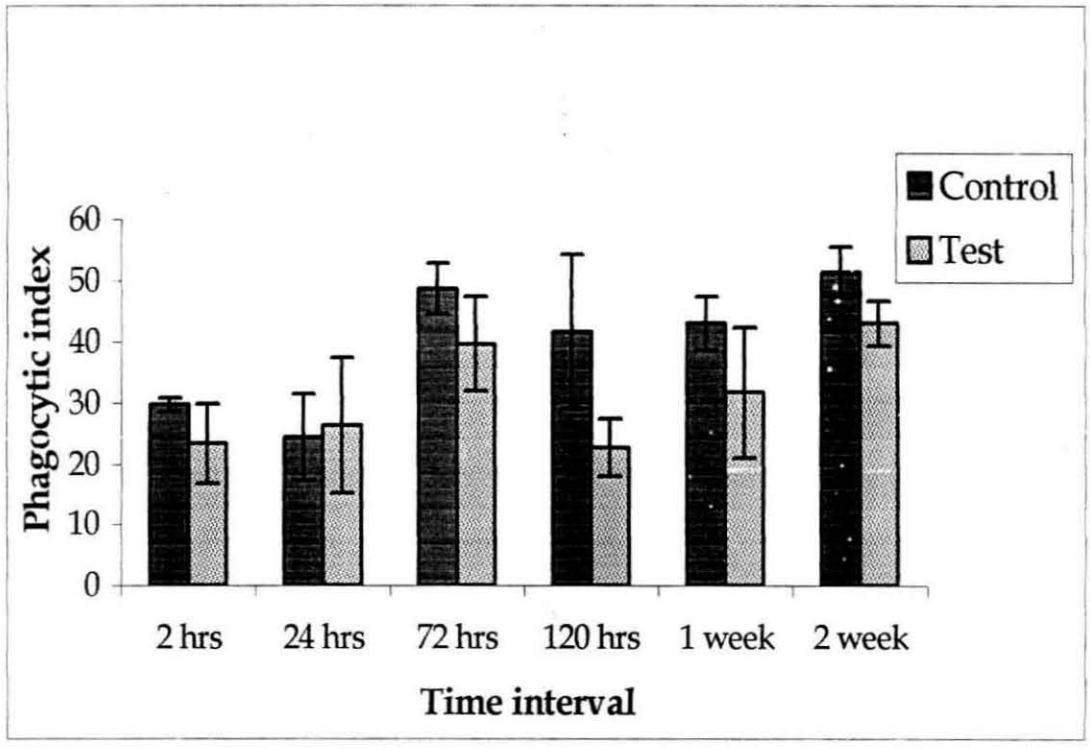


Figure 21: Mean phagocytic index of hemocytes of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

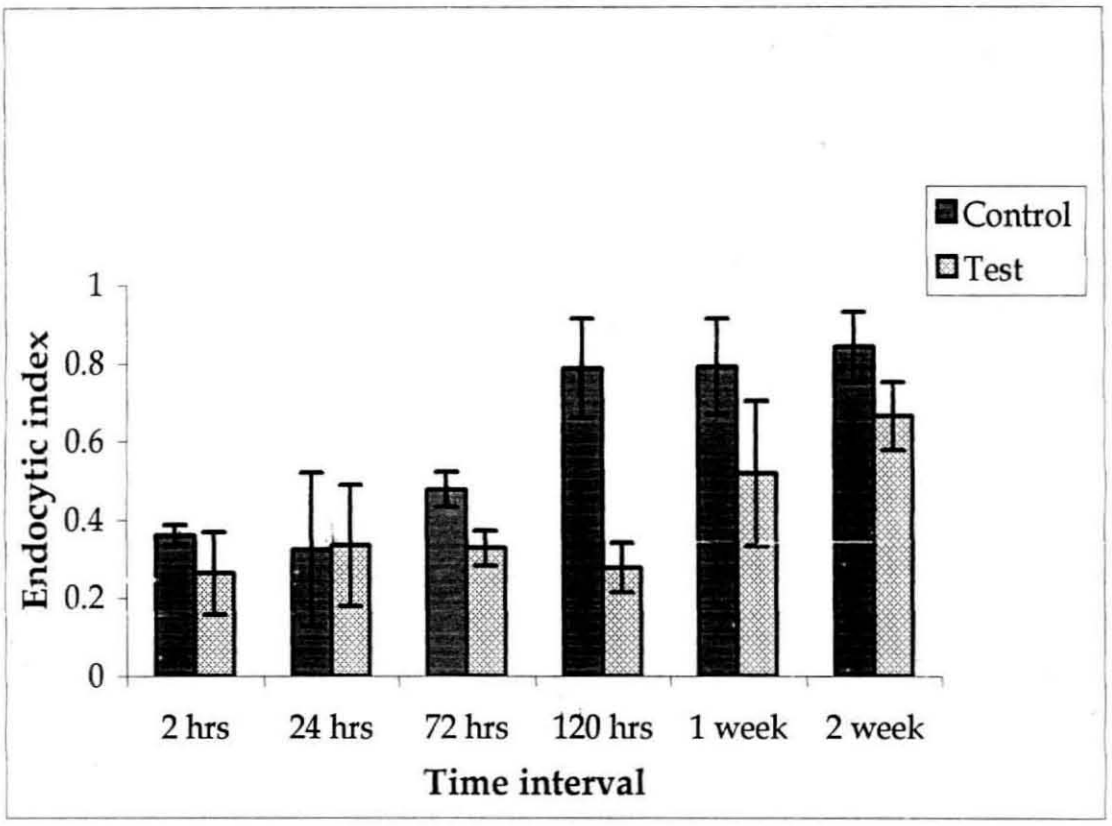


Figure 22: Mean endocytic index of hemocytes of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Table 24: Total serum protein (μg/ml of serum) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Treatments	Replications	2 hrs	24 hrs	72 hrs	120 hrs	1 week	2 week
Control	1	603.42	720.8	1082.62	709.4	558.4	503.6
	2	633.62	618.23	1088.32	695.16	524.22	476.91
	3	658.89	467.24	1099.72	689.46	495.73	511.55
	Mean	**631.98**	**602.09**	**1090.22**	**698.01**	**526.12**	**497.353**
	±SD	**27.77**	**127.55**	**8.7069**	**10.270**	**31.378**	**18.145**
Test	1	514.53	390.3	575.5	814.82	555.56	519.54
	2	548.15	367.52	552.7	1199.43	586.9	495.56
	3	547.01	401.71	618.23	1133.9	618.23	468.92
	Mean	**536.56**	**386.51**	**582.14**	**1049.38**	**586.897**	**494.67**
	±SD	**19.09**	**17.407**	**33.266**	**205.76**	**31.335**	**25.3216**

Table 25: Serum acid phosphatase (KA units) of *C. madrasensis* exposed *V. alginolyticus* at different time intervals

Treatments	Replication	2 hrs	24 hrs	72 hrs	120 hrs	1 week	2 week
Control	1	0.758	0.415	0.467	0.562	0.48	0.453
	2	0.702	0.456	0.444	0.532	0.48	0.406
	3	0.702	0.373	0.444	0.443	0.535	0.484
	Mean	**0.720**	**0.415**	**0.452**	**0.512**	**0.4983**	**0.4477**
	±SD	**0.032**	**0.0415**	**0.013**	**0.06189**	**0.0318**	**0.0392**
Test	1	2.13	2.93	1.238	0.769	0.553	0.5
	2	1.68	2.033	0.888	0.799	0.609	0.531
	3	1.994	2.49	0.981	0.621	0.48	0.344
	Mean	**1.935**	**2.484**	**1.036**	**0.73**	**0.547**	**0.458**
	±SD	**0.231**	**0.449**	**0.181**	**0.095**	**0.0647**	**0.1002**

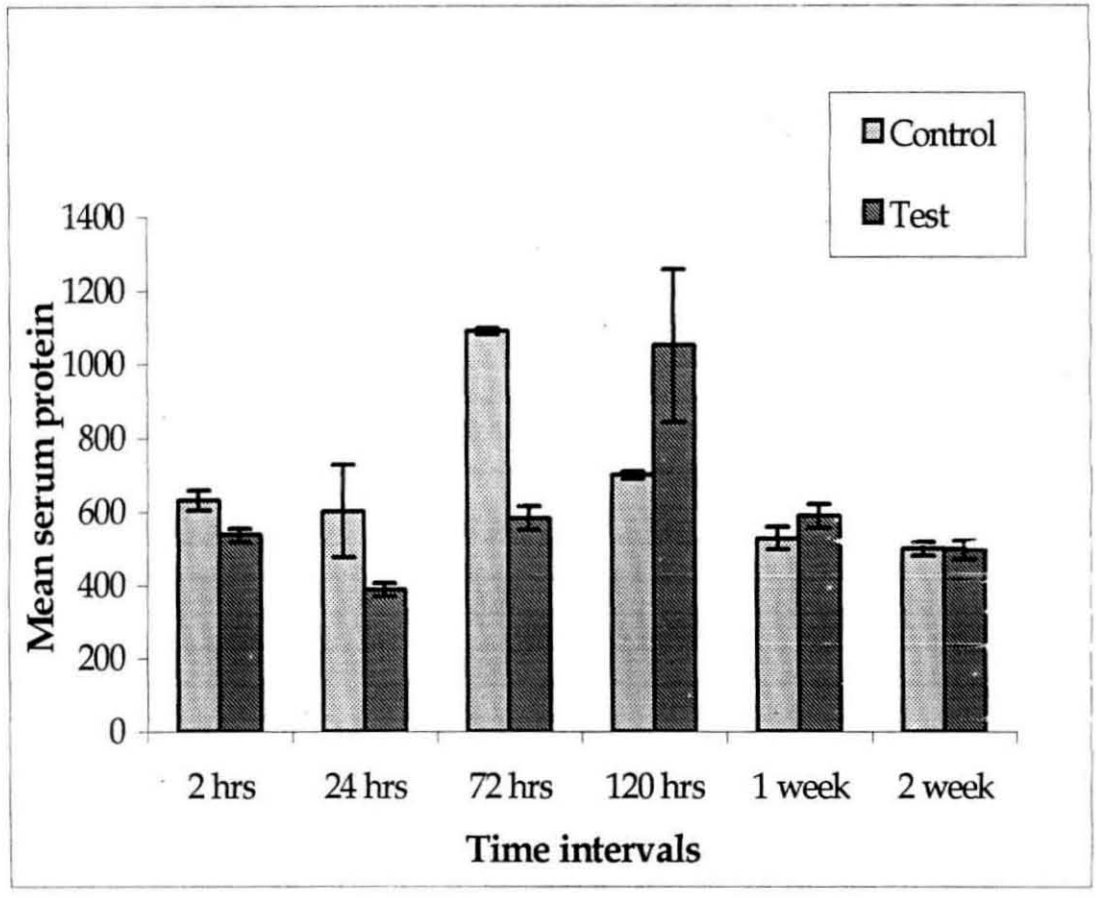
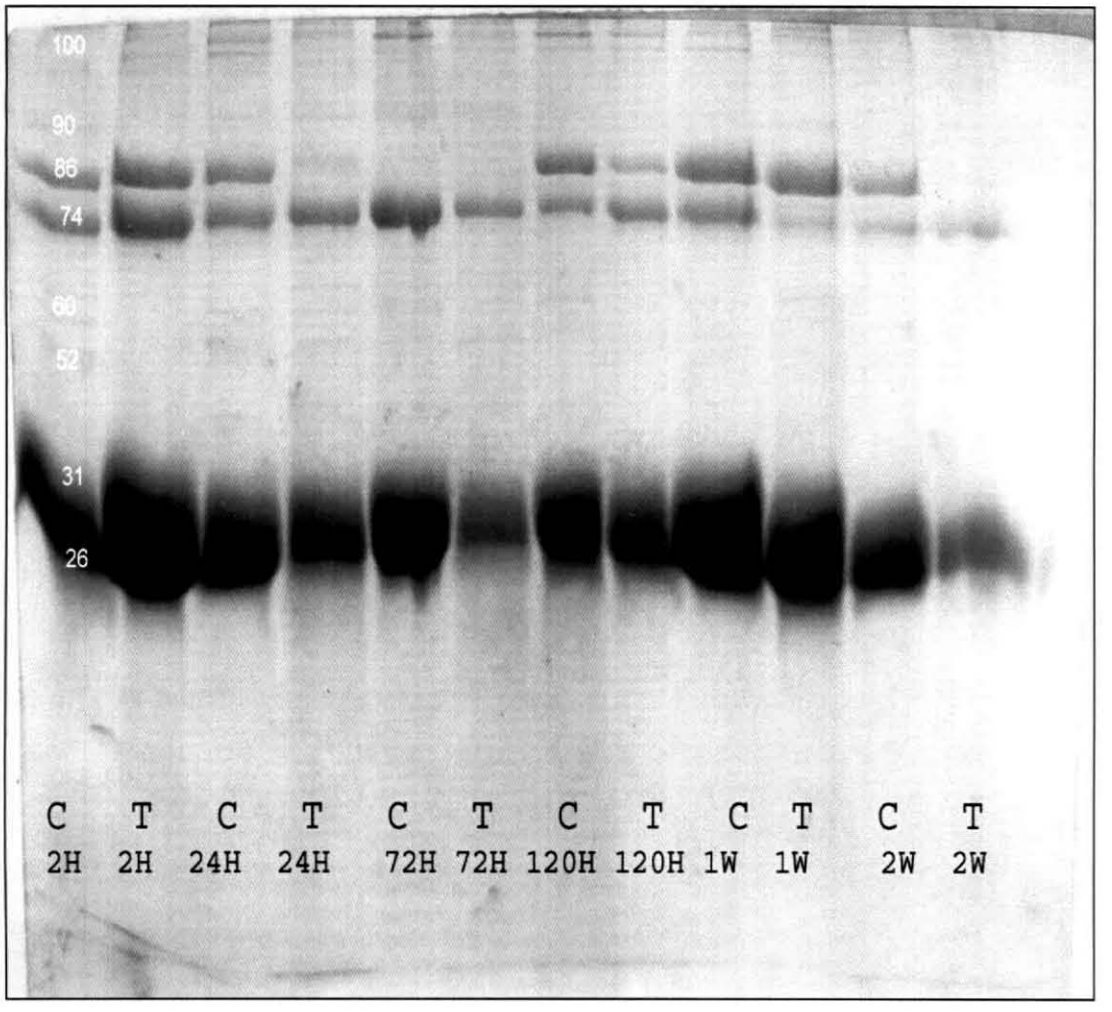


Figure 23: Mean serum protein (μg/ml of serum) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Plate 21: SDS - PAGE of the serum of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals


100
90
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26
C T C T C T C T C T C T
2H 2H 24H 24H 72H 72H 120H 120H 1W 1W 2W 2W

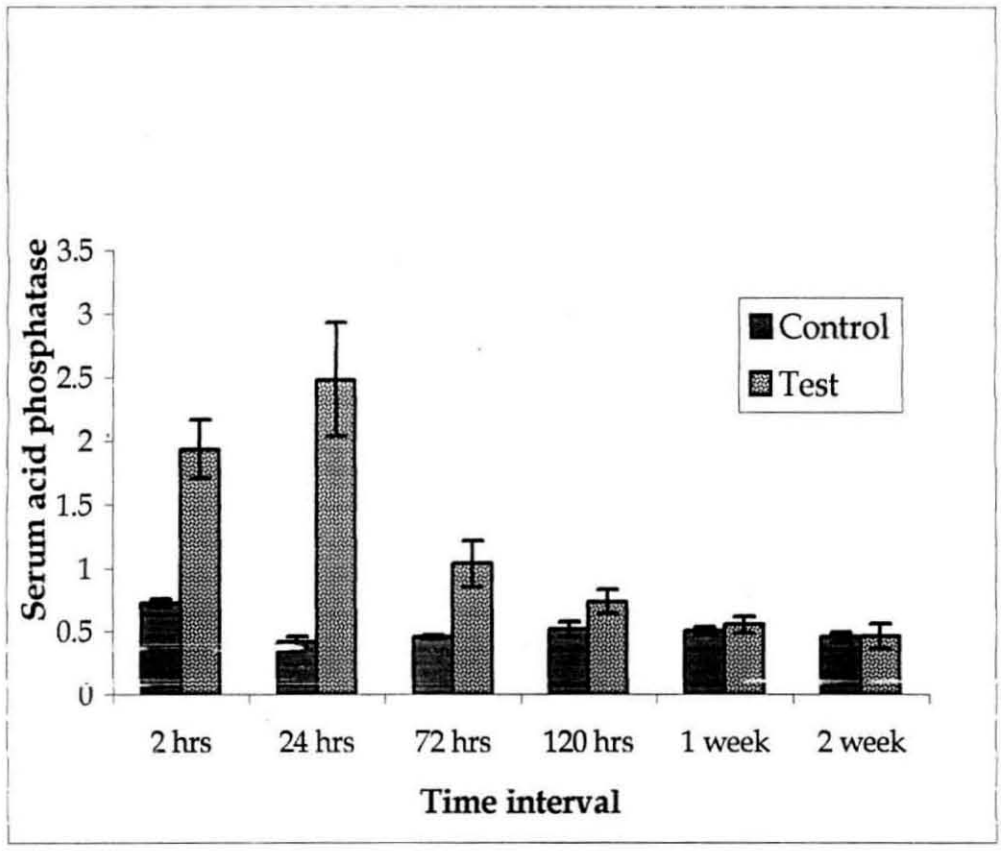


Figure 24: Mean serum acid phosphatase (KA units) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

kDa band was absent and the 86, 74, 31 and 26 kDa bands were more intense compared to control.

At 24 hours after the injection, the bands above 100 kDa were only feeble in test compared to control. One band of above 100 kDa was absent in both control and test. The 90 kDa band was absent in control but present in test. All the other bands (86, 74, 60, 52, 31 and 21 kDa bands) were present, but the intensity was less compared to the 2 hour sample.

At 72 hours, only 2 bands above 100 kDa were present in the control but 3 bands were present in the test. 90 kDa band was absent in both the control and the test. The major band 86 kDa was absent in both the control and the test. The 74 kDa band was less intense in the test compared to the control. The 60 and 52 kDa bands were present in both the control and the test. The 26 and 31 kDa bands were less dense both in control and test compared to the previous samples.

At 120 hours, the three bands above 100 kDa were present in both control and test. The 90 kDa band was absent both in control and the test. The 86 and 74 kDa bands were more intense in test compared to control. The 60 and 52 kDa bands were less visible in test compared to control. The 26 and 31 kDa bands were more intense in test.

