

Histopathological changes in the hepatopancreas of the penaeid shrimp *Metapenaeus dobsoni* exposed to petroleum hydrocarbons

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Abstract

Petroleum hydrocarbons are toxic to marine invertebrates when present above a threshold level in the marine ecosystem. The major detoxification organ in shrimps is the hepatopancreas, which has been used as an indicator organ for toxicity assessment. The effects of Bombay High Crude on the morphology of hepatopancreatic tubules of *Metapenaeus dobsoni* when examined under the light and electron microscopes reveal a series of changes indicating that the cellular compensatory mechanism is activated by low or sub lethal doses of hydrocarbons. At the high sub lethal dose of 8 ppm the cellular detoxification mechanisms fail resulting in severe structural damage. The changes indicate that they are dose and time related histopathological responses. Changes such as an increased presence of B – cells towards the distal end of the tubule, substantial increase in the number of F/B cells, extensive cytorrhhexis, reduction in the number of E- cells, vacuolation of E-, R- and F- cells are changes which can be detected under light microscope along with the presence of pyknotic nuclei, storage of large lipid droplet in combination with cells voided of other subcellular components, proliferation and distortion of Golgi apparatus and mitochondria which were detected in the ultrastructure study can be used as biomarkers of stress caused by petroleum hydrocarbon pollution.

Key words: *Metapenaeus dobsoni*, hepatopancreas, histopathology, petroleum hydrocarbons.

Introduction

Histopathological techniques are a rapid, sensitive, reliable and comparatively inexpensive tool for the assessment of stress response to xenobiotics. Cytological and histopathological alterations provide a direct record of trace effect. Evaluation of histopathological manifestations provides insight into the degree of stress, susceptibility and adaptive capability of the stressed organism. The route that the toxicant takes during its metabolism often dictates the choice of organs for examining the effect of xenobiotics. The visual means to study both local and systemic effect in crustaceans are effected by microscopic examination, particularly of organs that are involved in detoxification such as the hepatopancreas and gills of penaeids, which show general and specific responses to particular stressors.

Metapenaeus dobsoni is the dominant species in the shrimp catch landed along the southwest coast of India. Besides being commercially important their abundance make them an important link in the marine food chain. Contamination of the sea with petroleum hydrocarbons, especially in shipping channels and ports where crude and

refined petroleum products are transported, results in significant quantities of oil entering the water column and sediment. The hepatopancreas of crustaceans is the major site of petroleum hydrocarbons (PHC) detoxification. The main route for elimination of hydrocarbon metabolism is through faecal matter (Lee *et al.*, 1976).

Morphology of the shrimp hepatopancreas has been described by researchers from early 20th century (Hirsch and Jacobs, 1928) to present (Bliss and Mantel, 1983; Dall *et al.*, 1990). Bell and Lightner (1988) employed advanced photographic techniques to provide detailed photographs on penaeid histology including the hepatopancreas. The penaeid hepatopancreas is a large, compact, paired glandular organ occupying a substantial portion of the cephalothoracic cavity. It is enveloped by a thin membrane of connective tissue, the *tunica propria*. The hepatopancreas consists of several blindly ending tubules, held together loosely by basophilic connective tissue strands which provide an increased surface area for digestion and absorption. Each tubule has a lumen in the centre, which is lined by an epithelium of one cell thickness except in the distal blind end (Gibson and Barker, 1979). Five

types of cells have been identified in the hepatopancreatic tubule epithelium that take part in the digestion process, which has a 24 hour cycle in penaeids (Al-Mohanna, 1983). The E- (embryonic or Embryozellen) cells, F- (fibrillar) cells, B- (blister-like or Blastozellen) cells (Jacobs, 1928), R- (resorptive/absorptive or Restzellen) cells and the more recently discovered M- (midget) cells (Al-Mohanna *et al.*, 1984) play their individual roles in digestion, absorption and excretion. The undifferentiated E-cells are found at the distal tips of each tubule with proximal nuclei and conspicuous nuclear bodies. The multi- vacuolated R- cells occur throughout the hepatopancreas surrounded by a network of myoepithelial cells with prominent nuclei and associated contractile fibres. B-cells have smaller nuclei and nucleoli which are displaced to the periphery by a single large vacuole that forms by the aggregation of smaller vacuoles as digestion progresses. B-cells are absent from the middle regions of the hepatopancreas and are excretory in nature. F-cells are basophilic and intersperse between B- and R- cells in the middle region of tubules (Dall, 1967; Al-Mohanna *et al.*, 1985; Al-Mohanna and Nott, 1986, 1987). There is general agreement in the sequence of formation of cells within the hepatopancreatic tubules in the order E → R and E → F → B cell sequence (Loizzi, 1971; Hopkin and Nott, 1980). Al-Mohanna and Nott (1986) provided evidence through electron microscopy for the holocrine mode for digestion, rejecting any secretory role for the B-cells, which are recognized to be excretory in nature. Bunt (1968) confirmed the absorptive and lipid storage functions of the R-cells through electron microscopy and attributed an exocrine role to F-cells.

