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Inflammatory reactions of the Indian edible oyster, *Crassostrea madrasensis* (Preston) and its modulations on exposure to Nuvan and copper

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Abstract

Inflammatory responses in the Indian edible oyster *Crassostrea madrasensis* under apparently normal conditions and on exposure to an organophosphorous pesticide (Nuvan) and a heavy metal (copper) were studied. The inflammation was induced in the adductor muscle by injecting Freund's complete adjuvant. The animals were exposed to these pollutants for 14 days. Samples were examined histologically at 24 hours, and on the 3rd and 7th day. In the animals that were maintained in apparently normal conditions, initially there was mild to moderate infiltration of hemocytes, which secreted proteinaceous material. At later stages, the hemocytes were found to excrete fibrin like shreds, which were observed radiating from small cells. Finally, these hemocytes (fibrocytes) aggregated into nodules, in which some of the cells assumed a spindle shape and were arranged in concentric layers, forming a mass of cells. In Nuvan and copper exposed animals, the inflammatory reactions were very much subdued.

Keywords: *Crassostrea madrasensis*, copper, Nuvan, hemocytes, inflammation, edible oyster

Introduction

Invertebrates, including bivalves lack immunological memory mediated by the B and T lymphocytes unlike mammals. Primitive defense mechanisms like recognition and phagocytosis of non-self materials by the hemocytes play very important role in invertebrates. The hemocytes perform functions like nutrient digestion and transport, wound repair and excretion (Mounkassa and Jourdane, 1990). 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). The process of inflammation and wound repair in oysters include infiltration of hemocytes to the affected area, destruction of disease agents and aggregation of hemocytes *via* filopodia, which consequently form a plug to seal the wound. This prevents further bleeding until complete healing (Chu, 2000).

Molluscs frequently encounter diseases due to microbial infections. They become vulnerable to infections when they are physiologically weak and/ or the infecting organisms acquire virulence. The study of inflammatory reactions in molluscs will throw light on the evolution of host-pathogen interactions and will give information on adaptations of molluscs in hostile environments. Since many molluscs are candidates for aquaculture, it is essential to unravel the effect of different pollutants on the inflammatory reactions of bivalves. We made an attempt to study the inflammatory reactions in the Indian edible oyster, *Crassostrea madrasensis* and to understand how the defense system is affected in the presence of pollutants such as Nuvan (an organophosphorous pesticide) and copper (a heavy metal).

Materials and methods

***Experimental animals*:** The edible oysters, *Crassostrea madrasensis* (mean size 6.4 ± 1.2 cm x 4.3 ± 0.8 cm) were collected from the backwaters of Cochin around Vypeen Island during October

2000 when the salinity was 10-15 ppt. They were cleaned and maintained in filtered and aerated seawater at 12 ppt in tanks (capacity: 50 l). They were fed *ad libitum* on *Chaetoceros* sp. Faecal matter was removed and water partially exchanged regularly.

Experimental design: Four concentrations of Nuvan (Dichlorvos 1000 mg/ml), viz., 0 ppm, 0.05 ppm, 0.1 ppm and 0.2 ppm were chosen in triplicates and each treatment was with 15 animals. For the experiment with copper, 4 doses *viz*., 0 ppm, 0.1 ppm, 0.5 ppm and 1.0 ppm of copper were selected. Appropriate amounts of $CuSO_4.5H_2O$ was dissolved in water of 12 ppt salinity to get the respective concentrations of copper ions. Each treatment had three replicates with 15 animals each. The animals were exposed to Nuvan and copper at the respective concentrations for four weeks before the inflammatory studies. The sub-lethal doses of Nuvan and copper were selected based on previous studies by the same authors (Gijo Ittoop *et al*., 2005, 2006). One–tenth of the LC_{50} and below was taken as the sub lethal doses and at these doses there was normal feed intake, opening and closure of the shell valves.