After one week, the bands of above 100 kDa molecular weight were present in both control and test. The 90 kDa band was absent in control, but present in test. The 86 and 74 kDa bands were of high intensity compared to the previous time intervals. The 60 and 52 kDa bands were less intense in control compared to test. The 26 and 31 kDa bands were highly intensive compared to the previous time intervals.

After 2 weeks, all the bands of molecular weight above 100 kDa were present in control but only one band was present in test. The 90 kDa band was absent in both control and test. The major bands 86, 74, 26 and 31 kDa bands were normal in both control and test. The other minor bands of 60 kDa and 52 kDa were absent in both control and test (Plate 21).

4.4.2.2. Enzyme Assays

4.4.2.2.1. Serum acid phosphatase

Table 25 gives the mean serum acid phosphatase in the different treatments at different time intervals. Compared to the normal values, the value at 2 hrs for control was high. There was a significant reduction ($p< 0.05$) after that and the normal value was reached within 24 hrs. There were no significant differences between the values obtained at 24 hrs, 72 hrs, 120 hrs, 1 week and 2 weeks in control. The mean amount of acid phosphatase of test showed a significant increase (1.93 ± 0.23 KA units) at 2 hrs of injection compared to control. The mean value again increased significantly at 24 hrs (2.48 ± 0.44 KA units). There was significant decrease at 72 and 120 hours and the mean value reached almost the same as that of control (0.55 ± 0.06 KA units) by one week (Fig. 24).

4.4.2.2.2. Serum phenol oxidase

The mean serum phenol oxidase (ΔOD/mg serum protein/minute) of *C. madrasensis* exposed to *V.alginolyticus* at different time intervals are recorded in Table 26. Analysis of variance showed significant difference between the different treatments ($p< 0.05$) (Table 54). The result showed that there was a significant increase in the amount of serum phenol oxidase at 24 hours after injection in control and test. The test value (0.085 ± 0.004) increased much more when compared to the control. At 72 hours, both control and test value decreased, but test value (0.035 ± 0.004) was still high compared to control (0.015 ± 0.001). By 120 hours, both control and test reached normal values and remained stable until the end of experiment period (Fig. 25).

4.4.2.2.3. Serum lysozyme

The mean serum lysozyme values showed significant difference at 5% level according to analysis of variance between time and the treatments i.e. the control and the test (Table 27). There was a significant increase ($p< 0.05$) in the value obtained at 24 hrs in control compared to

Table 26: Serum phenol oxidase (Δ OD/mg serum protein/ minute) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Treatments	Replications	2 hrs	24 hrs	72 hrs	120 hrs	1 week	2 week
Control	1	0.0111	0.0379	0.016	0.0047	0.0072	0.0053
	2	0.0105	0.053	0.016	0.014	0.0102	0.0074
	3	0.0051	0.0457	0.015	0.00386	0.011	0.0052
	Mean	**0.0089**	**0.0455**	**0.0157**	**0.008**	**0.009**	**0.006**
	SD	**0.003**	**0.0076**	**0.001**	**0.0056**	**0.0020**	**0.001**
Test	1	0.0026	0.0872	0.037	0.0041	0.0072	0.009
	2	0.0043	0.087	0.0302	0.0056	0.008	0.008
	3	0.0049	0.0796	0.0378	0.0047	0.0086	0.0043
	Mean	**0.0039**	**0.08**	**0.035**	**0.0048**	**0.0079**	**0.007**
	SD	**0.0012**	**0.0043**	**0.0042**	**0.0008**	**0.0007**	**0.0025**

Table 27: Serum lysozyme (lysozyme units per ml of serum) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

Treatments	Replications	2 hrs	24 hrs	72 hrs	120 hrs	1 week	2 week
Control	1	24	30	20	30	20	20
	2	28	30	30	20	40	20
	3	16	60	40	60	30	10
	Mean	**22.667**	**40**	**30**	**36.667**	**30**	**16.667**
	SD	**6.1101**	**17.321**	**10**	**20.816**	**10**	**5.7735**
Test	1	52	80	60	40	40	10
	2	36	80	80	40	20	10
	3	48	40	60	30	30	10
	Mean	**45.333**	**66.667**	**66.667**	**36.667**	**30**	**10**
	SD	**8.3266**	**23.094**	**11.547**	**5.7735**	**10**	**0**

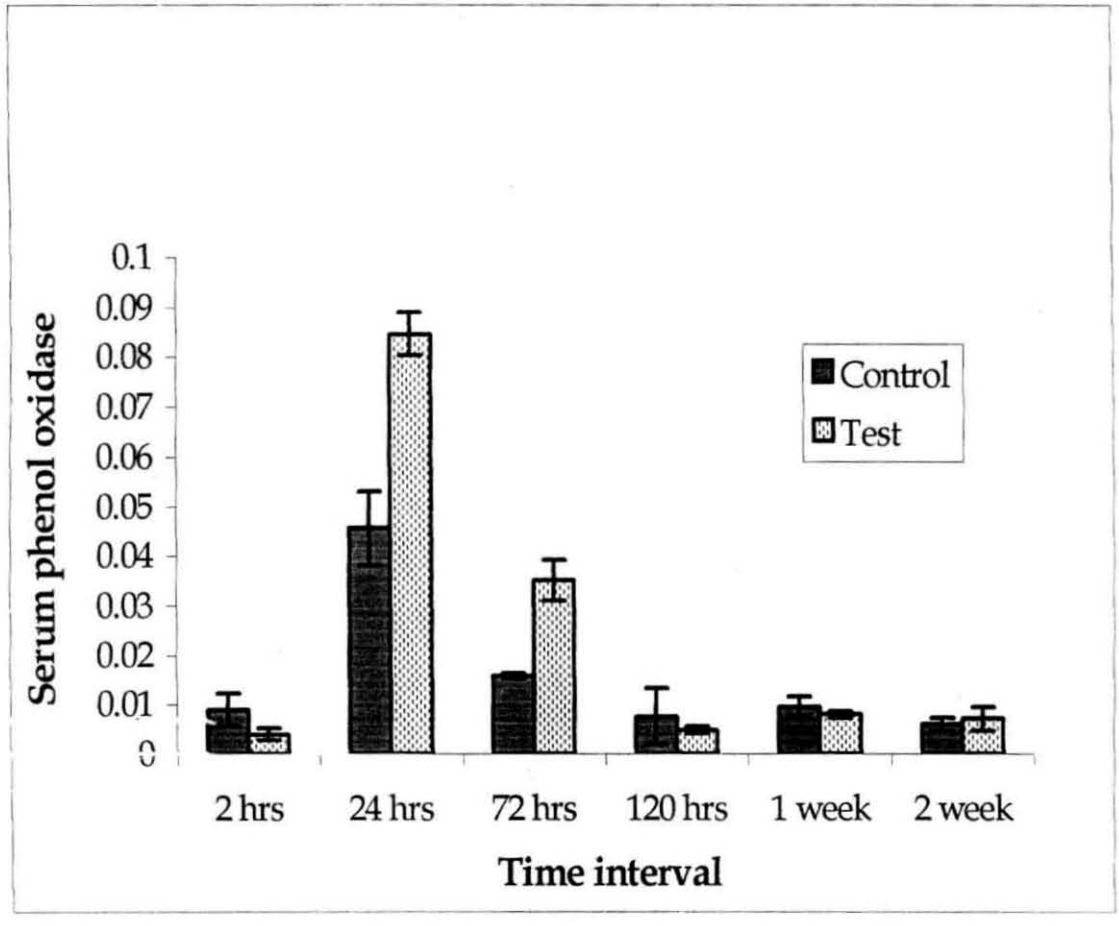


Figure 25: Mean serum phenol oxidase (Δ OD/mg serum protein/ minute) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

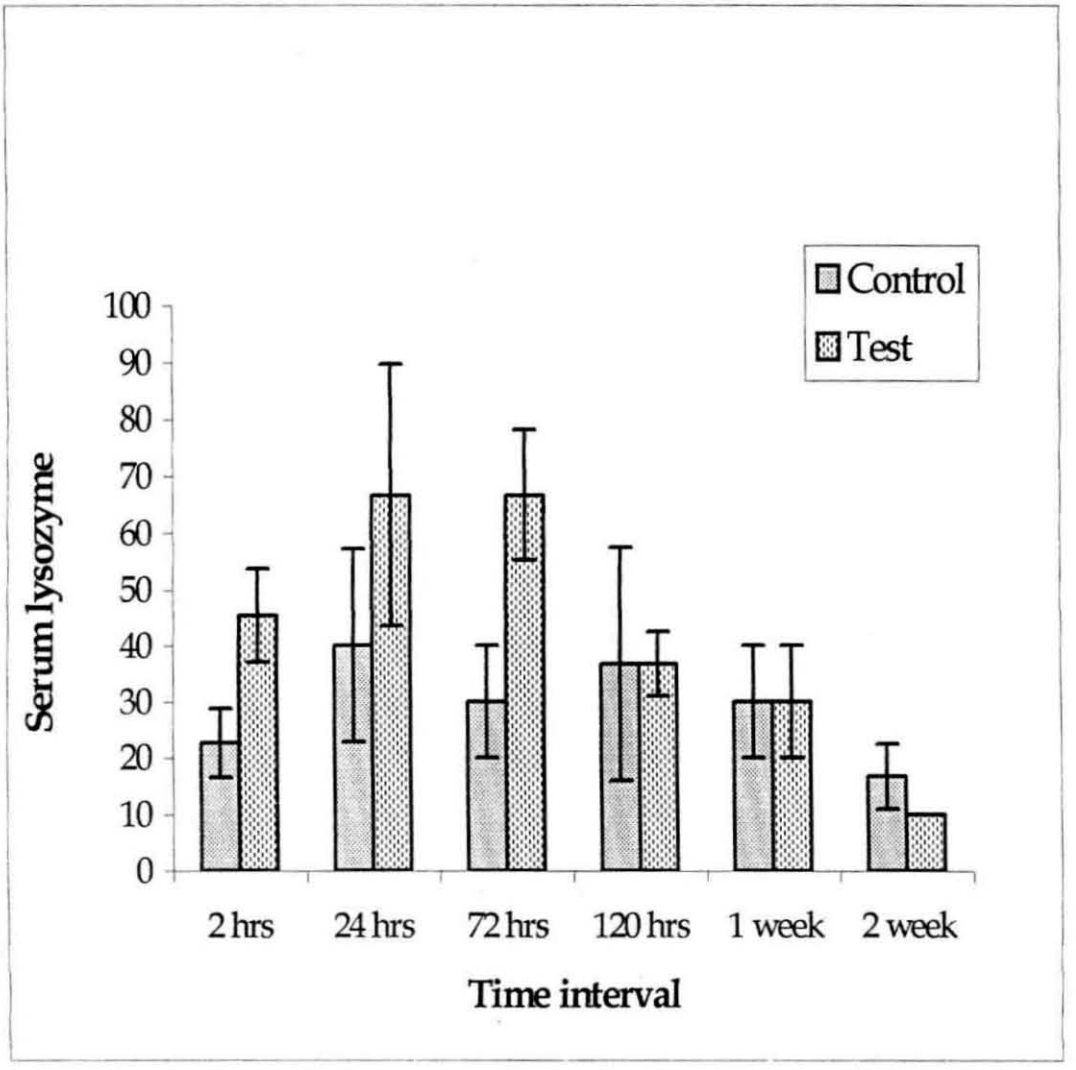


Figure 26: Mean serum lysozyme (lysozyme units per ml of serum) of *C. madrasensis* exposed to *V. alginolyticus* at different time intervals

the value obtained at 2 hrs. After that up to 1 week, there was no significant change in the values obtained. But at 2 weeks, again the value decreased significantly. Compared to the control, the test values were significantly high at 2 hrs, 24 hrs and at 72 hours. After that, both control and test gave almost similar values. At 24 hrs, the test value increased significantly compared to the value obtained at 2 hours. The value did not change at 72 hrs, but at 120 hrs, there was a significant reduction and the value returned to the normal range. However, there was a significant reduction again at 2 weeks (Fig. 26).

4.5. Histological changes as a result of exposure to pollutants

4.5.1. Histology of apparently healthy *C. madrasensis*

4.5.1.1. Adductor muscle

The light microscopic studies of the sections of adductor muscle revealed the following details (Plate 22). The adductor muscle fibers were enclosed by fascial sheaths of fibrous tissue, the epimysium. The epimysium was covered with a layer of ciliated pseudo stratified epithelial cells and below this; there was a layer of glandular cells.

4.5.1.2. Mantle

The mantle was composed mainly of muscular and connective tissue network. The columnar epithelium covered the mantle (Plate 23).

4.5.1.3. Gills

Gills of *C. madrasensis* consisted of filaments that were joined to each other by ciliary interfilamentar junctions. A branchial vein ran through each filament. The epithelium was composed of ciliated and unciliated cell types. The frontal cells were ciliated. Lateral cells were also ciliated. There were numerous glands in the abfrontal area (Plate 24).

4.5.2. Effect of exposure to Nuvan

4.5.2.1. Adductor muscle

In Nuvan treated animals, the thickness of epimysium appeared to have increased. The surface of epithelial cells showed focal denudation and loss of cilia (Plate 25).

4.5.2.2. Mantle

In Nuvan treated animals, the epithelial cells showed significant changes depending on the concentration of the Nuvan. In the mantle of the animals treated with 0.1 ppm Nuvan, the epithelial cells showed mild vacoulation and focal denudation (Plate 31), while in higher dose (0.2 ppm) areas of desquamation of the epithelial cells were noted (Plate 32).

4.5.2.3. Gills

In the gills of animals exposed to Nuvan, there was loss of lateral cilia and abfrontal cells (Plate 35), disorganization of filaments and frontal fusion. Some cells had become cuboidal. There was enlargement of mucous glands. The branchial vein was shrunken and there was interfilamentar fusion also.

4.5.3. Effect of exposure to copper

4.5.3.1. Adductor muscle

In the animals maintained in 0.5 ppm copper, the epimysium had become thickened and wavy, with a lot of fibrous tissue growth in the sub-epithelial layer (Plate 26). In the perimysial area, tissue growth was seen. The muscle fibers appeared fragmented and hyalinized (Plate 27). There were areas of muscle fibre necrosis with fibrous tissue growth (Plate 28).

In 1 ppm copper treated animals, the surface epithelial cells of epimysium became shrunken and cilia were lost (Plate 29). There was extensive necrosis and disappearance of muscle fibres with growth of fibrous tissue (Plate 30).

Plate 22: Section of adductor muscle of the normal, healthy *C. madrasensis* (40X)

Plate 23: Section of the mantle tissue showing columnar epithelium and sub-epithelial connective tissue (40X)

Plate 24: Structure of a normal gill of *C. madrasensis* (40X)

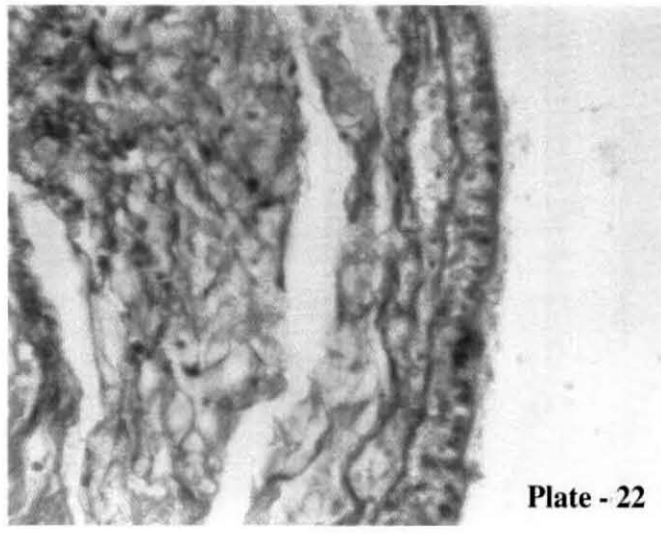


Plate - 22

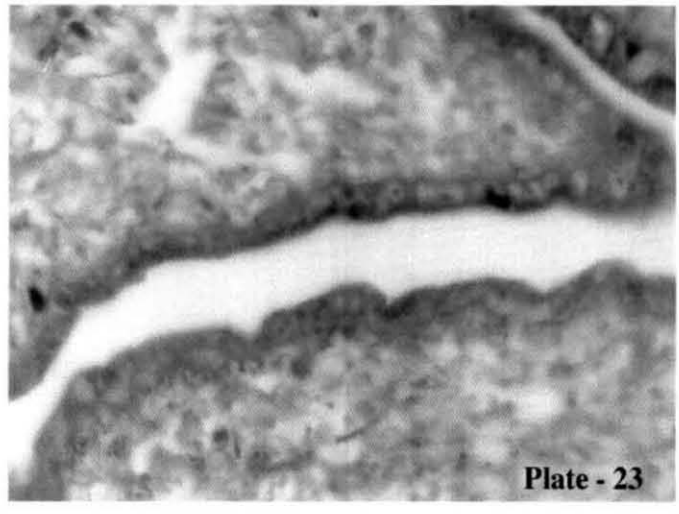


Plate - 23

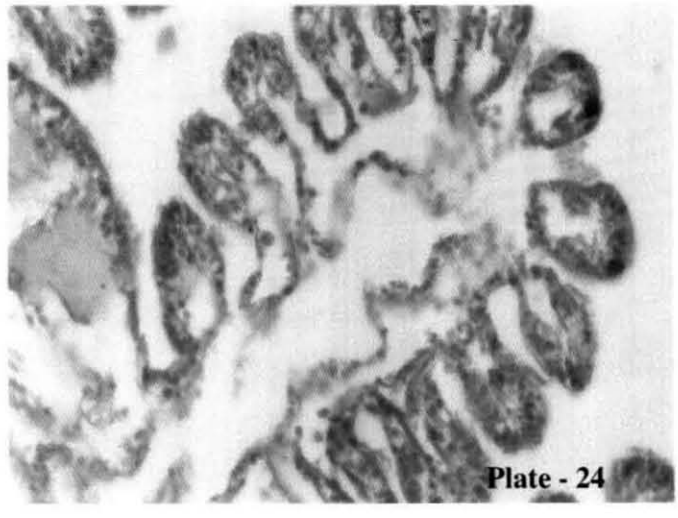


Plate - 24

Plate 25: Section of the adductor muscle of *C. madrasensis* showing thickened epimysium, focal denudation and loss of cilia in the animals exposed to Nuvan (40X).