Histopathological studies on crustaceans with regard to xenobiotic damage are few in comparison to those on molluscs. Such damage to mussel hepatopancreas has been investigated extensively, using both light microscopy (Bayne *et al.*, 1980; Moore *et al.*, 1982; Auffret, 1988; Cajarville *et al.*, 1992) and electron microscopy (Hawkins, 1980; Moore and Farrar, 1985; Nott *et al.*, 1985; Lowe and Pipe, 1994). Accumulation of heavy metals in shrimp hepatopancreas was reported by Dall and Moriarty (1983), Al-Mohanna and Nott (1987) and Manisseri and Menon (1995). Histopathological effects of different concentrations of Bombay High Crude oil in crabs *Charybdis lucifera* and *Scylla serrata* were described by Chandy and Kolwalker (1984) who found the degree of damage to correlate well with percentage mortality. Starvation is a stress factor that has been thoroughly investigated using histopathological methods in crustaceans, especially in aquaculture systems. Progressive suppression of metabolism and damage to hepatocytes were reported in starved *Penaeus japonicus* (Clifford and Bricks, 1983; Papathanassiou and King, 1984).

In the present study an attempt has been made to elucidate the response of hepatocytes of *M. dobsoni* to varying doses of sublethal concentration of PHCs and to identify histopathological biomarkers.

Materials and methods

Shrimps of the size group 30-35 mm were procured from shrimp farms in Vypeen (10° 00' N 76° 10' E) and acclimated in the laboratory. Intermoult stage shrimps were selected to avoid moult related changes interfering with the cytological assay and exposed to 1 ppm, 4 ppm and 8 ppm of PHCs using a semi flow through system and the water accommodated fraction of Bombay High Crude, the concentration of which was estimated using a fluorescent spectrophotometer at 310 (ex) - 360 (em) nm wavelengths by standard methods (IOC, 1984). A few shrimps were starved to study the effect of starvation, each in a separate tub to avoid cannibalism. One set of shrimps was also maintained as control animals. The salinity of the test medium was maintained between 18-20 ppt, temperature at 28 ± 2 °C and fed on boiled clam meat *ad libitum*. The test medium was renewed every 24 hours. Faecal matter and excess feed were siphoned out. Sampling was done 8 hours after feeding to avoid confusing the changes brought about during the course of active digestion within the hepatocytes with that of the effect of PHC exposure.

Samples of hepatopancreas taken from shrimps sacrificed on the 5th, 10th and 15th days in the case of those in the control, 1 ppm and 4 ppm doses and the 1st, 5th and 8th day in the case of 8 ppm and starved individuals were preserved in Bouin's fixative (Luna, 1968). Samples were made in triplicate for individuals exposed to each dose. After post fixative treatment with lithium carbonate and methyl benzoate the tissue were dehydrated using ethyl alcohol, cleared in benzene and embedded in paraffin wax at 58-50 °C. Sections of 5-7 µm thickness were cut from the embedded tissues, stained using Mallory's Triple stain and mounted in DPX following standard procedure (Humason, 1972). The sections were viewed and photographed under light microscope.

Hepatopancreas tissues for electron microscopy were fixed in 3 % glutaraldehyde in cacodylate buffer at 4° C at pH 7.2 for 12 hours. The tissues were washed in cacodylate buffer before and after trimming, and fixed in 1% osmium tetroxide at 4° C for 2 hours. The tissues were washed again in buffer after draining out the osmium tetroxide followed by three dips in double distilled water. The tissues were then dehydrated through an ascending series of ethanol concentrations (30%, 50%, 70%, 90% and absolute at 15 minutes each at 4° C (two washes

in each concentration). They were then infiltrated in a series of combinations of ethanol and DMAE (75:25, 50:50, 25:75) and finally embedded in Spurr's embedding resin in synthetic moulds at 70 °C for 24 hours. Ultrathin sections were cut from the blocks using an Ultracut E (LKB) ultramicrotome, the sections mounted on copper grids and stained with uranyl acetate (Watson, 1968) and lead citrate (Reynolds, 1963). The sections were examined and electron micrographs taken using Philips EM 300 transmission electron microscope operating at 60 and 80 KV.