Inflammatory studies: Freund's complete adjuvant (FCA) was used to induce inflammatory responses. The animals were injected 0.02 ml of FCA using a sterile hypodermic syringe into the adductor muscle under sterile conditions. The adductor muscle was dissected out at day 1, 3, 7 and 14 post injections and fixed in Bouin's fixative for 24 hours. The tissues were washed, dehydrated, embedded in paraffin wax and 5 ìm sections were cut and stained with hematoxylin and eosin (Sanders, 1974). The slides were observed and photographed using a compound microscope (Leica DMLS, Germany).

Results

Inflammatory reactions observed in the control animals at day 1 consisted of exudates containing proteinaceous material and moderate amount of hemocytes. These exudates contained fibrin-like shreds (Fig. 1). On the third day, large number of hemocytes were found to accumulate in focal areas and exudates contained more fibrin-like shreds (Fig. 2). The cells in 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 found radiating between the cells, criss-crossing the exudate. The hemocytes formed

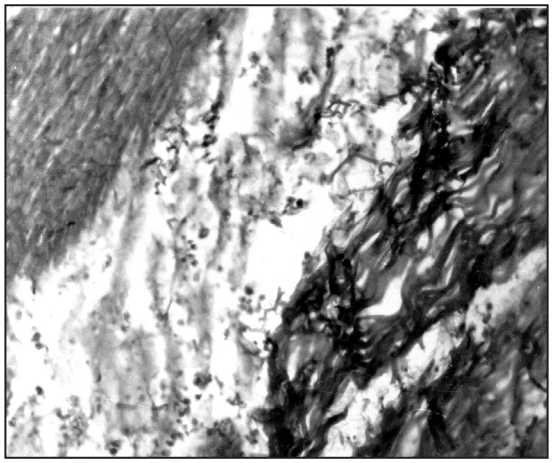

Fig. 1. Inflammatory reactions in the control animals after injection of FCA, at 24 hours show moderate number of hemocytes, proteinaceous exudates and the fibrin shreds (40x)

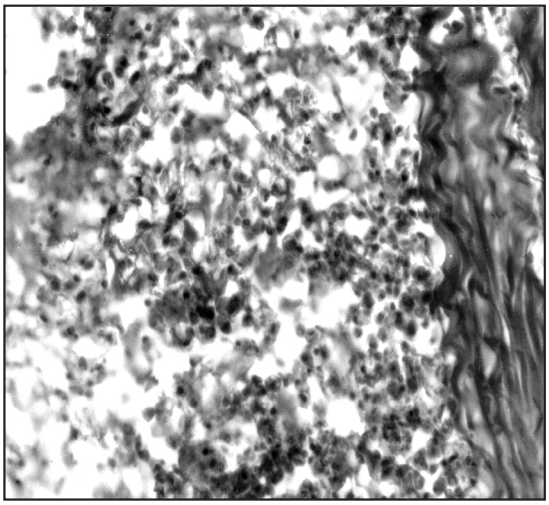

Fig. 2. Inflammatory reactions in the control animals on the third day after injecting with FCA, showing extensive infiltration of the hemocytes in the inflamed area. The fibrin like shreds increased in the matrix (40x).

nodules, which consisted of cells that had spindle shaped/elongated bodies and were arranged in concentric layers (Figs. 3 and 4). By the seventh day, the number of hemocytes in the intermysium was reduced and the mass of hemocytes found to fuse together to form a granuloma like structure (Fig. 5). 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 exposed animals (Fig. 6). There was extensive necrosis in the adductor muscle. The amount of hemocytes at the injected site was less at all time intervals.

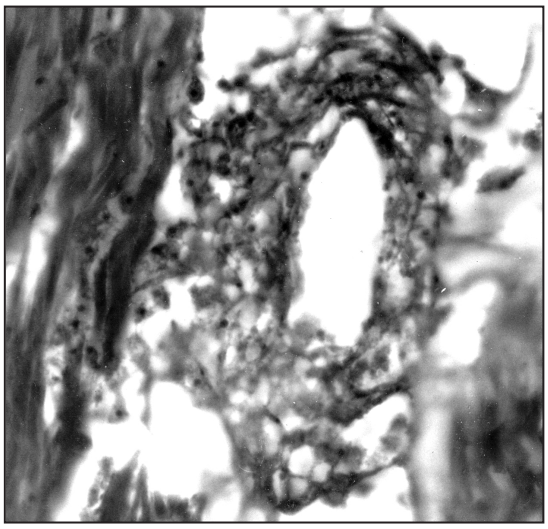

Fig. 3. Nodule formation in the third day of inflammation in the control animals (40x)