Plate 26: Section of the epimysium showing thickening and wavy appearance and sub-epithelial fibrous tissue growth in the animals exposed to copper (40X).

Plate 27: Section showing muscle fibre fragmentation and hyalinization in the animals exposed to copper (40X).

Plate 28: Section showing muscle fibre necrosis and fibrous tissue growth (F) in the animals exposed to copper (40X).

Plate 29: Section showing loss of cilia in the epithelial cells of epimysium in the animals exposed to copper (40X).

Plate 30: Section showing complete necrosis of muscle fibers and growth of fibrous tissue in the animals exposed to copper. A few islands of degenerating muscle tissue can also be seen in the section (40X).

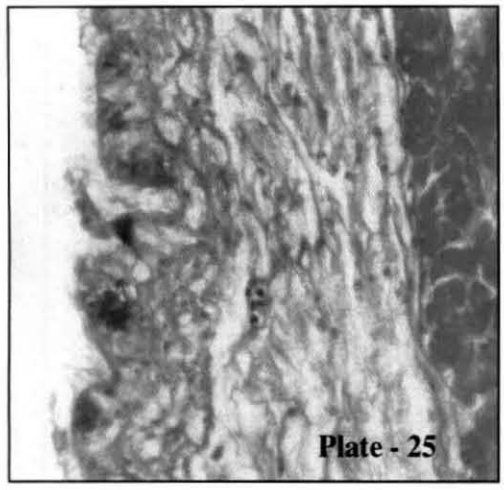


Plate - 25

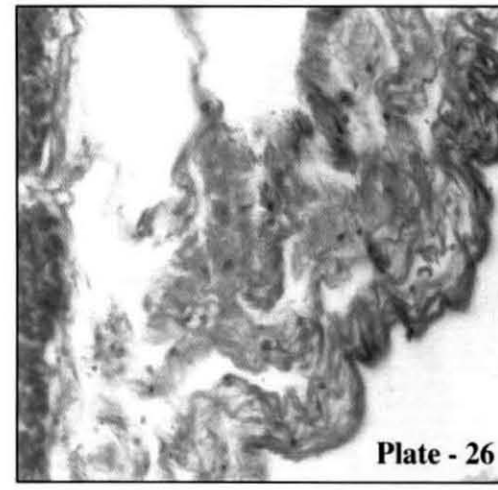


Plate - 26

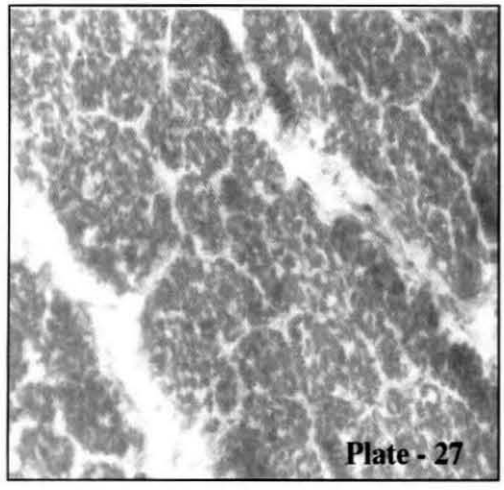
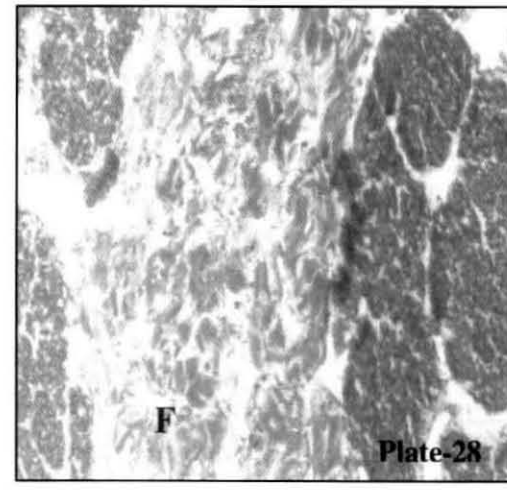


Plate - 27



F

Plate-28

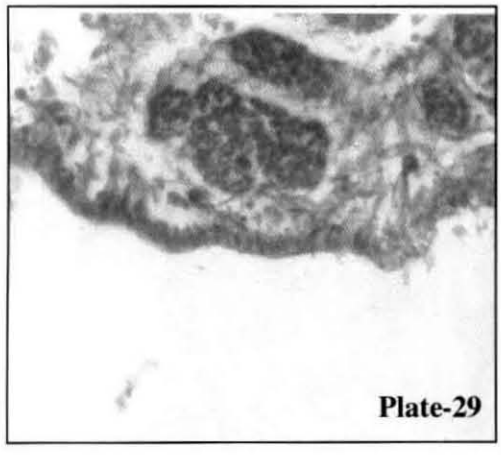


Plate-29

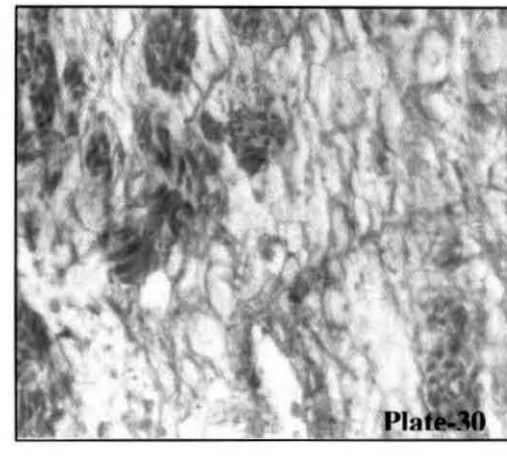


Plate-30

4.5.3.2. Mantle

The mantle surface epithelium in the 0.5 ppm treated animals revealed focal areas of necrosis and appearance of increased number of mucous secreting cells. In some areas, focal proliferation of the epithelial cells was also noticed (Plate 33). In the animals maintained in 1 ppm copper, extensive desquamation of epithelial cells was noticed and in other areas attempt for regeneration was also noticed (Plate 34)

4.5.3.3. Gills

The gills exposed to copper showed detachment of frontal, post lateral and abfrontal cells (Plate 36). There was vacoulation of post lateral cells (Plate 37) and extensive frontal and abfrontal fusion.

4.6. Inflammatory responses

4.6.1. Light microscopic studies

The control animals, which were injected with FCA in the experiments with Nuvan and copper, gave the following observations.

Inflammatory reactions observed at 24 hours consisted of exudates containing proteinaceous material and moderate amount of large hemocytes. These exudates contained fibrin like shreds (Plate 38).

On the third day, large hemocytes were found to accumulate in focal areas and exudates contained more fibrin like shreds (Plate 39). The perimysial layer covering the muscle fibre bundles became hypertrophied and proliferated. Small, nucleated cells appeared in many parts of the exudate from which, eosinophilic fibrils were radiating between the cells cris-crossing exudate. The hemocytes formed nodules, and the nodules consisted of cells that had spindle shaped/elongated cell body. These spindle shaped cells were arranged in concentric layers (Plate 40, 41).

By the seventh day, the number of hemocytes in the intermysium was much reduced and the mass of hemocytes was found to fuse together to form a granuloma like structure (Plate 42).

Plate 31: The epithelial cells of the mantle showing occasional vacoulation and the epithelial layer is detached from the connective tissue in the animals exposed to Nuvan (40X).

Plate 32: Section of mantle showing desquamation of the epithelial cells in the animals exposed to Nuvan (40X).

Plate 33: Mantle section showing areas of necrosis (N), increased number of goblet cells (G) and area of epithelial proliferation (E), in the copper exposed animals (40X).

Plate 34: Mantle epithelium showing extensive desquamation in copper treated animals (40X).

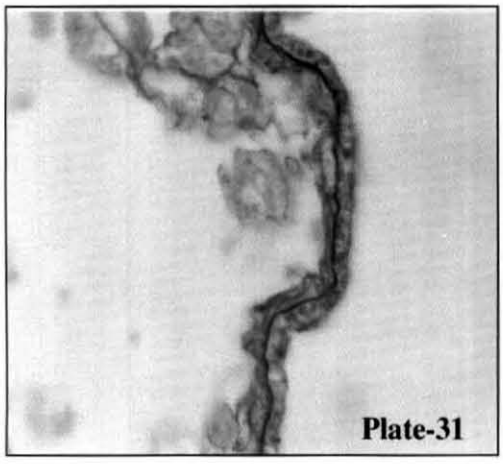


Plate-31

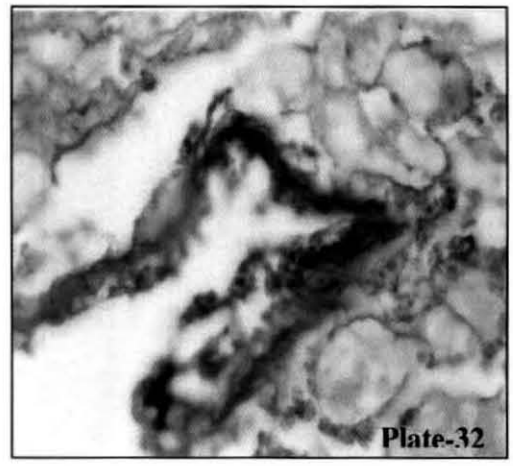


Plate-32

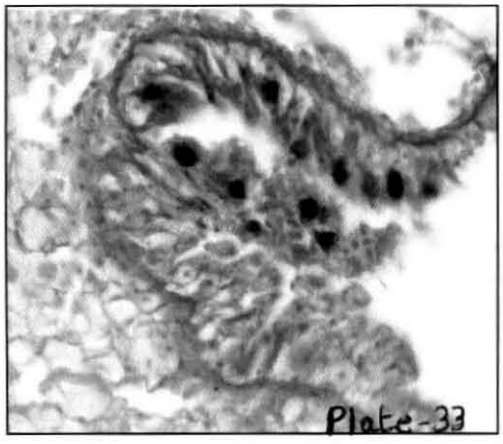


Plate-33

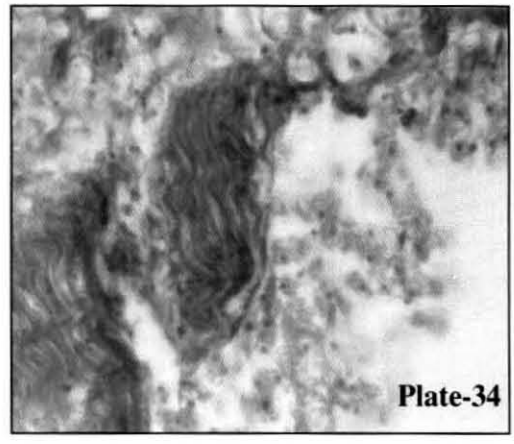


Plate-34

Plate 35: Gills of *C. madrasensis* exposed to Nuvan showing loss of lateral cilia (L) and abfrontal cells (A).

Plate 36: Gills of *C. madrasensis* exposed to Nuvan showing interfilamentar fusion and enlargement of mucous glands.

Plate 37: Gills of *C. madrasensis* exposed to copper showing detachment of frontal, postlateral and abfrontal cells.

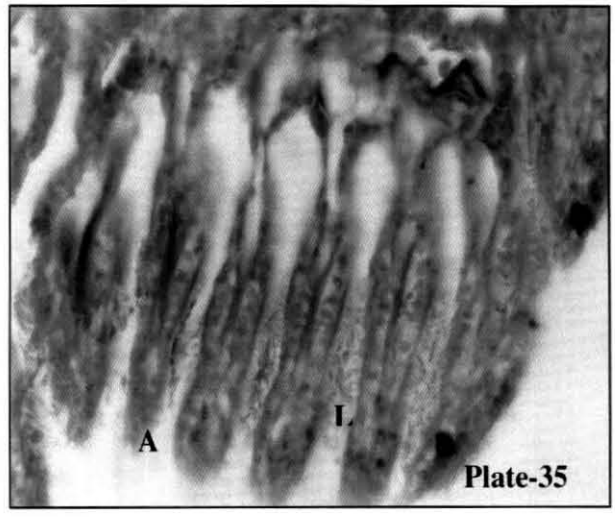


Plate-35

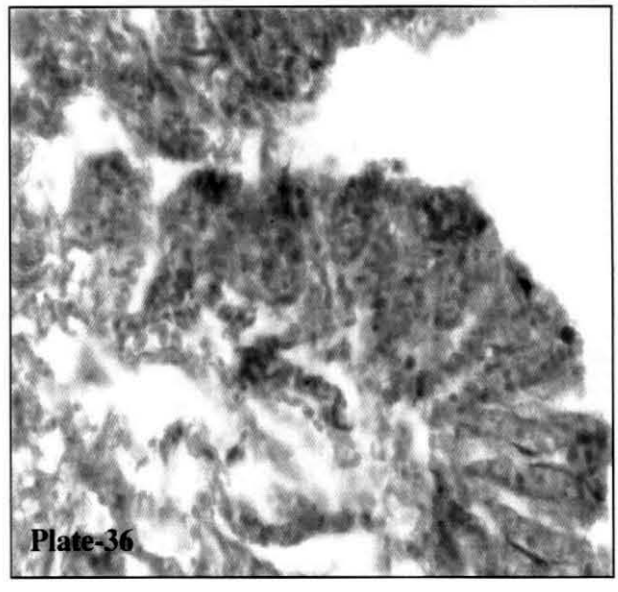


Plate-36

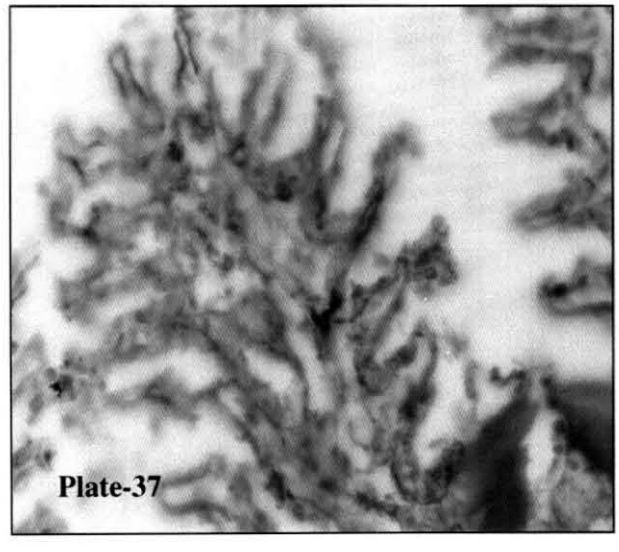


Plate-37

On the fourteenth day, the number of hemocytes was very much reduced. Intermysial exudate was also less, while epimysial exudates were moderately present and nodules disappeared.

The hemocytic infiltration in response to FCA injection was very less in the Nuvan and copper treated animals (Plate 43, 44, 45).

In the case of animals injected with *V. alginolyticus*, there was massive hemocytic infiltration to the injected area at 2hrs and 24 hrs after injection (Plate 47, 48); whereas the saline injection produced only mild hemocytic infiltration (Plate 46). There was complete healing within 120 hrs (Plate 49).

4.6.2. Ultrastructural studies.

At 2 hours of injecting with *V. alginolyticus*, disorganized areas containing fibrillar deposits indicated areas of necrosis. There were also vacuolated hemocytes. Bacteria like structures were seen in the vacuoles of hemocytes.

At 24 hours, a large number of hemocytes were seen in between muscles. The plasma membrane of the hemocyte was found to have vesicles budding off and these vesicles contained glycogen granules (Plate 50). Lysosomes were found to lay very close to plasma membrane and from these regions plasma membrane formed evaginations. In some regions, the plasma membrane lost its continuity and fine shreds of thin filaments were found to radiate from the discontinuous region to the outside (Plate 51). In many interstitial areas, free lysosomes, as well as, degenerating bacteria could be seen (Plate 52).

At 48 hours of injection, vesicles containing electron dense granules with outer membrane were seen. Hemocytes containing large number of lysosomes in the cytoplasm, which were being budded off from the cell membrane were seen. In these hemocytes also, lysosomes were found to discharge their contents by fusing with plasma membrane (Plate 53).

Plate 38: Inflammatory reactions in the control animals after injection of FCA, at 24 hours. See the moderate number of hemocytes, proteinaceous exudate and the fibrin shreds (40X).

Plate 39: Inflammatory reactions in the control animals on the third day after injecting with FCA, showing extensive infiltration of the hemocytes in the inflamed area. The fibrin like shreds increased in the matrix (40X).

Plate 40: Nodule formation in the third day of inflammation in the control animals (40X).

Plate 41: Nodule formation in the third day of inflammation in the control animals. Many of the cells in the nodule have assumed spindle shape (40X).

Plate 42: Granuloma formation in the seventh day at FCA injected site in the control animals (40X).