Results

Control specimen: Longitudinal and transverse sections of the hepatopancreas of shrimps maintained under control conditions sampled on the 1st and the 15th day did not show any differences in the number or structure of various digestive cell types or tubular structure and arrangement. The general structure of the hepatopancreatic tubules corresponded with that cited in literature for normal structure (Fig. 1 A, B). Electron micrographs revealed R- and F- cells with intact cytoplasm and organelles, a rigid cell membrane and undistorted microvilli in the brush border region.

Starved specimen: Hepatopancreatic tubules of shrimp examined under the light microscope after 8 days of starvation showed increased spacing between tubules due to shrinkage and an increased lumen within the tubules due to the reduction in the height of the epithelium (Fig. 1 H). A proliferation of R-cells was seen in the middle and proximal regions but these are reduced in size, though increased in numbers. At the end of 8 days of starvation, numerous vacuoles and reduced cytoplasm and organelles were seen under the electron microscope (Fig. 2 I). A dramatic increase in glycogen granules was also seen.

Dosed specimen: Under the light microscope no conspicuous change in the hepatopancreatic tubules was discernable until the 15th day in the dose 5 ppm PHCs, when a proliferation of F/B cells was noted. By the 15th day the presence of B- cells in the mid regions of the tubules was increasingly encountered. Vacuolation of the E-cell layers and proliferation of R-cells also were evident. The myoepithelium remained largely intact though in several areas the tubules did not remain as tightly packed as in comparison to control specimen. Ultrastructure examinations showed proliferation of the ER system and glycogen granules from the 5th day onwards (Fig. 2 A) and by the 15th day distortion in the structure and arrangement of Golgi apparatus in the F- and R- cells of shrimps exposed to 1 ppm PHCs (Fig. 2 B) was apparent. Desmosome-like structures were found to occur frequently on the microvilli of brush borders (Fig. 2 D).

Specimens exposed to 4 ppm of PHCs for 5 days showed an increased proliferation of F and F/B cells in the middle region of tubules, indicating enhanced secretion into tubules (Fig. 1 E). Interspaced between these are areas where cells have disintegrated, shrunk or eroded. In some shrimps dosed with 4 ppm of PHCs for 10 days only a single row of E-cells was seen (Fig. 1 C), indicating the formation of fewer new cells. Increased vacuolation, secretion in the lumens, breakage of tubule connective tissue lining and sloughing off of the tubule epithelium appeared to have set in by the 15th day (Fig. 1 D). Electron micrographs showed destruction of the ER (Fig. 2 G), proliferation of mitochondria (Fig. 2 C), lysosomes and small lipid droplet by the 10th day. Formation of excretory vesicles filled with electron dense material on the basal membranes of R-cells were also seen (Fig. 2 E).

The proliferation of vacuolated B-cells almost up to the distal end of the tubules and reduction in the number of E-cells were noticed by the 8th day of expose to 8 ppm of PHCs. The tubules appeared shrunk and disintegrated in a majority of cases. The *tunica proproria* lay detached from the tubules (Fig. 1 F). Collapse of structural integrity of the tubules was evident with the heavily vacuolated cells and cell debris piled up in the lumen. Shrinkage and breakage of tubules and enlargement of tubule lumen resulted in structurally void areas in the hepatopancreas. The tubules appeared shortened and the middle region of the hepatopancreas almost devoid of any distinct and defined morphological features.

Response to a 15 minute exposure to the lethal dose of 20 ppm showed an increase in the number of B-cell vacuoles and their presence right up to the distal region of the tubules accompanied by an extremely reduced number of F- cells. A number of cells were found extruded into the lumen in the proximal region. However, no shortening or distortion of tubules was noticed (Fig. 1 G).