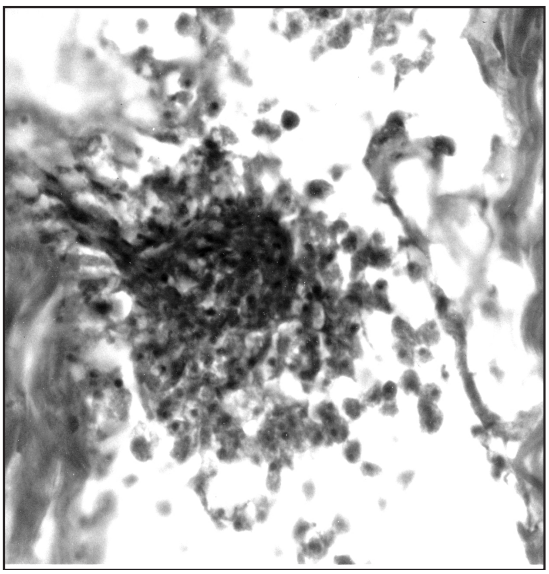

Fig. 4. Nodule formation in the third day of inflammation in the control animals. Many of the cells in the nodule exhibit spindle shape (40x)

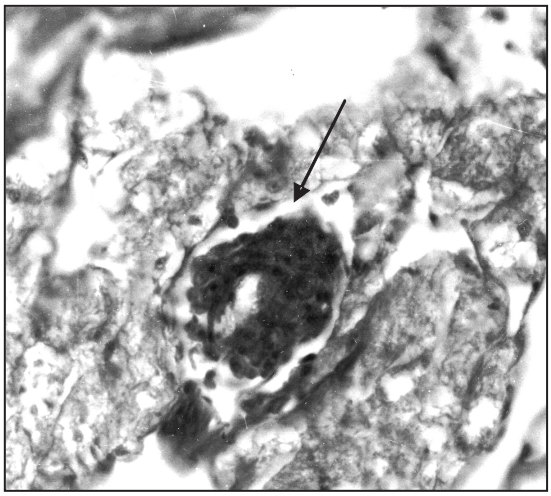

Fig. 5. Granuloma formation in the seventh day at FCA injected site in the control animals (40x)

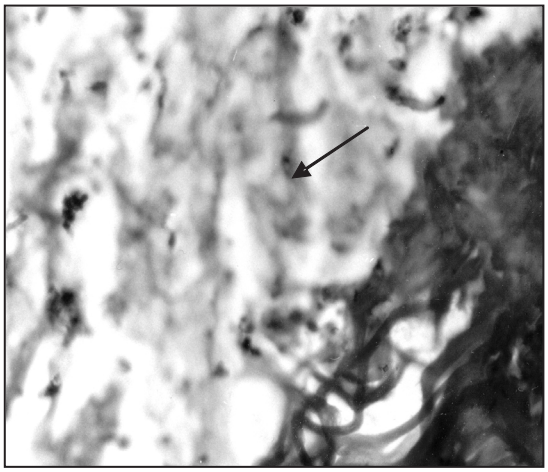

Fig. 6. Inflammation in Nuvan treated animals at 24 hours after FCA injection show reduced number of hemocytes (40x)

There was no nodule or granuloma formation. The secretion of exudates and fibrin formation were also not observed. The animals at 0.1 and 0.2 ppm died by the seventh day and those at 0.05 ppm Nuvan died in two weeks. Prior to mortality the animals exhibited poor feed intake, and the response to disturbance in water was also poor because the animals closed the shell valves.

In the copper exposed animals also, the inflammatory reactions were similar as in the case of Nuvan. Though there was moderate hemocyte infiltration by 24 hours (Fig. 7), the exudate secretion was very less and there was no granuloma formation. Healing, as observed in the control, was not observed in any of the samples studied. There was very high mortality in the animals exposed to 1 ppm copper from the very next day of injection and all the animals in that treatment died by the fifth day. In other concentrations of copper, there was complete mortality by fourteenth day.