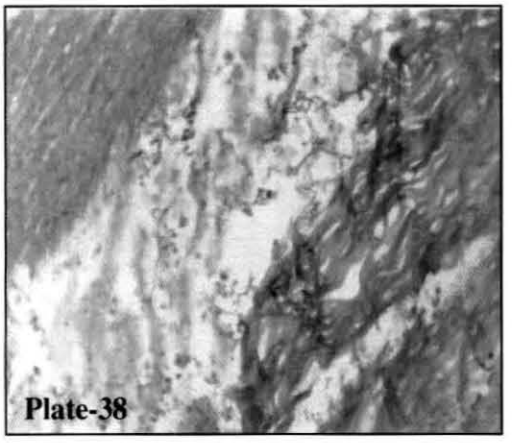


Plate-38

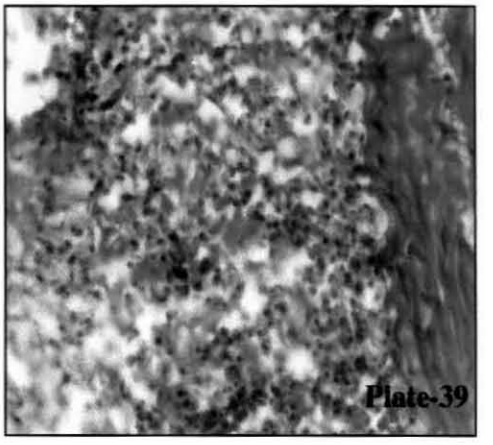


Plate-39

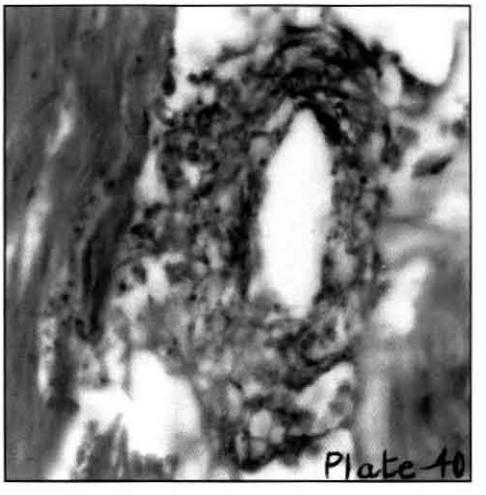


Plate-40

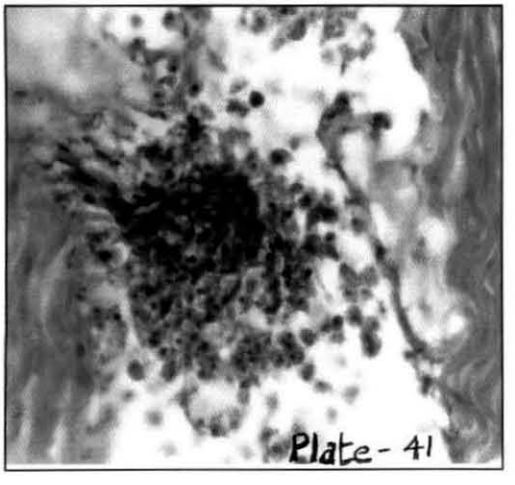


Plate - 41

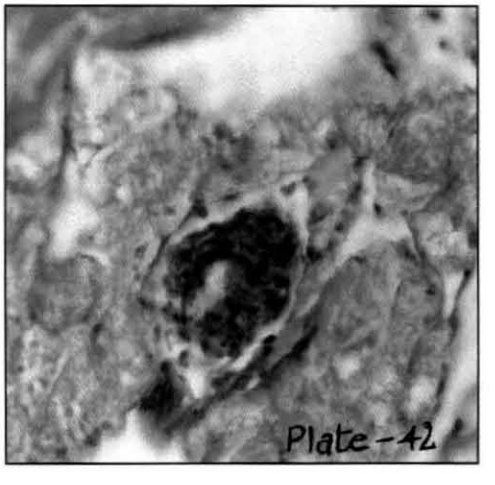


Plate - 42

Plate 43: Inflammation in Nuvan treated animals at 24 hours after FCA injection. Note the reduced number of hemocytes (40X).

Plate 44: Reduced inflammatory reactions in copper treated animals against FCA injection (40X).

Plate 45: Reduced inflammatory reactions in copper treated animals against FCA injection (40X).

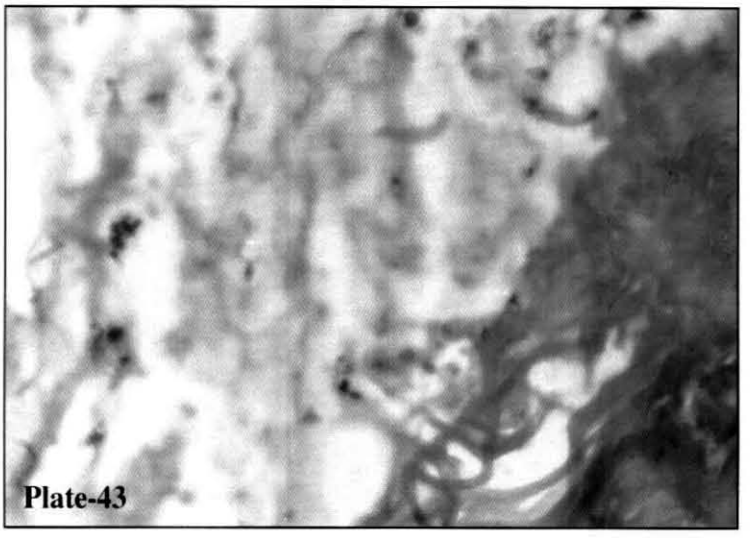


Plate-43

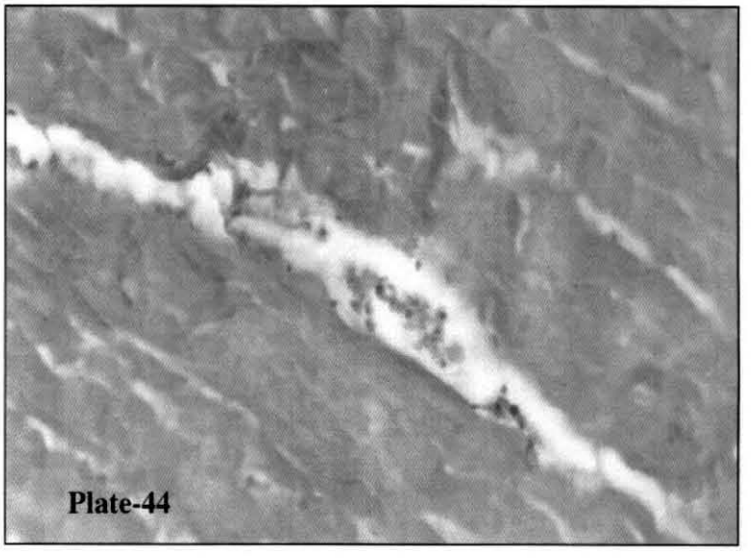


Plate-44

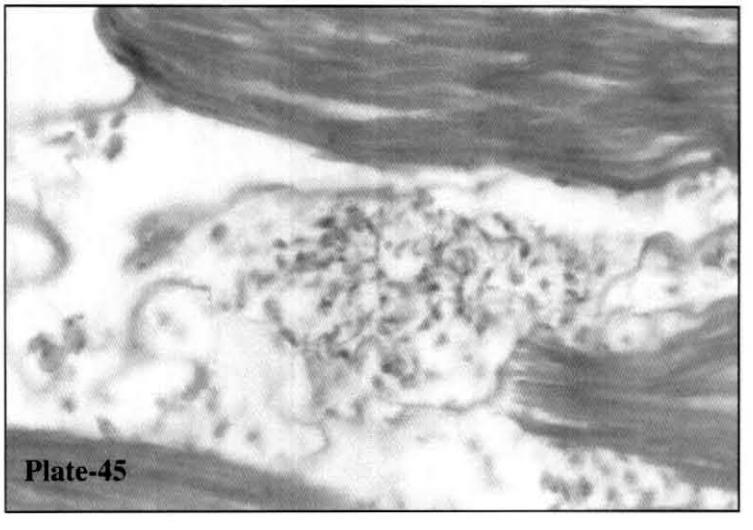


Plate-45

Plate 46: Section of adductor muscle showing mild hemocyte infiltration and exudate formation in the area of saline injection (40X).

Plate 47: Section of the adductor muscle showing multiplication of bacterial cells surrounded by hemocytes in *V. alginolyticus* injected sites after 2 hours (100X)

Plate 48: Hemocyte infiltration in the *V. alginolyticus* injected site at 24 hours (100X)

Plate 49: *V. alginolyticus* injected site 120 hours after injection showing complete healing (40X).

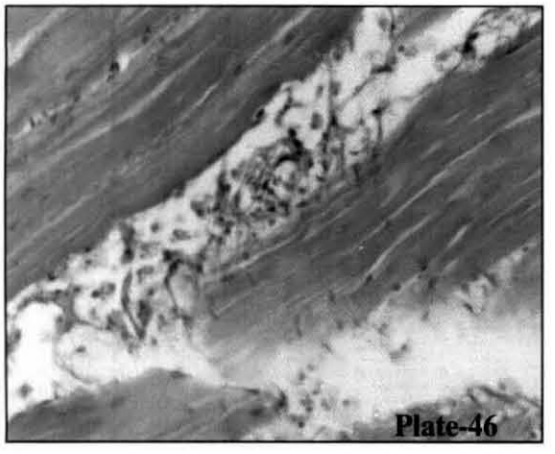


Plate-46

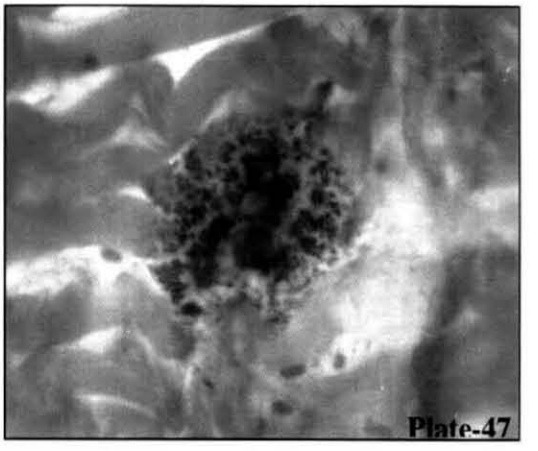


Plate-47

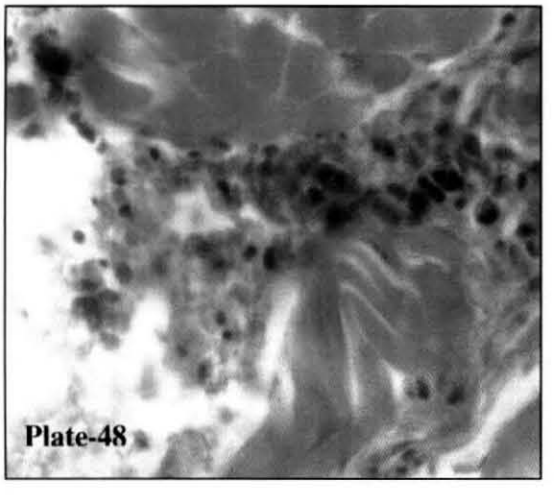


Plate-48

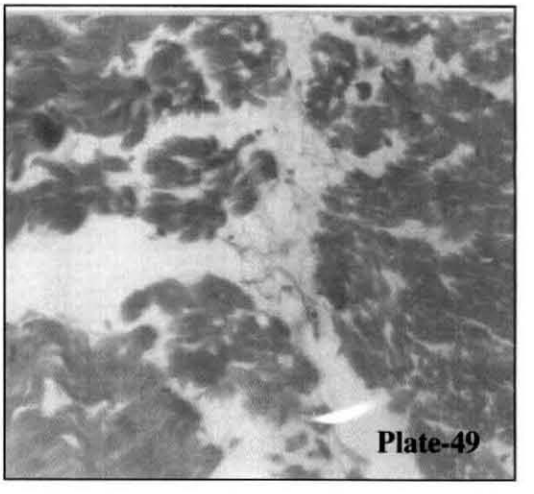


Plate-49

Filamentous materials had condensed into fibrin like materials. Degenerating bacteria were seen in the interstitial space (Plate 54). The hemocytes, probably granulocytes, with lot of vacuoles were seen at 72 hours (Plate 55). These vacuoles contained electron dense materials, which were at different stages of disintegration and some of these vacuoles contained granular materials, which appeared to have a structure similar to glycogen deposits. The buds from the plasma membrane also contained these granular materials.

The interstitial area contained large number of vesicles containing granular material, probably glycogen that was originated from the digestion of foreign material (Plate 56). Hemocytes, which were releasing lysosomes through budding, were visible in the interstitial space. The interstitial spaces were filled with filaments radiating in different directions, some of which were thickened to fibrin like material (Plate 57).

Plate 50: Hemocytes showing (B) budding of vesicles containing glycogen granules (D) degenerating bacteria like structures and (F) fibrin like thin filaments in the interstitial spaces. (3500 X)

Plate 51: Fibrin like materials originating from hemocytes. (17000 X)

Plate 52: Presence of lysosomes in the interstitial space. (17000 X)

Plate 53: Hemocyte with cytoplasm containing large number of lysosomes. Discharge of lysosome contents by fusion of lysosomes with plasma membrane can be seen. Fibrin filaments are also visible (5000 X).

Plate 54: Degenerating bacteria in the interstitial space. (12000 X)

Plate 55: A granulocyte that shows intracellular degradation of bacteria. Note the glycogen granules (G) in the lumina of secondary phagosome. Presence of vesicles (V) and mitochondria (M) can also be visualized. (10000X)

Plate 56: Hemocyte showing budding off of lysosomes (B). (5000 X)

Plate 57: Interstitial space with thin filaments as well as thick fibrin likes materials. Note also some lysosomes (L) present in the area. (6000X)

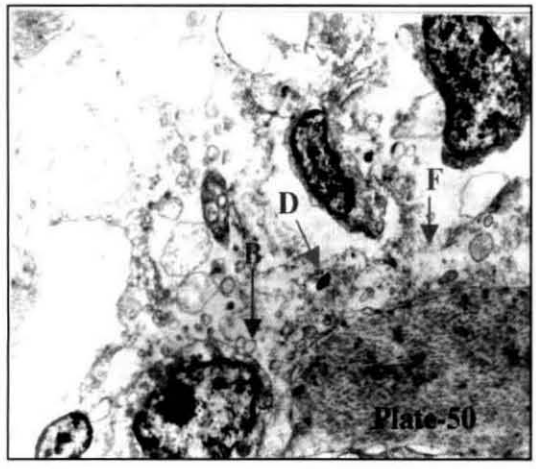


Plate-50

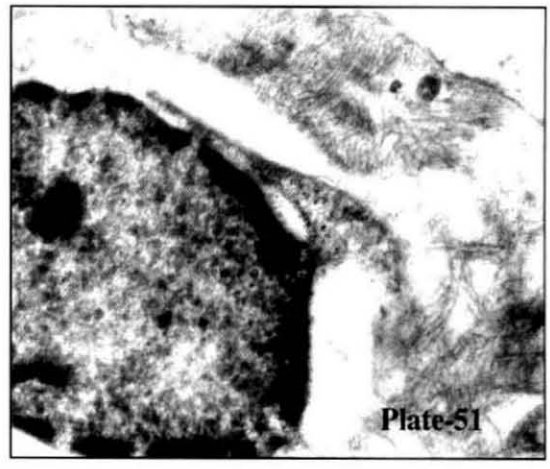


Plate-51

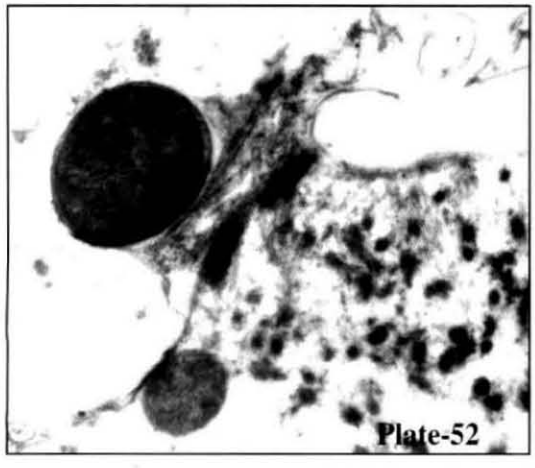


Plate-52

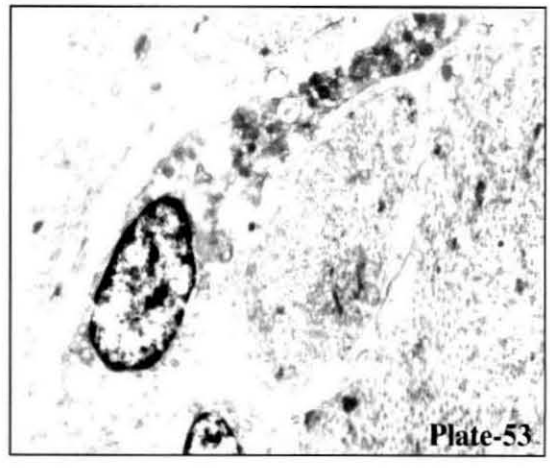


Plate-53


Plate-54

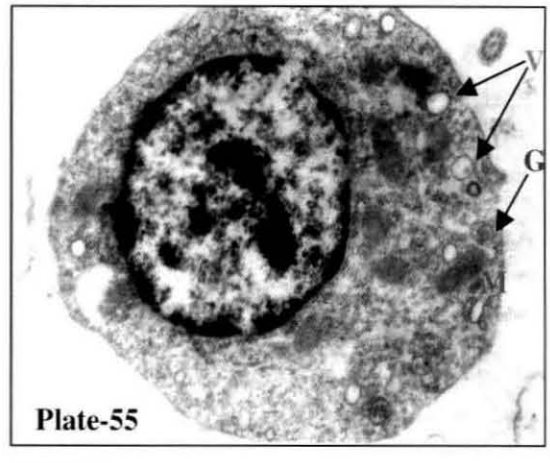


Plate-55

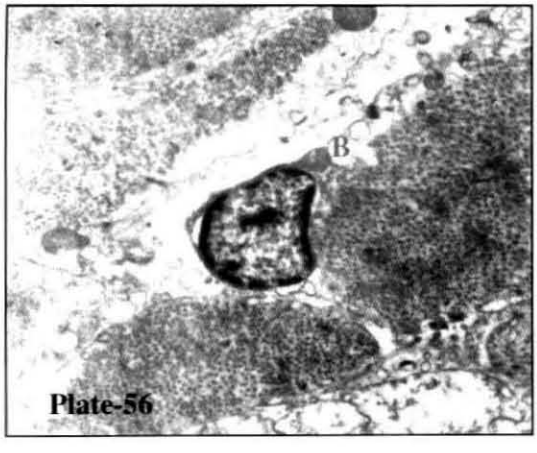


Plate-56

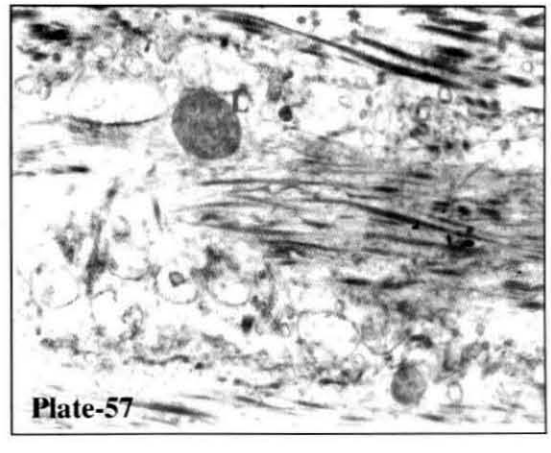


Plate-57

5. DISCUSSION

5.1. Hemolymph factors of apparently, healthy *C. madrasensis*

5.1.1. Cellular factors

5.1.1.1. Characterisation of the hemocytes

5.1.1.1.1. Microscopic studies

During the present investigations, both light and electron microscopic observations revealed the presence of mainly three types of hemocytes. Under light microscopy, the cells which appeared smaller with a large nucleus and small amount of cytoplasm without any granules were designated as hyalinocytes. The second type of cells was comparatively larger with small, oval to round, eccentric nucleus having large amount of cytoplasm containing granules. Among these cells, those with a few basophilic granules were named as semigranulocytes. Similar cells with abundant eosinophilic and basophilic granules in the cytoplasm were called as granulocytes. The electron microscopic studies also revealed three types of cells. One type of cells consisted of abundant cytoplasm with rough endoplasmic reticulum, mitochondria and a few vesicular structures. The second type of cells contained electron lucent granules that were scanty. The third type of cells had abundant number of electron lucent granules. All these cell types were characterized by the presence of nucleoli, rough endoplasmic reticulum, mitochondria and some vesicular structures. All the granules were electron lucent. In the tissues, among the granulocytes, cells with fibrin like material, which may be termed as fibrocytes, were also noticed.