Discussion

In the present study, one of the most evident changes is a proliferation of B-cells in the dosed shrimps, indicating a high rate of excretion from the hepatopancreas. The accumulation and elimination of the xenobiotic entering the hepatopancreatic tubules is perhaps effected with a large number of F-cells converting into B-cells. This inference may be drawn from the increase in the proliferation of F- and F/B cells in the mid region of the tubules from shrimps exposed for 5, 10 and 15 days to 1 ppm PHCs and those exposed for 5 and 10 days to 4 ppm PHCs. The presence of B-cells close to the distal end in the 1 ppm dose at 15 days and 4 ppm dose at 10 days

and almost up to the distal end in the shrimps exposed to lethal concentration are further indicative of the attempt by the shrimp to excrete as much of the metabolized xenobiotic as possible. The photomicrographs provide evidence that as the dose and time duration of exposure increase the shrimp is unable to keep up with the production of more F- and B- cells from E- cells, with the E-cells themselves suffering atrophy and extensive vacuolation. In the 4 ppm (x 15 days) and 8 ppm doses, cytorrhesis and delamination of hepatocytes may be the combined effect of cell necrosis and dysfunction of the connective tissue surrounding the tubule. The disintegration of the *tunica propria* is apparent as the dose and time of exposure increases. While the extrusion of B-cells from the proximal end of the tubules and the presence of darkly staining inclusions within the hepatocytes is part of the natural progression of the digestive cycle, the mass delamination of hepatocytes, presence of clumps of dislodged cells within a tubule lumens and the proliferation of darkly staining bodies can only be attributed to endotoxic response of the hepatocytes. Influx of water and ions were cited as the cause for swelling and damage of cell membranes with leaked intracellular ions, enzymes and other proteins in snail *Planorbis corneus* exposed to PCP (Klobucar *et al.*, 1997). Low pH inside the cell was also seen to stimulate lysosomal enzymes and cellular lesions in turn increasing lipid peroxidation within the cells. A deficiency in ATP which stimulates glycolysis promotes the accumulation of lactic acid and a shift towards acidosis. A deficiency in ATP also inhibits the cationic pump within cells allowing and influx of water and ions leading to cytorrhesis. Destabilisation by lysosomal membranes may be linked to lysosomal enlargement and lipofuscin accumulation, both of which are indicative of autophagy. Autophagy is directly linked to digestive cell atrophy (Moore, 1988; Lowe and Pipe, 1994). Osmotic and ionic exchanges between the cell and the extracellular milieu is mediated by the cell membrane and is essential for maintenance of cellular integrity and for such physiological functions as neurotransmission, muscle contraction and osmoregulation. Aromatic hydrocarbon induced changes in the surface organisation of membranes may interfere with these essential processes. Cellular injury can induce dramatic alteration of ER with swelling and rupture of other membranous structures like the mitochondria, which additionally show lack of cristae as well as general cytoplasmic disorganization (Bayne, 1985). Activated derivatives of xenobiotics are retained within the lipoprotein membranes of the endoplasmic reticulum where they may enter a self sustaining redox cycle and give rise to potentially damaging oxyradicals which react with many biological molecules leading to protein degradation, lipid peroxidation, DNA damage and cell death (Winston and

Di Giulio, 1991). The resulting bulging and disintegration of the ER may have deleterious effects on the vital function of this organelle. Hinton and Lauren (1990) found that because of exposure to xenobiotics ER the cells can undergo proliferation, markedly altering the internal organization. Proliferation of SER is an indication of induction and increased activity of the MFO system. Detoxifying enzymes of PAH mixed function oxidases are localized in the microsomes in the SER of the hepatopancreas of bivalves (Varanasi *et al.*, 1989).

The findings of Viarengo *et al.* (1987) that the rate of protein synthesis could be related to the rate of RNA synthesis in the case of the organisms exposed to cadmium, mercury and copper and in reactions to the RER integrity probably indicate that these organelles are directly involved in dealing with all xenobiotics. Bayne (1985) opined that injury of cell induces structural alterations in the mitochondria. Early morphological changes preceding cell death are cytoplasmic oedema, dilation of ER and disaggregation of polysomes accompanied by the accumulation of triglycerides as fat droplets. Late changes are progressive swelling of mitochondria, cristae destruction, cytoplasmic swelling, dissolution of organelles and nucleus and rupture of plasma membranes (Bridges *et al.*, 1983). Similar changes were seen in the present study also.