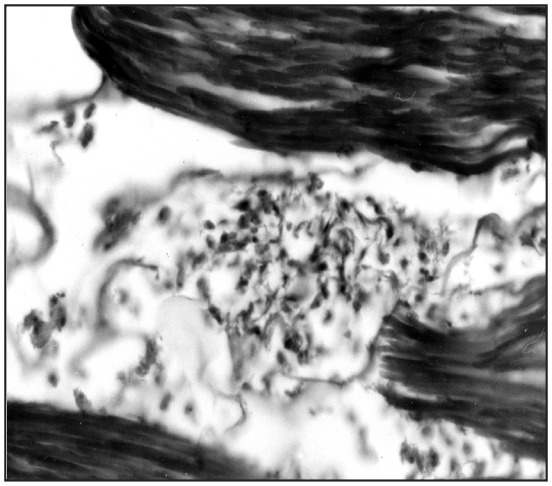

Fig. 7. Reduced inflammatory reactions in copper treated animals against FCA injection (40x)

Discussion

There was large-scale infiltration of hemocytes in the inflammatory tissue of *C. madrasensis*, which secreted the exudate containing proteinaceous material and fibrin-like shreds. In vertebrates, the fluid part of the inflammatory exudate originates from the blood plasma (Sindermann, 1993), whereas, in molluscs the entire exudate is formed from cells. Many workers have reported massive infiltration of the hemocytes in the inflammatory tissue of molluscs (Cheng, 1983; Balquet and Poder, 1985; Elston *et al.,* 1987; Jourdane and Cheng, 1987; Uhazy *et al.,* 1988; Friedman, 1991; Fisher *et al.,* 2000). The present work has established the significant role of hemocytes in the formation of inflammatory exudate. The formation of hemocyte nodules by concentric accumulation of hemocytes, which assume a spindle shape, was noticed in animals that were not exposed to pollutants. Such nodule formation was absent in the case of animals exposed to Nuvan and copper. The formation of nodules towards the later stages of infection was reported by Elston *et al.* (1987) in *Crassostrea gigas* and fibrosis was reported by Uhazy *et al.* (1988) in *Biomphalaria glabrata*. Foley and Cheng (1972) also observed formation of nodules by arrangement of concentric layers of granulocytes in *Crassostrea virginica*, as observed in the present study. Fisher (1986) and Feng (1988) have observed the hemocytes encircling abiotic particles or invading organisms that are too large to be phagocytosed by a single hemocyte, a process referred to as encapsulation in *C. virginica*. The hemocytes surrounding the foreign particle grow into layers and eventually isolate it from the host tissue. This has also been demonstrated by Rifkin and Cheng (1968) and Rifkin *et al.* (1969). The results observed in *C. madrasensis* are similar to those observed in *C. virginica.*

In Nuvan and copper exposed animals, the amplitude of hemocyte infiltration and subsequent exudate formation and fibrin 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. However, exposure of *Mytilus edulis* (Sunila, 1988) and *C. virginica* (Oliver and Fisher, 1997) to heavy metals resulted in increased inflammatory response. This is contrary to the result obtained in the present study. This may be because of the higher concentration of copper used in the present study

compared to the above studies, which might have resulted in a general reduction of the hemocyte population in these animals.

The reduction of hemocyte infiltration can be attributed to the general reduction of circulating hemocytes. The reduction in the hemocyte number as a result of exposure to organic pollutants was reported by Fisher *et al.* (1990), Pipe *et al.* (1995) and Fournier *et al.* (2002), whereas, copper is reported to be lethal to the hemocytes (Cheng, 1988). 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.

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 an increased susceptibility to *Perkinsus marinus* infection.

The present study revealed the inflammatory responses in *C. madrasensis* and the significant role played by hemocytes in exudate and nodule formation. It was also observed that the primary defense mechanism like inflammation is very much reduced in Nuvan (organophosphorous pesticide) and copper (heavy metal) exposed animals which could ultimately result in increased disease susceptibility of animals exposed to these pollutants.

Acknowledgements

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