Many authors have classified the hemocytes of molluscs. The cells are mainly divided into hyalinocytes and granulocytes (Cheng and Foley, 1975; Rasmussen *et al.*, 1985; Rodrich and Ulrich, 1984; Seiler and Morse, 1988; Suresh and Mohandas, 1990a). The granulocytes are further classified into granulocytes and semigranulocytes (Foley and Cheng, 1972; Moore and Lowe, 1977; Rasmussen *et al.*, 1985). The present observations are in agreement with the above findings. The hyalinocytes are generally smaller

cells and granulocytes are larger cells as reported in the case of *C. virginica* (Foley and Cheng, 1972; 1974) and *M. edulis* (Rasmussen *et al.*, 1985). The granules of the granulocytes have either eosinophilic/acidophilic (Nakayama *et al.*, 1997; Bayne *et al.*, 1979) or a mixture of acidophilic and basophilic granules (Foley and Cheng, 1972). The present study revealed that the granulocytes contained both acidophilic and basophilic granules, whereas, semigranulocytes contained only basophilic granules. This finding is very important, since granulocytes are believed to be originated from semigranulocytes (Foley and Cheng, 1972; Moore and Eble, 1977; Rasmussen *et al.*, 1985; Balquet and Poder, 1985). The fibrocytes are reported to be degranulated granulocytes by Foley and Cheng (1975) and Cheng (1981).

The electron microscopic studies also revealed three types of cells similar to light microscopic studies. The structure of the granulocytes is similar to that in *M. edulis* (Rasmussen *et al.*, 1985), where the cells contain cytoplasm completely filled with granules, short strands of rough endoplasmic reticulum and few mitochondria and unlobate nucleus. The granules are filled with electron dense materials. However, the granules of *C. madrasensis* are electron lucent as in the case of *C. virginica* (Feng *et al.*, 1971). Seiler and Morse (1988) have reported three types of hemocytes in *M. arenaria*. One type contains electron lucent granules, second type contains electron opaque granules and the third type has only vesicles in the cytoplasm. In our study, only electron lucent granules were observed.

Scanning electron microscopic studies of the molluscan hemocytes are very rare. As observed by Morona and Mingye (1989) in *O. hupensis*, in *C. madrasensis* also, the unspread hemocytes were spherical. As they began to spread on the glass slide, the initial pseudopodia had a lobe like appearance. The fully spread hemocytes had pseudopodia with terminal swellings. These hemocytes had several pseudopodia and the cells lacked a spherical shape.

5.1.1.1.2 Cytochemical studies

In the present study, the activities of various enzymes such as acid phosphatase, prophenol oxidase and peroxidase were demonstrated in the hemocytes of *C. madrasensis*. These enzymes have been reported in other bivalves also. Acid phosphatase belongs to the group of lysosomal enzymes, which are responsible for dephosphorylation, degradation of mucopolysaccharides (a constituent of bacterial cell wall), hydrolysis of acylglycerol and hydrolyzation of peptides bearing free amino acid groups (Chu, 2000). It has been identified in the hemocytes of *M. edulis* (Moore and Lowe, 1977), *M. californianus* (Bayne *et al.*, 1979), *B. glabrata* (Granath and Yoshino, 1983b), *M. mercenaria* (Moore and Gelder, 1985), *L. luteiola* (Jyothirmayi and Rao, 1988), *C. virginica* (Cheng and Downs, 1998; Cheng, 1989), *V.ater* (Frachini and Ottaviani, 1990), *S. scripta, V. cyprinoides* var *cochinensis* (Suresh and Mohandas, 1990c), *M. arenaria* (Beckmann *et al.*, 1992), *T. crocea* (Nakayama *et al.*, 1997) and *R. decussatus* (Lopez *et al.*, 1997). However it is also reported that the acid phosphatase activity is not present in all the granules, which are believed to be lysosomes (Yoshino and Cheng, 1976). This is evident in the present study also, as the number of granules with acid phosphatase activity was very less.

Peroxidase is an enzyme associated with the release of toxic reactive oxygen intermediates. The involvement of myeloperoxidase system in the production of reactive oxygen intermediates and the subsequent killing of the foreign material is reported in *M. edulis* (Schlenk *et al.*, 1991) and *C. virginica* (Wojcik and Paynter, 1996). Peroxidase is reported in *T. crocea* by Nakayama *et al.* (1997), which is suggested to play a role in aggregation and release of the anti-bacterial agents. However, in a number of bivalves, the production of reactive oxygen intermediates is absent (Anderson, 1994; Lopez *et al.*, 1997; Torreilles *et al.*, 1996). The demonstration of strong reaction of peroxidase in granulocytes and semigranulocytes of *C.*

madrasensis indicates that the production of reactive oxygen intermediates is a major anti-bacterial mechanism in this species also.

Phenol oxidase is the terminal enzyme in the pro-phenol oxidase system, a complement-like enzyme cascade, and is responsible for the synthesis of melanin, which is deposited in the presence of microbial invaders (Söderhäll, 1982). Quinones are formed as by products in the melanin synthesis and they also play an important role in the destruction of the pathogen. The enzyme is also associated with the generation and release of opsonins in the self and non-self recognition (Smith and Söderhäll, 1991). Prophenol oxidase is reported to be present in the plasma and hemocytes of *M. edulis, Argopecten irradians, Placopecten magellanicus* and *Perna viridis* (Coles *et al.*, 1994; Jordan *et al.*, 1997; Asokan *et al.*, 1997). However, it is not detected in *M. arenaria, M. edulis, Biccinum undatum,* and *Patella vulgata* (Smith and Söderhäll, 1991). Phenol oxidase has not been reported in oysters, so far. So its presence in *C madrasensis* is a significant finding.

5.1.1.2. Maintenance in artificial media

The present findings suggest that the hemocytes of *C. madrasensis* can be maintained in viable condition upto about 72 hours in artificial media like HBSS and media M 199. Kent *et al.* (1989) and Friebal and Renwrantz (1995) have used balanced isotonic salt solutions like EDTA buffer and ringer solution respectively for maintaining the cells, to study *in vitro* phagocytosis and cytotoxic assay respectively for short duration. They have not studied the effect of the media on the viability of the cells. In Leibovitz L-15 medium supplemented with antibiotics and salts the hemocytes of *M. galloprovincialis* were maintained for more than 20 days in viable condition (Asunción *et al.*, 2003).

The medium M 199, not only maintained the cells, but also supported the growth of the cells as indicated by the proportional increase in the number of cells at 48 hrs. It was found that, at 24 and 48 hrs,

the increase in number of cells was mainly contributed by small hyalinocytes and their numbers are proportionately high. The hyalinocytes are reported to be immature cells that mature into granulocytes and the intermediate stage during maturation is the semigranulocytes (Foley and Cheng, 1972; Moore and Eble, 1977; Rasmussen *et al.*, 1985; Balquet and Poder, 1985). So, in the present study, there is a possibility that the hyalinocytes under suitable conditions have undergone division.

During the present investigations, the addition of foetal calf serum (FCS) did not cause any significant difference in both the media tested. Since the hemolymph was used as such for the culture, the media contained some native serum. So, it has to be assumed that like FCS, the serum of the molluscs contains certain unknown growth factors that contribute to cell viability. Such an assumption was also made by Asunción *et al.* (2003).

5.1.1.3. Total and differential hemocyte count

The total hemocyte count in the present set of experiments varied from 100-300 x 10^4 cells per ml of the hemolymph. The present values are less, when compared to species like *S. scripta* and *V. cyprinoides* var. *cochinensis* (Suresh and Mohandas, 1990a) and *Tapes semidecussata* (Cheney, 1971). This difference may be due to species variation or environmental and nutritional factors. The percentage of hyalinocytes in the normal *C. madrasensis* was less, compared to the percentage of granulocytes and semigranulocytes. This result is in agreement with the result obtained in *M. mercenaria* (Moore and Eble, 1977), *S. scripta, V. cyprinoides* var *cochinensis* (Suresh and Mohandas, 1990a), and *M. edulis* (Rasmussen *et al.*, 1985). However, in species like *C. virginica* (Cheng, 1981) and *R. philippinarum* (Oubella, 1996), hyalinocytes constitute much of the cell population. These findings indicate that there is considerable variation in differential cell counts in different species.

5.1.1.4. Phagocytosis

Phagocytosis is the most common method of internal defense in molluscs (Pipe and Coles, 1995; Nakayama *et al.*, 1997). If the foreign body is too large to be internalized by the hemocytes, encapsulation occurs, which is actually an aborted attempt of phagocytosis (Cheng, 1990). After internalization, there will be intracellular degradation (Jourdane and Cheng, 1987). Evidences for all these stages were obtained in the present experiment.

Yeast cells were used as the target for phagocytosis in the present study. Most of the previous workers preferred these cells compared to bacteria may be because of the better visualization of the result and its cheap availability (Bayne *et al.*, 1979; Cheng, 1989; Fryer *et al.*, 1989; Beckmann *et al.*, 1992). The phagocytic index of apparently healthy animal ranged from 36 to 63 and endocytic index varied from 0.55 to 0.93. The results almost confirm to that of *C. virginica* (Cheng, 1988b). But the value obtained for endocytic index is higher compared to that of *C. madrasensis*, which may be because in the other study, bacteria are used as the target. Since the size of bacteria is lesser than the yeast, the hemocytes of *C. virginica* would have internalized more cells. Phagocytosis depends on the adhesion of the phagocyte membrane to the heterogeneous particle, which is mediated through lectins and carbohydrates. There is difference in the carbohydrate molecules in the cell wall of yeast and bacteria, which may be another reason for the difference in endocytic index.

5.1.2. Humoral factors

5.1.2.1. Protein profile

The total serum protein in apparently healthy *C. madrasensis* varied from 392.02 to 551.51 µg/ml of serum. The values obtained by Ford (1986a; b) and Chu and Peyre (1989) in *C. virginica* (9-10 mg/ml of hemolymph) reveal a protein concentration much higher than the protein concentration obtained in *C. madrasensis*. The value obtained in *B.*

glabrata by Loker and Hertel (1987) is still higher (25 to 35 mg/ml). In the same species, Wolmarans and Yssel (1988) got a very low protein concentration by using a different method (0.85 to 0.95 mg/ml). In *M. edulis,* the value ranged from less than 1 to 3 mg/ml of the hemolymph (Mulvey and Feng, 1981). Thus, the total hemolymph protein varies from species to species and also on the method used. In the present study, the concentration of protein was very low, which may be because, in this study, only the serum was used for the estimation of protein, after the removal of hemocytes.

The SDS-PAGE revealed four prominent bands of approximately 86, 74, 26 and 31 kDa molecular weight. In addition there were 6 to 8 minor bands of molecular weights from 52 to above 100 kDa. In a study to find out the changes in the protein profile in the serum of *C. madrasensis* in response to changes in salinity, all the four prominent bands as obtained in the present study were reported. However, there were noticeable differences in the number of minor bands obtained. Although all the minor bands reported in the previous study were also recorded in the present experiments, the bands of 90 kDa and above were absent in the previous study (George *et al.,* 2001).

According to Muramoto *et al.* (1996), the PAGE map makes it possible to compare the proteins in animals. Bayne *et al.* (1979), based on electrophoretic separation of the plasma protein in *M. californianus,* have reported 16 protein bands, which are unaffected by bleeding, starvation and foreign implants. In *B. glabrata,* hemoglobin is the major protein (160 kDa), with about 20 other minor proteins most of which are visible only by more sensitive silver staining procedure (Granath *et al.,* 1987). The stress proteins reported in bivalves are of hsp (heat shock protein) 70 and hsp 60 families and of 29 kDa (Lawerence and Nicholson, 1998; Clayton *et al.,* 2000). The major bands of *C. madrasensis* are having a molecular weight similar to these stress proteins.

5.1.2.2. Enzyme assays

In apparently healthy *C. madrasensis,* the amount of acid phosphatase in the serum varies from 0.37 to 0.465 KA units. The amount of phenol oxidase varies from 0.007 to 0.019 Δ OD/mg serum protein/minute and the amount of lysozyme in the serum varies from 20 to 40 lysozyme units per ml of hemolymph.

Acid phosphatase is reported in the plasma and tissue extracts in *C. virginica* (Chu *et al.,* 1996). It is reported in the plasma and hemocytes of *M. edulis* (Moore and Lowe, 1977). The enzyme is reported to be present in the hemocytes of *M. mercenaria, M. arenaria* and *R. decussatus* (Moore and Gelder, 1985; Beckmann *et al.,* 1992; Lopez *et al.,* 1997).

Phenol oxidase is reported to be present in the plasma and hemocytes of *A. irradians* and *P. magellanicus* (Jordan *et al.,* 1997) and *P. viridis* (Asokan *et al.,* 1997). However, it is not detected in *M. arenaria, M. edulis, B. undatum,* and *P. vulgata* (Smith and Söderhäll, 1991).

Lysozymes, which hydrolyse peptidoglycan, the principle structural polymer of the bacterial cell wall (Salton, 1957) is reported to be present in the plasma of *C. virginica, M. edulis* and *M. mercenaria* (Chu and Peyre, 1993; Hardy *et al.,* 1976; Coles *et al.,* 1994; Cheng, 1975).

Most of these enzymes are demonstrated in the granules of the hemocytes. Hence, it is assumed that the enzymes are having their origin in the hemocytes. They are restricted to the lipoprotein lysosomal membrane in the latent phase. When they are challenged by abiotic and biotic factors, the lysosomes become destabilized and the enzymes are released from lysosomes into the surrounding cytoplasm by a process called degranulation (Moore and Gelder, 1985; Cheng and Mohandas, 1985). Further, the electron microscopic study of the inflammation also reveals the release of enzymes from hemocytes into extracellular space by budding as well as by fusion of the lysosomal membrane with plasma membrane of the

cell. The role played by lysosomal marker enzymes and prophenol oxidase in the defense system is already explained in the section 5.1.1.1.2.

5.2. Hemolymph factors of *C. madrasensis* exposed to Nuvan

5.2.1. Cellular factors

5.2.1.1. Total and differential hemocyte count

The mean total hemocyte count showed a significant reduction in the animals, when exposed to 0.05 ppm, 0.1 ppm and 0.2 ppm of Nuvan. Since hemocytes are the major components of disease resistance, their reduction will significantly affect the disease resistance of the animals. Hence, the presence of organophosphorous compounds, even at trace levels is likely to affect the animals adversely. However, there was no change in the percentage of granulocytes. The percentage of semigranulocytes showed a decreasing trend as the concentration of Nuvan increased. The percentage of hyalinocytes showed significant increase in Nuvan treated animals, compared to control. The decrease in the percentage of semigranulocytes is a significant observation. These cells are supposed to be the intermediate stage of cells maturing into granulocytes (Foley and Cheng, 1972; Moore and Eble, 1977; Rasmussen *et al.*, 1985). Since the percentage of granulocyte is unaffected, it has to be assumed that the semigranulocytes might have matured into granulocytes, resulting in its decrease and the granulocytes might have lysed in defending against pollutants. The decrease in percentage of semigranulocytes may result in a decrease in the percentage of granulocytes in the long run. Though hyalinocytes are increasing, there is no corresponding increase in semigranulocytes, which indicates its inability to mature into semigranulocytes as a result of exposure to Nuvan. Since total hemocyte count is very much reduced, the increase noticed in the hyalinocytes can be explained as a relative increase due the reduction of semigranulocytes. The granulocytes are the phagocytes in molluscs and the hyalinocytes do not have any functional and immunological correlations (Balquet and Poder, 1985). So a relative increase of hyalinocytes indicates the

decreased ability to phagocytose foreign particles. These results indicate that there is an overall reduction in defense capacity of the animal as a result of exposure to Nuvan.

Studies by Egidius and Moster (1985) reveal that the compound Nuvan has relatively little effect on the survival of bivalves even at a concentration of 1 ppm. In the present study also, the survival of *C. madrasensis* was not affected even at a concentration of 10 ppm. But the result shows that, at a very low concentration of 0.05 ppm there is a significant reduction in the total hemocyte count. According to Serrano *et al.* (1995) the toxic effects of the organophosphorous pesticides are moderate to nil in bivalves. This high resistance towards organophosphates is reported to be because of the inability of the chemical to inhibit esterase activity in the mollusc (Dauberschmidt *et al.*, 1977a; b). The reduction in the hemocyte number as a result of the organic pollutants is reported by Fisher *et al.* (1990), Pipe *et al.* (1995) and Fournier *et al.* (2002). The reduction in the hemocyte count may be because of lysis, diapedesis, less recruitment or movement of the hemocytes from circulation to tissues (Pipe and Coles, 1995). Since there is no change in the percentage of granulocytes it has to be assumed that the ability of the granulocytes to undergo hyper synthesis of lysosomal enzyme in response to the changed environment is reduced thereby reducing the ability of immune system (Mohandas *et al.*, 1985). The result is in agreement with the findings of Sami *et al.* (1992) who reported a significant reduction in the large hemocytes and a concomitant increase in the small hemocytes in *C. virginica* exposed to polycyclic aromatic hydrocarbon. However, Seiler and Morse (1988) reported a significant increase in the percentage granulocytes in *M. arenaria* specimens collected from the polluted area compared to those collected from unpolluted area. This may be because of the difference in the pollutants in the two studies. So, different pollutants may have different effects on the hemocyte profile.

5.2.1.2. Phagocytosis

At low concentration (0.05 ppm), Nuvan did not cause any significant change in Phagocytic index, however, the phagocytic index declined as the concentration increased to 0.1 ppm. The endocytic index was not significantly affected at any of the Nuvan concentrations used.