The pattern of atrophy and vacuolation caused by starvation were distinctly discernable from that caused by PHCs as there were conspicuous absence large quantities of electron dense material, lipid droplets and delamination of cells. A proliferation of hematocytes, which was not seen in the PHC dosed shrimps, was recorded in the starved ones. However, logical conclusion can be drawn that digestion was not effective in hepatopancreas of shrimps in doses where there was extensive damage to tubules and some changes would have been compounded by the effect of starvation. However, there is no means to clearly demarcate the above in the present study.

The intracellular disintegration of the cytoplasmic organelles is further evidenced by the observations under the electron microscope. The sequestering of electron dense material along basal membranes and within vacuoles as well as the presence of large lipid filled vacuoles in cells with pyknotic nuclei elucidate the effect of toxicants at the subcellular level. A proliferation in desmosomes on microvilli, glycogen granules, mitochondria and lysosomes and exocytosis of electron dense granules by the basal membrane in lower doses and at lesser duration of exposure reflect the activation of the detoxification mechanism. Kohler (1990) found that large lipid droplets resembling large vacuoles filled with finely granular material

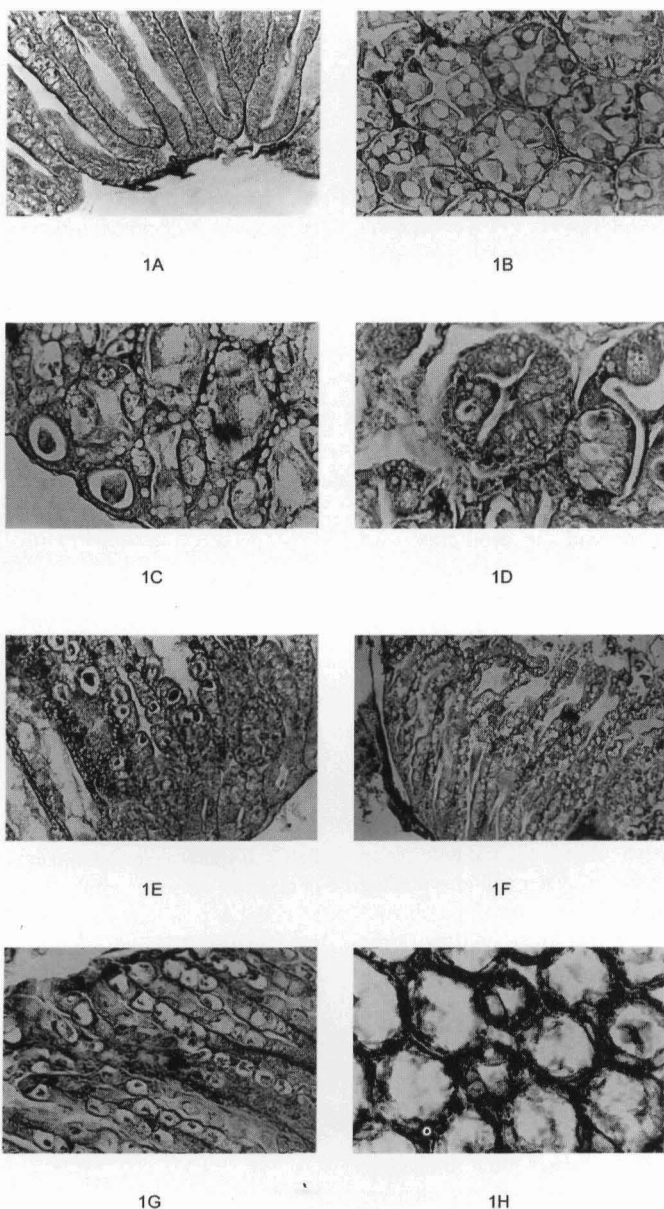


Fig. 1. *Metapenaeus dobsoni* Miers: Hepatopancreas: Structure under light microscope:

- A. Digestive tubules: L. S. of hepatocytes in shrimp maintained under control conditions with E-, F- and B-cell regions demarcated (x 80).
- B. Proximal region of digestive tubules: T. S. showing a predominance of B- and F/B-cells in shrimp maintained under control conditions (x 200).
- C. Digestive tubules: T. S. of hepatocytes in shrimp exposed to 4 ppm PHCs for 10 days showing reduction in E-cell layer thickness (x 200).
- D. Distal region of digestive tubules: T. S. of hepatocytes showing increased vacuolation in E-cell layer in shrimp exposed to 4 ppm PHCs for 15 days (x 320).
- E. Digestive tubule: L. S. of hepatocytes in shrimp exposed to 4 ppm for 5 days (x 80).
- F. Digestive tubule: L. S. of hepatocytes in shrimps exposed to 8 ppm PHCs for 8 days (x 80).
- G. Digestive tubules: L. S. of hepatocytes in shrimp exposed to 20 ppm PHCs for 15 minutes (x 80).
- H. Proximal region of digestive tubules: T. S. of atrophied hepatocytes in a shrimp starved for 8 days (x 200)