Suppression in the phagocytic activity as a result of exposure to organic pollutants is reported by Fries and Trip (1980) in *M. mercenaria*, Fournier *et al.* (2002) in *M. arenaria* and *M. polynyma*, and Dyrynda *et al.* (1997) in *M. edulis*. The suppression of phagocytosis has serious repercussions on animal's health, since phagocytosis is a major immune response in molluscs. The present study has revealed serious impact of Nuvan on the immune system of bivalves.

5.2.2. Humoral factors

5.2.2.1. Protein profile

The exposure to Nuvan did not cause any significant change in the serum protein at lower concentrations. However, at higher concentrations there was significant elevation in the total serum protein concentration.

In the SDS-PAGE, it was possible to observe that the minor protein bands were very faintly stained in animals exposed to Nuvan and some of the bands above 90 kDa were absent; whereas, the major protein bands (86, 74, 26 and 31 kDa) were more intense in the Nuvan treated groups compared to the control.

Stress proteins (heat shock proteins) are induced by many environmental stresses including exposure to heavy metals or organic pollutants, changes in temperature or osmolarity, hypoxia/anoxia, and exposure to ultraviolet radiation. Stress proteins, especially hsp70 and hsp60, have been used as biomarkers in a range of algae, invertebrates, fish, and higher vertebrates (Lewis *et al.*, 1999). Studies conducted by Cruz-Rodriguez *et al.* (2000) and Clayton (2000) reveal that stress proteins can be used in

oysters as an indicator of exposure to proteotoxic stress that include organic pollutants and heavy metals. According to Lawrence and Nicholson (1998), chlorine treatment is the strongest inducer of 68-72 kDa stress proteins and it also induced the synthesis of 29 kDa protein. The role of these proteins for detoxification of pollutants has been recognized, though the exact mechanism is not known (Bhattacharya, 2001). In the present study, the increase in the intensity of 86, 74, 31 and 26 kDa bands in response to Nuvan indicates its use as indicator of pollution. The exact role played by these proteins in stress modulation needs further study.

5.2.2.2. Enzyme assays

Exposure to Nuvan had resulted in a significant increase in the amount of serum acid phosphatase in all the treated groups compared to the control without Nuvan. The amount of phenol oxidase in the serum of *C. madrasensis,* although increased at lower concentration of Nuvan, the exposure resulted in significant decrease of the enzyme at higher concentrations. The Nuvan exposure resulted in a significant increase in the amount of serum lysozyme. Among the three enzymes studied, the lysosomal enzymes namely acid phosphatase and lysozyme showed an increase, at higher concentrations of Nuvan. So the exposure to Nuvan had resulted in an increase in the synthesis of these enzymes and their subsequent release into the serum. This may another reason for the increased survival rate of the animals even at a high concentration of 10 ppm Nuvan. The phenol oxidase synthesis is however, suppressed at higher concentrations of Nuvan.

5.3. Hemolymph factors of *C. madrasensis* exposed to copper

5.3.1. Cellular factors

5.3.1.1. Total and differential hemocyte count

Copper also induced a significant dose-dependent reduction in total hemocyte count in all the treated groups compared to the control. The percentage of granulocytes decreased significantly in all the

copper treated groups. At the same time, the percentage of semigranulocytes increased significantly in copper exposed groups compared to control, especially at 0.5 and 1 ppm. The percentage of hyalinocytes also increased.

In most of the earlier studies, total hemocyte count has been reported to increase by heavy metal pollution (Pickwell and Steinert, 1984; Pipe *et al.*, 1995; Pipe *et al.*, 1999; Fisher *et al.*, 2000). In contrast, George *et al.* (1983) have reported a decrease in hemocyte count in cadmium exposed *O. edulis*. According to Cheng (1988a), the exposure to 1 ppm copper does not alter hemocyte number in *C. virginica;* whereas the same concentration of cadmium significantly reduces the hemocyte count at a salinity of 30 ppt. In another study by Suresh and Mohandas (1990b), it is found that the total hemocyte count does not vary in *S. scripta* at a concentration of 1 ppm, which may be because of the lower uptake of copper at high salinity (30 ppt). They have also reported that the count decreases significantly in *V. cyprinoides* var. *cochinensis* because of the higher uptake of copper at low salinity (15 ppt) and also because the concentration of copper used in the experiment is closer to the LC_{50} value. In the case of *S. scripta,* since the concentration of copper employed is much lower than the LC_{50} value, the effect is very less. Philips (1977) and Elfing and Tedegren (2002) have also reported the high toxicity of copper at low salinity. This may be because of the ability of the low saline water to maintain the metals in solution or suspension. A significant increase in the percentage of granulocytes (Pickwell and Steinert, 1984) and a significant decrease in the percentage of hyalinocyte (Cheng, 1988a) are reported in bivalves exposed to copper. These results are also contrary to the result obtained in the present experiment. It may be concluded that, since the present set of experiments are carried out at a lower salinity of 12 ppt, the uptake of copper by the animal tissue would have been more.

Copper is lethal to the hemocytes (Cheng, 1988a). Hence, the effect of copper on the immune system is much more, which results in the decreased hemocyte count and decreased percentage of granulocytes.

According to Cheng (1990), since granulocytes are more actively phagocytic, a reduction in the percentage of granulocytes in the hemolymph may affect the animals' immunity.

5.3.1.2. Phagocytosis

In animals exposed to copper, the phagocytic index slightly increased at 0.1 ppm, but it significantly decreased as the concentration increased to 0.5 ppm. The endocytic index showed significant increase at low doses of copper, but significantly reduced at 0.5 and 1 ppm.

The effect of exposure to heavy metal on phagocytosis depends on the species used (Sauvé *et al.*, 2002) and also on the metal used and its concentrations (Cheng and Sullivan, 1984; Cheng, 1988b). The result obtained in the present experiment is similar to that reported by Cheng (1988b) in *C. virginica*. According to him, copper at a concentration of 1 ppm inhibited phagocytosis, but stimulated endocytosis. In the present experiment, the availability of copper is increased at low salinity, which has affected the phagocytic index and endocytic index at different concentrations. The endocytic index has increased at low concentration of copper, while it is decreased at higher concentration of copper. The stimulation of phagocytosis at low concentration of heavy metals and its suppression at higher concentration are reported (Cheng and Sullivan, 1984). The reduction in the percentage of granulocyte may be one of the reasons for reduced phagocytosis (Balquet and Poder, 1985).

5.3.2. Humoral factors

5.3.2.1. Protein profile

In the animals exposed to low doses of copper, there was a significant increase in the total serum protein concentration; whereas at higher copper concentrations, the protein concentrations significantly declined.

SDS-PAGE revealed that all the minor bands were clearly visible and the major bands were more intense at lower doses of

copper. At higher concentrations of copper, the minor bands were less visible and some of them especially those above 100 kDa were absent. However, at these concentrations (0.5 and 1 ppm copper) the major bands (86, 74, 26 and 31 kDa) were highly intense compared to the other treatments. In *M. edulis* also, the increased production of stress protein of hsp 60 and 70 family in response to copper exposure had been reported (Sanders *et al.*, 1994). It is believed that these proteins play an important role in the detoxification of heavy metals. However, the exact mechanism by which, they detoxify pollutants has to be studied in detail (Bhattacharya, 2001).

5.3.2.2. Enzyme assays

The exposure to copper resulted in a significant decrease in the acid phosphatase concentration in *C. madrasensis*. In contrast, increased serum acid phosphatase is reported in copper exposed bivalves by Cheng and Mohandas (1985) and Suresh and Mohandas (1990b). This may be because of the difference in the concentrations of copper used and also the difference in experimental conditions. Inhibition of the release of acid phosphatase from granulocytes in copper stressed bivalves is also reported (Cheng 1989; 1990). During the present investigations, since the percentage of granulocytes significantly reduced at higher concentrations of Nuvan, it has to be assumed that the ability of the hemocytes to synthesize the enzyme has reduced, which subsequently resulted in the decreased amount of acid phosphatase in the serum.

The exposure to copper resulted in a significant reduction in the amount of serum phenol oxidase in all the treatments. An increase in the percentage of cells containing phenol oxidase is reported in *M. edulis* (Coles *et al.*, 1994). The present result may be because of decreased synthesis of the enzyme in the hemocytes or inhibition of its release into serum.

In copper exposed animals, there was no significant change in the serum lysozyme. The result is in agreement with the report of

Cheng (1989) in *C. virginica*. This indicates that copper is not having any effect on lysozyme synthesis and its release into the serum.

5.4. Hemolymph factors of *C. madrasensis* exposed to *V. alginolyticus*

5.4.1. Cellular factors

5.4.1.1. Total and differential hemocyte count

Various species of bacteria belonging to the genus *Vibrio* are known to cause a number of diseases in both fish and shellfish. In the case of bivalves, the bacteria attached to the periostracum with invasion into the mantle and soft tissues characterize the vibriosis. These events cause irreversible injury leading to the death of the animals (Elston and Leibovitz, 1980). *V. alginolyticus* and *V. anguillarum* are reported to cause severe larval mortality in the scallop, *Argopecten purpuratus* (Riquelme *et al.*, 1996).

In the present experiment, the sham injection and injection with bacteria had resulted in a significant increase in the hemocyte count much more than the normal values soon after injection, followed by a significant reduction at 24 hrs after injection. The increase at 2 hrs of injection and decrease at 24 hrs of injection were more pronounced for test compared to the control (sham injected ones). This indicated that the handling of animals itself caused increase in the hemocyte count followed by a fall. But the presence of bacteria resulted in a significant reduction of the hemocyte count. In animals that received sham injections, the normal values of hemocyte count were reached by 120 hrs, where as in test animals, it took 2 weeks.

Even though the hemocyte count increased significantly at 2 hrs and then decreased significantly at 24 hrs, the percentage of granulocytes was within the normal range in the sham injected ones. However, after 24 hrs, there was significant reduction in the percentage of granulocytes with a concomitant increase in the percentage of hyalinocytes that reached maximum at 120 hrs, when the total count became normal.

After that, the values obtained for percentage of granulocyte, semigranulocyte and hyalinocyte were within the normal range.

In the test, however, the percentage of granulocytes was very high at 2 hrs and 24 hrs. This indicated that the increase in the total count at these time intervals was mainly constituted by granulocytes. The value was reduced at 72 hrs and 120 hrs but again increased significantly at 1 week. It came to the normal range by 2 weeks.

Pauley *et al.* (1972) and Granath and Yoshino (1983b) have reported a decrease in the number of circulating hemocytes after pathogenic challenge. According to them, the decrease in the hemocyte count soon after injection is because of the encapsulation and phagocytic reactions. It is proposed that the phagocyte, after phagocytosis might migrate across the epithelial borders leading to its elimination. Suresh and Mohandas (1990c) have performed a similar experiment with *S. scripta* and *V. cyprinoides* var. *cochinensis.* Apart from sham and bacteria injected group, they also maintained untampered control. They got significantly high total hemocyte count in sham-injected and bacteria-injected group, compared to the untampered control. However, the value returned to normal by 48 hrs in the sham injected ones; where as the value remained high in the *V. alginolyticus* injected ones in the late periods also. The increase in the total hemocyte count subsequent to pathogenic challenge is also reported elsewhere (Mounkassa and Jourdane, 1990; Anderson *et al.*, 1992; Oubella *et al.*, 1993; 1996). It is proposed that the increase in the hemocyte count is the result of the mobilization and migration of the hemocytes to the hemolymph compartments from the tissues in response to the pathogen (Oubella *et al.*, 1996). In the present experiment, the increase in the total hemocyte count soon after the injection may be because of the mobilization of the host response against pathogen. The subsequent decrease may be the result of the elimination of the hemocytes with the phagocytosed bacteria through epithelial borders.

The granules in the hemocytes of *A. californica* are proposed to be phagocytosed bacteria (Pauley *et al.*, 1972). Oubella *et al.* (1996) have reported a significant decrease in the number of hyalinocytes and a concomitant increase in the granulocytes after bacterial challenge. A decrease in the percentage of granulocytes is found in *C. virginica* with juvenile oyster disease with a probable bacterial aetiology (Paillard *et al.*, 1996). Uzhazy *et al.* (1988) have reported the formation of granuloma by the granulocytes subsequent to pathogenic challenge. The involvement of granulocytes in the formation of focal abcesses is reported by Balquet and Poder (1985). In the present experiment, the increase in percentage of granulocytes and the corresponding decrease in percentage of hyalinocytes may be in response to the bacterial challenge. The decrease at 72 and 120 hrs may be due to the migration of the granulocytes with phagocytosed bacteria from circulation to the tissues and subsequently towards outside. Further increase at 1 week can be attributed to the formation of new granulocytes for clearance of the remaining bacteria.

5.4.1.2. Phagocytosis

The phagocytic index using yeast as the target was not significantly different at any of the time intervals in the present experiment. The mean phagocytic index for both control and test, at 2 hrs and 24 hrs were lower than the normal value of 40% to 60%. At 72 hrs, the control and the test reached normal range. But at 120 hrs, the mean phagocytic index reduced. It increased by 1 week and reached almost normal value by 2 weeks. The endocytic index was significantly less during the early time periods, which became normal during the late periods. This showed a reduced ability of the hemocytes of *C. madrasensis* to phagocytose yeast at early time periods, but the ability was restored within 72 hrs.

In phagocytosis, the recognition and attachment processes involve self and non-self discrimination leading to finding and subsequent internalization of non-self materials. These processes are

believed to be receptor mediated and lectins play an important role in the binding and subsequent internalization (Chu, 1988; Olafasen, 1988; Adema *et al.*, 1991). Probably these lectins/receptors are engaged in binding bacteria, hence, are not available for binding yeast particles. This may be the reason for the reduced phagocytic activity in animals injected with bacteria in the early time periods.

In the present experiment, there was no mortality due to injection of *V. alginolyticus*. This indicates that the animals are able to resist the infection by *V. alginolyticus*. The hemocytes of eastern oyster, *C. virginica* are able to kill *Vibrio* sp (Harris Young, 1995; Genthner *et al.*, 1999). In this study, the hemocytes were probably engaged in a process of killing the bacteria. So the ability to recognize yeast particles for phagocytosis might have decreased. The reduced ability of the hemocytes to adhere and ingest yeast cells is also reported in diseased *M. arenaria* (Beckmann *et al.*, 1992). All these studies indicate that multiple infections are highly dangerous for the animals. Animals may not be able to resist, when one infection is followed by infection with another pathogen.

It is evident that the presence of organophosphorous compounds and heavy metals in rearing water adversely affect the phagocytic ability of the hemocytes. Phagocytosis is one of the primary resistance factors in molluscs affected with infection. Hence, the presence of these compounds in the rearing water may have serious effects on the immunity of bivalves, especially when there is an infection.

5.4.2. Humoral factors

5.4.2.1. Protein profile

Exposure to bacteria had resulted in a significant reduction in the total serum protein concentration during the early hours. The reduction of the total protein in response to infection is also reported by Lee and Cheng (1972), Ford (1986a) and Loker and Hertel (1987). The decrease is followed by an increase in the total serum protein by 72 hrs. This

may be a reaction to the infectious agent. The animal may be producing antibacterial proteins in response to bacterial challenge. Lectins and other antibacterial proteins are produced against bacterial challenge (Vasantha, 1996; Hubert *et al.*, 1996). By 1 week, the value reduced to normal range, which indicated a recovery.

The major bands of 86, 74, 26 and 31 kDa showed a decrease in intensity during short term exposure but increased significantly by 120 hours. Change in the protein bands in response to bacteria is reported by Cheng (1969). A change in the ratio of two protein bands in response to infection by *M. velsoni* is reported by Feng and Canzonier (1970) in *C. virginica*. However, Ford (1986a) reported that there is no correlation between the protein bands and the infection in *C. virginica*. According to Loker and Hertel (1987), in *B. glabrata*, there are no changes in the SDS-PAGE pattern in response to infection. The studies by Tirard *et al.* (1995) in *C. virginica* reveal that the proteins of 29, 63, 79 and 86 kDa are produced in response to infection by *P. marinus*. In *C. madrasensis*, the role played by 86, 74, 26 and 31 kDa in protecting animals against bacteria needs further study.

5.4.2.2. Enzyme assays

The amount of acid phosphatase in the serum of control animals increased significantly at 2 hrs of injection and then reduced and reached normal values by 24 hrs; whereas, in the test, the amount increased significantly at 2 hrs compared to the normal values, followed by a further increase at 24 hrs and the value reached normal values by 1 week. An increase in the acid phosphatase in response to pathogen is also reported by Granath and Yoshino (1983b), Cheng and Mohandas (1985) and Jyothirmayi and Rao (1988). Even though the saline injection had resulted in an increase in the acid phosphatase, the percentage of increase was much high in the test where the bacteria was involved. The elevated level continued upto 120 hrs may be for the complete removal of the bacteria. By one week, the value returned to normal range, which indicated a probable recovery.

The amount of phenol oxidase in the serum increased significantly compared to normal values in both control and test at 24 hrs and 72 hrs of injection. However, the increase was more significant in the test compared to control. By 120 hrs, the value reached within the normal range both in control and in test. The role played by the enzyme in the removal of bacteria is clearly evident from the fact that the amount of enzyme is much higher in the test compared to the control upto 120 hrs. The published reports of similar studies in other species are scanty.

In the control group, the amount of serum lysozyme was within the normal range at all the time periods tested, except at 2 weeks. In the test, the value was high compared to the normal values upto 72 hrs and after that, the amount decreased and reached within the normal value range by 120 hrs. The results of the present experiment are in agreement with McDade and Trip (1967) and Rodrick and Cheng (1974), who have reported elevated levels of lysozyme in molluscan serum and its role in the defense reactions against pathogen. The increased serum lysozyme as a result of exposure to bacteria is also reported by Cheng *et al.* (1975) in *M. mercenaria.* In *C. virginica,* infection with *M. velsoni* decreases serum lysozyme, whereas the infection with *Bucephalus* sp. increases the same (Feng and Canzonier, 1970). Lysozyme is unaffected by infection by *P. marinus* in the same species (Chu and Peyre 1989). Thus the response of the lysozyme towards an infection varies with species and also with pathogen.

The three enzymes viz. acid phosphatase, phenol oxidase and lysozyme have their origin from hemocytes. There are reports that granulocytes secrete these enzymes when there is an infection (Moore and Gelder, 1985; Cheng, 1990). In the present study, there is an increase in the granulocytes in the hemolymph and a corresponding increase of enzymes in the serum. Thus, there is an indication that the enzymes are released from hemocytes.

5.5. Histological studies

5.5.1. Histology of apparently healthy animals

The histology of the adductor muscle, mantle and gills of *C. madrasensis* are studied in order to characterize the inflammatory responses in these tissues when exposed to pollutants. The adductor muscle of apparently healthy animal reveals a structure similar to that described for *Tridacna gigas* (Norton and Jones, 1992). The fibres are enclosed in a fascial sheath of fibrous tissue. The epimysium is covered with a layer of ciliated pseudo stratified epithelial cells and there is a layer of glandular cells below this layer. All these structures are also noticed in *T. gigas* (loc.cited). The mantle is covered by columnar epithelium and is composed of muscular and connective tissue network. The structure is very similar to that described for *T. gigas* (loc.cited). The gills of *C. madrasensis* consist of filaments that are joined to each other by ciliary interfilamentar junctions. A branchial vein runs through the filaments. The epithelium is composed of ciliated and unciliated cell types. The structure of gills observed in the present study is very similar to the observation in *M. edulis* (Sunila, 1988).

5.5.2. Histological changes after exposure to Nuvan

In Nuvan treated animals, the thickness of the epimysium of adductor muscle increased and there was focal loss of cilia from the surface epithelial cells. The epithelial cells of the mantle tissue showed vacoulation and focal necrosis in the initial stages that became extensive at higher doses. The lateral cilia and the cilia of the abfrontal cells of gills were lost. In higher doses, complete disorganization of the filaments and frontal fusion of the gills were observed. There was enlargement of mucous gland and branchial vein was shrunken. Interfilamentar fusion was also observed. Similar results are reported by Sunila (1988) in the gills of *M. edulis*. Hemocytic infiltration was not noticed in the area.

Very few studies on the effects of organophosphorous compounds and other organic compounds on the molluscan defense system

are available in the literature. The lack of information on this subject may be due to the less importance given to these compounds on pollution studies. However, the present study has revealed that Nuvan has very serious deleterious effect on the mollusc. The harmful effect of Nuvan on the gills may be affecting the respiration and thus the oxygen availability for the animal. The loss of cilia and necrosis of the surface epithelial cells may cause the break down of natural barriers against pathogens and may make the animals susceptible to infection. As already noticed, the immune system of the animal is highly affected by the exposure to pathogen. This will affect the general health of the animals grown in pesticide contaminated areas (Chu, 2000).

5.5.3. Histological changes after exposure to copper

In the adductor muscle of the copper treated animals, low doses caused epimysial thickening due to fibrous tissue growth and such growth was also observed in perimysium. There was fragmentation and necrosis of muscle fibres. In high doses of copper, the epimysium became shrunken and cilia were lost and further necrosis of the muscle fibres was also noted. The changes were very severe. However, hemocytic infiltration was not observed here also, as in the case of Nuvan.

There is scanty information on the effect of copper on the adductor muscle of bivalves. Reduction in metabolic rate in response to copper has been observed in the adductor muscle of *H. rufescens* (Viant *et al.*, 2002). The adductor muscle is an important appendage used for opening and closing of shell valves. In the present study, the muscle was severely damaged and there was a possibility that the muscle function was affected. The impact of this damage on the physiology of animal needs further study.

In copper treated animals, initially the mantle revealed area of necrosis in the epithelium and increase in the number of goblet cells. Focal proliferation of the epithelial cells was also noted. In high dose (1 ppm), extensive necrosis was observed and attempts for regeneration were

also noticed. These changes indicate that copper produces mild to moderate irritation, which causes proliferative and secretory responses. Mantle is one of the sites where highest accumulation of heavy metal occurs (George *et al.*, 1978) in bivalves. The increased response of mucous secreting cells in the gills is reported in response to copper pollution (Viant *et al.*, 2002). However, there is no such report in the case of mantle tissue. There were no hemocytes in the section of mantle studied.

The gills of the copper exposed animals revealed detachment of frontal, postlateral and abfrontal cells and there was vacoulation of the post lateral cells. In high doses, extensive frontal and abfrontal fusion was observed. Sunila (1986, 1988) has also reported the above changes in *M. edulis*. Since gills are involved in the respiration and gas exchange of the animals, the damage caused is likely to influence the physiological functions. Hence, the present observations are highly significant. Further studies are essential to find out the extent of physiological effect on animal due to gill damage. However, the hemocyte infiltration observed by the above-cited author is not observed in our study.

Copper is an ingredient in a number of fungicides that are used in agriculture. The extensive use of fungicides is taking place in plantations and horticulture. These fungicides, during rains collect in the run off water and reach the water bodies that are used for molluscan cultivation. The present study has indicated very serious repercussions on molluscan cultivation. This point to the need for an integrated approach to the whole problem of agriculture, animal husbandry and aquaculture. All these wings are essential for the well being of the human population. Hence a balanced approach has to be evolved for the use of agrochemicals.

5.6. Inflammatory responses

5.6.1. Light microscopic studies

The inflammatory reactions observed in the control animals injected with FCA at 24 hours, on the 3rd day and 7th day indicated

initial mild to moderate infiltration of hemocytes followed by massive infiltration of hemocytes and these hemocytes probably secreted proteinaceous material. The hemocytes at later stages were found to excrete fibrin like shreds. Such shreds were found to radiate from small cells. Later these hemocytes (fibrocytes) aggregated into nodules of cells, some of which assumed a spindle shape and these cells were arranged in concentric layers and formed a mass of cells. In the animals injected with *V. alginolyticus,* massive infiltration of hemocytes was noticed at the injected site at 2 hrs and at 24 hrs. Thereafter, the infiltration was reduced and the tissues appeared normal by 120 hrs. Nodule formation was not noticed in this study.

The massive infiltration of the hemocytes in the site of inflammation is also reported by Balquet and Poder (1985), Elston *et al.* (1987), Jourdane and Cheng (1987), Cheng (1983), Uhazy *et al.* (1988), Friedman (1991) and Fisher *et al.* (2000). The formation of nodules towards the later stages of infection is reported by Elston *et al.* (1987) in *C. gigas* and fibrosis is reported by Uhazy *et al.* (1988) in *B. glabrata.* Foley and Cheng (1972) also observed formation of nodules by arrangement of concentric layers of granules. Feng (1988) and Fisher (1986) have observed the hemocytes encircle abiotic particles or invading organisms that are too large to be phagocytosed by a single hemocyte. The hemocytes surrounding the foreign particle grow into layers and eventually separate it from the host tissue. Complete healing is found to occur by 15 th day in FCA injected ones, whereas, in *V. alginolyticus* injected ones, the healing occured by 120 hrs.

In Nuvan and copper exposed animals, the amplitude of hemocyte infiltration and subsequent exudate formation and collagen formation were minimal. In the case of Nuvan, our result is in agreement with Winstead and Couch (1988), who have reported a lack of hemocytic infiltration in *C. virginica* exposed to organic pollutant. But in the case of heavy metals, our results are contrary to the results obtained in *M. edulis*

(Sunila, 1988) *and C. virginica* (Oliver and Fisher, 1997), where the exposure to heavy metals has resulted in increased inflammatory response.

Chu (2000) has suggested that pollutant exposure reduces disease resistance by causing physiological stress in the host or suppressing certain host defense mechanisms, thus enhancing disease susceptibility and progression. He found that in *C. virginica,* the exposure to organic pollutants has resulted in increased susceptibility to *P. marinus* infection. In the present study, it is noticed that the primary defense mechanism like inflammation is very much reduced in organophosphorous and heavy metal exposed animals.

The reduction of hemocyte infiltration can be attributed to the general reduction of circulating hemocytes in these animals. This is supported by the fact that, in Nuvan and copper treated animals, we have observed a reduction of total hemocyte count. Since, hemocytes are the major soldiers fighting against infectious agents, and inflammatory process is one of the mechanisms by which these cells are activated to perform their function, a reduction in the hemocyte count and lack of hemocyte infiltration in the presence of pollutants, can affect the immunity adversely.

5.6.2. Ultrastructural studies.

The ultrastructural studies of the adductor muscle site injected with *V. alginolyticus* reveal the presence of hemocytes in the area, with cytoplasm filled with lysosomes, lysosomes budding off from the hemocytes, presence of lysosomes and degenerating bacteria in the interstitial space and secretion of thin filaments, which formed thick fibrin like material in the interstitial spaces.

The release of lysosomal hydrolases from the cells into serum is studied in a number of species of bivalves (Rodrick, 1979; Cheng and Mohandas, 1985; Mohandas *et al.*, 1985; Cheng, 1990). The injection of *V. alginolyticus* produced inflammatory response at the site of injection, which attracted hemocytes to the site. In inflammatory areas, the hemocytes are

known to release hydrolases (Rodrick, 1979; Moore and Gelder, 1985; Cheng, 1990). Mohandas *et al.* (1985) have demonstrated the budding off of lysosomes by scanning electron microscopy. During the present study, we have demonstrated that the lysosomes release enzymes by fusing with cell membrane and a process of exocytosis releases the material. The lysosomes are also found to bud off from cell membrane and such lysosomes are seen in the intracellular spaces. The release of hydrolases is accompanied by formation of primary and secondary phagosomes in which bacteria are digested to form glycogen deposits as is reported in the case of *C. virginica* (Cheng and Rudo, 1976). The filamentous material, which subsequently formed thick fibrin like material is also released from the hemocytes. This may in turn result in fibrosis as reported by Uhazy *et al.* (1988) in *B. glabrata.* The light microscopic study of inflammation also reveals the presence of fibrils radiating from hemocytes. All these indicate that the origin of fibrin seen in inflammation is from hemocytes itself, which are produced by the action of lysosomal enzymes of the hemocytes. The ultrastructural observations in the present study are highly significant, since it has brought out the exact role played by the hemocytes in molluscs.

The present study reveals some important information regarding the cellular and humoral immune mechanisms in *C. madrasensis.* The cellular immune mechanism is constituted by granulocytes, semigranulocytes and hyalinocytes, of which, granulocytes are actively phagocytic and produce various enzymes necessary for the degradation of non-self materials. These cells digest non-self materials internally as well as by secreting the enzymes extracellularly. The humoral factors are composed of enzymes like acid phosphatase, phenol oxidase, lysozyme etc. and also other proteins, which are yet to be characterized. The animal is able to increase the production of proteins of 86, 74, 26 and 31 kDa in response to pollutants. The source of these proteins is probably hemocytes.

Organic pollutants like pesticides and inorganic pollutants like heavy metals affected the cellular and humoral immune mechanisms of *C. madrasensis*. Though there was variation in the effect of each pollutant on individual factors, generally they adversely affected the defense capacity of the animals.

In bacterial infections, the animals were able to withstand the infection by mounting a strong inflammatory reaction, which involved phagocytosis and action of enzymes. The animals were able to ward off infection within 7 days. The present study revealed that the total and differential hemocyte count, phagocytic index and estimation of stress proteins and enzymes can indicate the health status of cultured bivalves.

SUMMARY

1. The hemocytes of *C. madrasensis* are composed of granulocytes, semigranulocytes and hyalinocytes. The granulocytes have a cytoplasm with large number acidophilic and basophilic granules; the semigranulocytes have scanty basophilic granules and hyalinocytes have little or no granules at all in their cytoplasm.
2. Cytochemical studies revealed the presence of three enzymes, namely, acid phosphatase, prophenol oxidase and peroxidase in the hemocytes.
3. The mean total hemocyte count of apparently healthy *C. madrasensis* is 211.21± 44.4 x 10^4 cells/ml of hemolymph with a higher mean percentage of granulocytes compared to semigranulocytes and hyalinocytes.
4. The mean phagocytic index of hemocytes using yeast as substrate is 47.83± 10.67 and the mean endocytic index is 0.68± 0.135.
5. Among the humoral factors the mean serum total protein (µg/ml) is 470 ± 61.54, the mean serum acid phosphatase in KA units is 0.418 ± 0.032, the mean serum phenol oxidase is 0.0123 ± 0.005 Δ OD/mg serum protein/minute and the mean serum lysozyme is 31.67 ± 9.832 lysozyme units per ml of serum.
6. In the animals exposed to Nuvan, there is a significant reduction in the total hemocyte count. Although the percentage of granulocytes does not change significantly, the percentage of semigranulocytes and percentage of hyalinocytes increased.
7. There is a significant reduction in the *in vitro* phagocytosis of the yeast by the hemocytes of the animals exposed to Nuvan, but the endocytic index is unaffected.
8. There is a significant increase in the total protein concentration in the hemolymph, associated with a rise in acid phosphatase and serum lysozyme, but the amount of phenol oxidase in the serum decreased as a result of exposure to Nuvan.
9. There is an increase in the intensity of protein bands of molecular weight 86, 74, 26 and 31 kDa in the SDS-PAGE in response to Nuvan.

10. The inflammatory responses against foreign particles, including hemocytic infiltration into the affected areas are reduced in the animals exposed to Nuvan and the healing process is delayed.
11. The exposure to Nuvan results in a general atrophy of cells especially epithelial cells in the gills, adductor muscle and mantle. There is no hemocytic infiltration in the above tissues, in response to Nuvan.
12. In copper exposed *C. madrasensis,* the total hemocyte count and percentage granulocytes decreased, but the percentage semigranulocytes and the percentage hyalinocytes increased.
13. The phagocytic index and endocytic index were significantly reduced in copper exposed animals.
14. The humoral factors in the copper exposed animals such as, total serum protein concentration, serum acid phosphatase and serum phenol oxidase showed a decrease. However, the amount of serum lysozyme is unaffected by the copper exposure.
15. The intensity of hemocyte infiltration into the area of inflammation is considerably reduced and the healing is delayed in the copper exposed animals.
16. There is extensive necrosis in the adductor muscle, gills and mantle of animals exposed to copper. The hemocytic infiltration is absent in these tissues.
17. The total count of the hemocytes increased significantly during the early hours in the animals exposed to *V. alginolyticus,* associated by an increase in the percentage granulocytes. There is a corresponding decrease in the percentage semigranulocytes and hyalinocytes.
18. The phagocytic index with yeast is not significant at any of the time intervals. The endocytic index is significantly less during the early time periods, but becomes normal during the late hours in the animals exposed to *V. alginolyticus.* This is believed to be because; the phagocytes

are already engaged in fighting the bacteria and so are unavailable for *in vitro* phagocytosis.

19. There is significant reduction in the total protein in the early time periods, but all the enzymes studied in the present experiment increase at early time periods.
20. All the different parameters studied came to the normal range within 120 hrs to 2 weeks in the animals exposed to *V. alginolyticus.*
21. The inflammatory response against FCA and *V. alginolyticus* includes extensive hemocytic infiltration and secretion of fibrinaceous exudates.
22. In FCA injected animals, there is granuloma formation and formation of nodules towards the healing stage.
23. The ultrastructural studies reveal the origin of enzymes and fibrin in the hemocyte.
24. The ultrastructural study also reveals the killing of bacteria intracellularly, production of glycogen granules and extracellular release of enzymes.

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Maintenance of haemocytes of edible oyster, *Crassostrea madrasensis* (Preston) in artificial media

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ABSTRACT

The haemocytes of *Crassostrea madrasensis* were maintained in Hank's balanced salt solution (HBSS), HBSS with 10% foetal calf serum (FCS), tissue culture media M 199, and M 199 with 10% FCS. Total counts of viable haemocytes were taken at 0 hr, 24 hrs, 48 hrs, and 72 hrs. HBSS and HBSS with 10% FCS were found to maintain cell viability upto 72 hrs without much reduction in the count of viable cells. In M199 and M199 with 10% FCS, it was found that a substantial increase of small hyalinocytes occurred at 24 hrs and 48 hrs. This was followed by a steep fall in the number of viable cells at 72 hrs in both the media. The HBSS, HBSS with 10% FCS, M199 and M199 with 10% FCS were found to be good for maintaining the cells in viable state. However, only M 199 and M 199 with 10% FCS indicated multiplication of haemocytes.

Introduction

In bivalves, haemocytes constitute the first line of defense against potential pathogens. In order to study the functional characteristics of these cells *in vitro*, they have to be suspended in a medium, in which, they can function normally. Maintenance of the haemocytes in media is essential for studies such as density gradient centrifugation and cell separation, phagocytosis, chemotaxis, cell aggregation, adhesion, etc.

According to Bachere *et al.* (1995), a modified Alsever's solution is efficient in preventing cell degradation and maintaining hemocytes in a quiescent state. They have suggested a medium, which contain calcium and magnesium, where the haemocytes can recover their attachment and spreading behaviour. Bayne *et al.* (1979) has used 0.1% N-ethyl maleimide in millipore filtered seawater for cytological and cytochemical examination of haemocytes of *Mytilus californianus*. Tissue culture media M 199 and Bge media in 1:1 proportion were used by Noda and Loker (1989) for suspending haemocytes of *Biomphalaria glabrata*, whereas, M199 with foetal calf serum was used for haemocytes of *Crassostrea virginica* by Chen and Rudo (1976). Chen (1996) used

calcium magnesium tris buffered saline for the maintenance of haemocytes of abalone, *Haliotis diversicolor*. Kent *et al.* (1989) used EDTA buffer and Friebal and Renwrantz (1995) used Mytilus ringer solution for the maintenance of haemocytes of *Mytilus edulis*.

In all these cases, the suitability of the media for the maintenance of haemocytes and its effects on the functions of haemocytes have not been studied except for M 199, which has been tested and found suitable for the haemocytes of *B. glabrata* and *C. virginica*.

In the present study, an attempt was made for the maintenance of haemocytes of *Crassostrea madrasensis* in different media so as to find their suitability to support these cells.

Materials and methods

C. madrasensis specimens of shell length 4 to 6 cm were obtained from Vypeen Island, Cochin and maintained in filtered and aerated sea water of 25 ppt salinity, in 10L basins with 5 numbers in each basin at room temperature. They were fed with *Chaetoceros* sp. *ad libitum* every morning, after the removal of waste. About 50% of the water was exchanged with fresh seawater every alternate day.

Four types of media were used for the study. They were, 1) Hank's balanced salt solution (HBSS) 2) HBSS with 10% foetal calf serum (FCS) 3) Tissue Culture media M 199 and 4) M 199 with 10% FCS. The HBSS, FCS and M 199 were obtained from HIMEDIA, Mumbai, India. The media were prepared as follows: One vial (9.76gm of HBSS 9.6 gm of M 199) of entire dehydrated media was added to 900 ml of double glass distilled water and stirred until it was dissolved. 0.35g and 2.2g of sodium bicarbonate were added to HBSS and M 199 respectively. 5 units of heparin and 100µg of streptomycin were added to each of the media. The pH was adjusted to 7.4 and made up to 1000 ml using double distilled water. The media 2 and 4 were prepared by adding 10% FCS under sterile conditions. The media were sterilized by filtration through millipore 0.22µ membrane filter and stored in dark at 4°C.