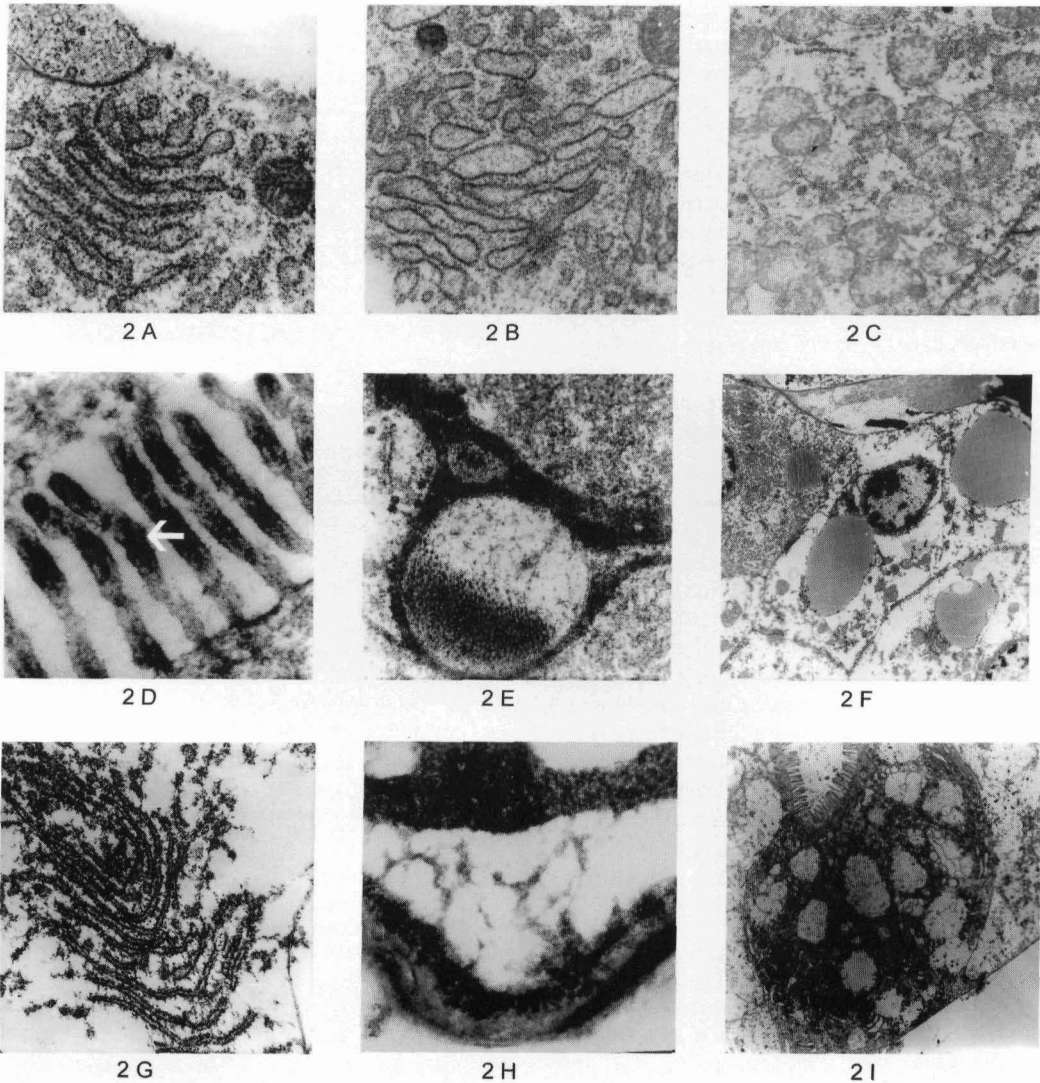


Fig. 2. *Metapenaeus dobsoni* Miers: Hepatopancreas: Ultrastructure of hepatocytes

- A. R-cell: Golgi apparatus in a shrimp exposed to 1 ppm PHCs for 5 days. (x 44000).
- B. R-cell: Golgi apparatus: Swollen cisternae in a shrimp exposed to 1 ppm PHCs for 15 days (x 44000).
- C. R-cell: Proliferation of mitochondria in a shrimp exposed to 4 ppm PHCs for 10 days (x 44000).
- D. R-cell: Microvilli: Formation of desmosomes-like structures (↑) in a shrimp exposed to 1 ppm PHCs for 5 days (x 69000).
- E. R-cell: Basal membrane: Extrusion of a vesicle filled with electron dense material of a shrimp exposed to 4 ppm PHCs for 10 days (x 93000).
- F. F- and R-cells: Large lipid droplets and a pyknotic nucleus in an F-cell adjacent to less damaged R-cell in a shrimp exposed to 4 ppm PHCs for 10 days (x 6900).
- G. R-cell: Rough endoplasmic reticulum: Damaged membranes and ribosomes in a shrimp exposed to 4 ppm PHCs for 15 days (X 37800).
- H. R-cell: Basal membrane: Separation from cytoplasm in a shrimp exposed to 8 ppm PHCs for 8 days (x 93000).
- I. R-cell: Extensive vacuolation in a shrimp starved for 5 days (x 12000).

accompany accumulation of lipophilic substances in flounder *Pleuroichthys flesus* caught from a highly contaminated site and suggested that these droplets might play a role in the transport of lipoproteins. Such conspicuous droplets were recorded during the current study also.

An inability to cope with the xenobiotic load results in increased sequestration of the hydrocarbons or metabolites within the hepatocytes, bringing about increased lysosomal activity and instability, ultimately resulting in vacuolated cells emptied of the cellular components. The conspicuous absence of lysosomes in the higher doses and at greater duration of time suggest the destabilisation of lysosomal membranes resulting in autophagy and explicate the emptying of cells or the presence of damaged cell organelles such as highly pyknotic nuclei.

The presence of some undamaged cells or areas within the hepatopancreas even within severely damaged hepatopancreas indicates that there is some mechanism where by the hepatocytes selectively induct the xenobiotic for metabolizing. The total disruption of subcellular organisation is seen only in the shrimps exposed to the 8 ppm dose or towards the 15th day of expose to the 4 ppm dose. The shrimps did not survive in the 8 ppm for more than 9 days on an average as this was close to the LC₅₀ concentration of 9.2 ppm for the size range (Paul *et al.*, 2000). Accumulated hydrocarbons were found to interact with cellular hydrocarbons leading to different histopathological lesions with the highest necrosis index of cells being recorded in the digestive tract in a study conducted in oyster samples off the site of the wreck of the Amoco Cadiz in Brittany after seven years of the incident (Berthou *et al.*, 1987).

Cellular and subcellular histopathological changes have proved to be reliable biomarkers in toxicological assays (Moore, 1990, 1993; Moore and Simpson, 1992; Cajarville *et al.*, 1993; Lilius *et al.*, 1995; Sandbacka *et al.*, 1999). Varanasi *et al.* (1992) proffered that the mode of intake, transport within, biotransformation and elimination are key factors influencing toxicokinetics. Histological alternations can be a consequence of these functions or a result of failure, inadequacy or malfunction of defense by compensatory mechanism induced by the presence of the xenobiotic. Hypertrophy, atrophy of cells, changes in the lipid content, proliferation of smooth ER, increased lysosomal autophagy and accumulation of lipofuscin were noted in shrimp hepatopancreas exposed to heavy metals (Manisseri, 1993). Positive correlation between levels of sediment PAHs and prevalence of liver lesions in *Parophrys vetulus* from contaminated sites in Puget Sound (Myers, 1992) and flounder *Pleuroichthys flesus* from similar regions in the Elbe Estuary (Kohler, 1990) were reported.

Bayne *et al.* (1980) listed several histological changes such as hyaline degeneration of the collagenous connective tissue of the gills, occurrence of parasites, production of mucus by gills, gonadal and haemopoetic neoplasma, granulocytomas, haemocyte infiltration of the tissues and loss of synchrony in digestive cells as useful for monitoring pollution effects. The noted changes elucidated in the present work can therefore be taken as biomarkers for xenobiotic damage in shrimps.

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