Haemolymph was withdrawn from the posterior adductor muscle of the oyster using a sterile hypodermic syringe. 0.5ml of haemolymph was drawn into the syringe containing 0.5ml media and transferred into sterile tubes in three replicates. The tubes were incubated at 18°C.

Cell viability was tested using trypan blue dye exclusion technique. 0.1% trypan blue was used for staining. The cells with stained nuclei were considered dead. Viable cells were counted in a haemocytometer. The total viable haemocyte count was taken at 0 hr, 24 hrs, 48 hrs and 72 hrs.

Results

The haemocyte counts obtained at different intervals in different media are given in Table 1. In HBSS the initial haemocyte count ranged from 63 to 64 x 10^4 cells per ml with a mean of 63.67 ± 1.58 x 10^4 cell's per ml. At 24 hrs, the count was between 64 and 86 x 10^4 cells per ml with a mean of 73 ± 11.53 x 10^4 cells per ml. The haemocyte count at 48 hrs ranged from 66 to 72 x 10^4cells per ml with a mean of 68.67 ± 3.06 x 10^4 cells per ml. On completion of 72 hrs the count varied from 50 to 76 x10^4 cells per ml with a mean of 65.33 ± 13.61 x 10^4 cellsper ml.

HBSS with 10% FCS contained 63 to 65 x 10^4 cells per ml initially with a mean of 64 ± 1 x 10^4 cells per ml. The count was between 59 and 63 x 10^4 cells per ml at 24 hrs and mean was 61.67 ± 2.31 x 10^4 cells per ml. The haemocyte count again decreased to a range of 52 to 63 x 10^4 cells per ml with mean of 58.67 ± 5.86 x 10^4 cells per ml by 48 hrs. At 72 hrs the count

TABLE 1. *Total haemocyte count (X 10^4 cells per ml.)*

Time	HBSS	HBSS with FCS	M199	M199 with FCS
0 hr	64	63	85	71
	63	65	67	73
	64	64	60	64
24 hr	64	63	69	116
	69	63	100	102
	86	59	53	98
48 hr	66	61	132	158
	72	52	158	140
	68	63	137	115
72 hr	70	49	64	120
	50	67	91	90
	76	63	123	80

ranged from 49 to 67 x 10^4 cells per ml with mean of 59.67 $\pm$ 9.45 x 10^4 cells per ml.

The tissue culture media M 199 had an initial haemocyte count between 60 and 85 x 10^4 cells per ml with a mean of 70.67 $\pm$ 12.9 x 10^4 cells per ml. The count ranged from 53 and 100 x 10^4 cells per ml with a mean value of 74 $\pm$ 23.9 x 10^4 cells per ml at the end of 24 hrs. At 48 hrs the count almost doubled and ranged from 132 to 158 x 10^4 cells per ml with mean value of 142.33 $\pm$ 13.8 x 10^4 cells per ml. In all the three replicates small hyalinocytes were abundant. With the completion of 72 hrs, the count was reduced to a range of 64 to 123 x 10^4 cells per ml with mean value 92.67 $\pm$ 29.54 x 10^4 cells per ml.

M 199 with 10% FCS showed almost similar results as M 199. The initial count varied from 64 to 73 x 10^4 cells per ml with mean value 69.3 $\pm$ 4.73 x 10^4 cells per ml. The count increased to a range of 98 to 116 x 10^4 celk per ml with average of 105.3 $\pm$ 9.45 x 10^4 cells per ml at 24 hrs. At 48 hrs the value doubled to a range of 115 to 158 x 10^4 cells per ml with a mean of 137.67 $\pm$ 21.59 x 10^4 cells per ml. In this treatment also small sized hyalinocytes were abundant in all the three replicates. The count again decreased to a range of 80 to 120 x 10^4 cells per ml with mean value of 96.67 $\pm$ 20.82 x 10^4 cells per ml at 72 hrs.

The variation in mean haemocyte count with time in the four different media is given in Fig. 1. The result was analyzed using analysis of variance technique. The results of analysis show significant difference between the different media and within the media with different time interval at 5% level of significance. The analysis of variance of the data is given in Table 2.

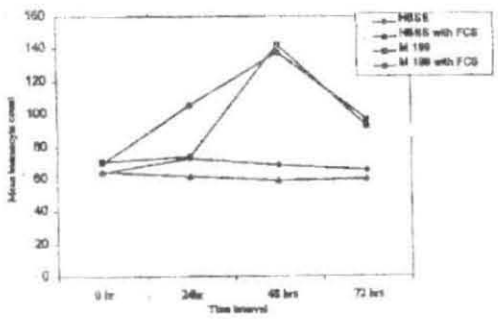

Fig. 1. Variatiom of mean haemocyte count with time in different media (x 10^4 cells per ml.).

Discussion

The haemocytes that were maintained

TABLE 2. *Analysis of variance table for the differences within and between media with time interval*

Source	Degrees of freedom	Sum of squares	Mean sum ofsquares	F value
Media	3	14666.0834	4888.695	4.66 •
Time interval	3	7723.4167	2574.47	
Interaction	9	9451.749	1050.194	5.1396 •
Error	32	6538.6676	204.333	
Total	47	38379.9167		

• Significant at 5% level at 3 and 9 degrees of freedom.

• Significant at 5% level at 9 and 32 degrees of freedom.

in the four different media gave significant results. The HBSS with and without FCS, though maintained the viable cells, the viability decreased with time. The addition of FCS did not improve the quality of HBSS media. Kent *et al.* (1989) and Freibal and Renwrantz (1995) used balanced isotonic salt solutions like EDTA buffer and ringer solution respectively for maintaining the cells, to study *in vitro* phagocytosis and cytotoxic assay respectively for short duration. They have not examined the effect of the media on the viability of cells. In HBSS there was no increase in the number of cells at 24, 48 and 72 hrs. The number of viable cells was more or less maintained upto 72 hrs. This phenomenon is surprising, because in the other media used, there was a steep decline of viable cells at 72 hrs.

Foetal calf serum is supposed to provide colloidal osmotic protection and additional nutrient factors that are available in native plasma. As the different treatment groups already contained some amount of native plasma, FCS may not have any additional benefits in the present experiment.

The medium M 199, not only maintained the cells, but also supported the growth of cells as indicated by the proportional increase in the number of cells at 48 hrs. The increase in the number of cells up to 48 hrs indicated a probable multiplication of the cells in the media. In order to prove the multiplication of the cells, incorporation of radiolabelled thiamidine in the cells has to be tested. In the present study this has not been done. However, the substantial increase in the number of cells in all replicates could not be explained otherwise. There is a fall in the number of cells at 72 hrs. As the media has not been supplemented, the' nutrients in the media must have been exhausted. This, coupled with the high metabolic rate of the dividing cells and accumulation of waste products contributes to the deterioration of the quality of the media. The decrease in the number of viable haemocytes can be explained by the above-described phenomenon.

Cheng and Rudo (1976) and Noda and Loker (1989) have observed the suitability of M 199 as a medium for maintenance of haemocytes. Cheng and Rudo also found that the medium with FCS with or without plasma of oyster supported the viability of cells even up to one week. The results of the present study also support this view, although they have not reported multiplication of cells.

The haemolymph fraction contains immature cells, partially mature cells and mature cells. The hyalinocytes were reported to be immature cells that mature in to granulocytes and the intermediate stage during maturation is the semigranulocytes (Foley and Cheng, 1972). The increase in number of cells at 24 and 48 hrs were mainly contributed by small sized hyalinocytes, and their numbers were proportionately very high. There is a possibility that hyalinocytes under suitable condition had undergone division.

In media M 199 when FCS was added, there was an increase in the number of haemocytes at 24 and 48 hrs. Here also there was a fall in the number of cells at 72 hrs. Though there was an increase in number of cells at 24 and 48 hrs, the increase did not vary significantly from those samples maintained in M 199 alone. Cheng and Rudo (1976) have studied chemotaxis of haemocytes in media M 199 with foetal calf serum. They have found that foetal calf serum enhances chemotactic movement of haemocytes. The addition of foetal calf serum did not have any beneficial effects on the viability of the cells in the present study. This could be due to the presence of substantial amount of native plasma in the media.

In conclusion, of the four types of media tested, namely, HBSS, HBSS with FCS, M 199 and M 199 with FCS, HBSS and HBSS with FCS maintained cell viability, but did not support the multiplication of the cells. In the media M 199 and M 199 with FCS, there was proportional increase in number of cells that indicated a probable multiplication of the cells in the media. The addition of FCS did not alter the quality of the media as far as the viability of haemocytes are concerned. Among the media tested, M 199 appeared to support the multiplication of the haemocytes whereas HBSS maintained the cells. However further detailed studies using 3_H thiadmidine has to be done for the confirmation of multiplication of haemocytes *in vitro*.

Acknowledgments

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Haemolymph protein profile of the edible oyster, *Crassostrea madrasensis* (Preston) exposed to different salinities

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ABSTRACT

The haemolymph total protein concentration and protein profile of *C. madrasensis* exposed to different salinities were studied. The animals were maintained at salinities 6 , 12, 24 and 36 ppt for one month. The serum total protein concentration showed significant reduction at 6 and 36 ppt. The haemolymph protein profile obtained by SDS-PAGE also varied with difference in salinities. There were four bands with molecular weight 86, 74, 31 and 26 kDa in all the four groups. The intensity of bands was higher in groups maintained at 12 and 24 ppt compared to other groups. The haemolymph of animals maintained at 6 ppt also showed three additional bands of 60, 52 and 13 kDa that were feebly expressed.

Introduction

The immune mechanisms of molluscs include cellular and humoral factors. Several workers have studied the cellular defense system of bivalves. These include studies on *Crassostrea virginica* (Cheng 1989), *Ruditapes philippinarum* (Oubella *et al.*, 1996), *Tridacna crocea* (Nakayama *et al.*, 1996), *Mytilus edulis* (Rasmussen *et al.*, 1985) etc.

Studies on humoral factors of bivalves are very limited. The haemolymph proteins of marine invertebrates are unique in composition, as they do not contain immunoglobulin or albumin like proteins and the protein composition vary in relation to physiological and functional state of the animal. This in turn may depend on exposure to pollutants and pathogens and also on environmental factors correlating with the reproductive cycle of the animal (Muromoto *et al.*, 1996).

The difference in total protein concentration in copper and cadmium stressed *C.virginica* has been studied by Cheng (1989). Chu and Peyre (1989) studied the effect of environmental factors and parasitism on haemolymph protein of *C.virginica*.

Granath *et al.* (1987) have analyzed the haemolymph of *Biomphalaria glabrata* by sodium dodecyl sulphate polyacrylamide

gel electrophoresis (SDS-PAGE). Two dimensional gel electrophoresis was used by Muramoto *et al.* (1996) to study the haemolymph composition of *Megabalanus rosa*. The change in *C. virginica* serum composition associated with parasitic infection was studied using SDS-PAGE by Ford (1986).

In the present experiment an effort was made to study the effect of variation in salinity on total protein concentration and protein profile.

Materials and methods

The edible oyster, *C.madrasensis* were collected from Cochin backwaters. They were cleaned and maintained in filtered and aerated seawater of 22 to 24 ppt salinity. About 60 animals of the same stage of gonadal development were chosen for the experiment, 15 each for each salinity group of 6, 12, 24 and 36 ppt. They were slowly acclimatized to the respective salinity during a one-week period and maintained in the same salinity at a temperature of 31°C for one month before collecting the haemolymph samples.

The haemolymph was withdrawn using a 2 ml syringe with 27-gauge needle from the adductor muscle through an adjacent notch in the shell valve without harming the animals. It was centrifuged at 5000 rpm for 10 minutes to remove cells and the supernatant serum was used for the experiment. Nine replicates for each group were taken. The total protein was estimated using the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Serum proteins were separated by SDS-PAGE as described by Laemmli *et al.* (1970). Separating gel of 11.5% was used for electrophoresis. The samples mixed with equal volume of sample buffer were loaded along with standard protein marker (Genei). Electrophoresis was carried out at 140 V for 4 to 5 hrs and the gel was stained with coomassie brilliant blue.

Results

The mean total protein concentration of haemolymph of *C. madrasensis* varied in different salinity groups (Fig. 1). In group 1 which was maintained at 6 ppt salinity, the total protein concentration varied from 0.82 to 1.12 mg/ml of

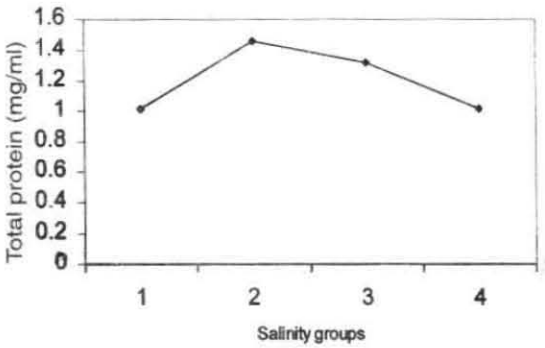

Fig.1. SDS-PAGE of *C. madrasensis* haemolymph maintained at different salinities.

haemolymph with a mean of 1.01 ± 0.09 mg/ml haemolymph. The group that was maintained in 12 ppt showed a total protein concentration ranging from 1.18 to 1.62 with a mean of 1.46 ± 0.15 mg/ml haemolymph. Group 3 showed a total protein concentration varying from 1.22 to 1.46 with a mean of 1.32 ± 0.1 mg/ml haemolymph. Group 4 had a mean total protein value of 1.01 ± 0.11 mg/ml with a range of 0.88 to 1.14 mg/ml haemolymph. The maximum total protein value was observed at 12 ppt salinity and minimum at 6 and 36 ppt. The result of total protein concentration at various salinities is given in Table 1.

The analysis of variance of the results reveals significant difference between total protein concentrations at different salinities. Students t test revealed the following facts : Group 1 and 4 behaved similarly with respect to total protein concentration. Likewise, group 2 and 3 showed similar total protein concentra-

TABLE 1. *Total protein concentration (mg/ml) of* C.madrasensis *haemolymph maintained at different salinities.*

Replications	Gr.1	Gr.2	Gr.3	Gr.4
1	1.02	1.52	1.23	1.1
2	1.06	1.44	1.24	1.12
3	0.96	1.46	1.28	0.88
4	0.96	1.36	1.26	1.14
5	0.98	1.62	1.30	0.92
6	1.12	1.60	1.22	1.06
7	1.10	1.60	1.44	1.08
8	1.06	1.18	1.45	0.89
9	0.82	1.33	1.46	0.9
Mean±SE	1.01±0.09	1.46±0.15	1.32±0.1	1.01±0.11

tions. However group 1 and 4 had a significantly lower total protein concentration when compared to group 2 and 3.

The SDS-PAGE of the proteins in the serum of *C.madrasensis* maintained at different salinities showed 4 to 7 bands. The haemolymph of animals maintained at 6 ppt, showed 5 to 7 bands of molecular weight 86, 74, 60, 52, 31, 26 and 13 kDa. In some animals 86 and 60 kDa bands were not expressed. In group 2, which was maintained at 12 ppt, and group 3 at 24 ppt only 4 bands with molecular weight 86, 74, 31 and 26 kDa were expressed. The 31 and 26 kDa bands were seen as a single zone. The haemolymph of animals maintained at 36 ppt salinity also expressed protein bands of 86, 74, 31 and 26 kDa. The 60, 52 and 13 kDa bands were feebly expressed only in group 1 at 6 ppt salinity (Fig. 2).

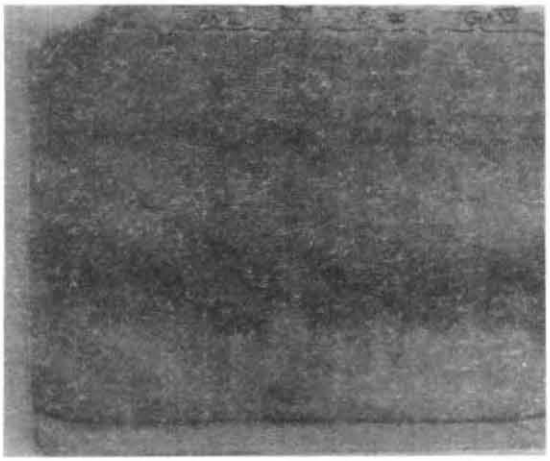

Fig. 2. SDS-PAGE of haemolymph *C. madrasensis* maintained at different salinities.

Discussion

Very limited studies have been done on the total protein concentration of bivalves. Studies by Chu and Peyre (1989) on *C.virginica* showed that the total protein concentration did not vary with salinity. The total protein value was also much higher than that of the present observation. *C. virginica* is a species which is cultured extensively in temperate zone. The difference in response to various environmental factors in these two species may be due to species variation or adaptation in different climatic conditions. The study by Ford (1986) indicated a variation in total protein value with relation to temperature and gonadal development. In the present study, both at high and low salinities, there was a significant reduction in total protein

concentration which may be due to the stress caused by these salinities. This reduction in total protein concentration in *C. madrasensis* needs further study.

The SDS-PAGE of haemolymph of *B. glabrata* (Granath *et al.*, 1987) and *C. virginica* (Ford, 1986) showed bands ranging from 10 kDa to more than 500 kDa. Though majority of bands in the above cited works were comparable to bands obtained in the present study, some bands were not at all comparable.

In the present study, we noticed differences in polypeptide bands in relation to salinity. The animals kept at 12 and 24 ppt salinity showed 4 bands of protein of molecular weight 86, 74, 31 and 26 kDa which are comparable to protein bands obtained in *C. virginica* by Ford (1986). He also noticed 12 bands having molecular weights above 500 kDa, the number of which varies seasonally. These bands were not observed in the present study. Probably this may be due to the fact that *C. virginica* were maintained in a temperate climate, which is entirely different from the climatic condition in which *C. madrasensis* were kept.

At 6 ppt salinity, three additional bands of 13, 52 and 60 kDa appeared and there was a significant reduction in 26 and 31 kDa fractions. In 36 ppt salinity also, there was a reduction in 26 and 31 kDa bands, but 13, 52 and 60 kDa protein bands were totally absent. Our study indicated that the proteins of 26 and 31 kDa are affected by the stress conditions of high and low salinities which had caused a reduction in total protein concentration. The appearance of 13, 52 and 60 kDa bands in low salinity group is a significant finding that has not been reported elsewhere. The protein fractions of *C. madrasensis* in relation to various environmental and stress condition need further study.

Acknowledgements

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