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ULTRASTRUCTURAL CHANGES ASSOCIATED WITH MOLLUSCICIDE HEPATOTOXICITY IN FISHES

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Key words : Mosquito fish, *Gambusia affinis*, Bayluscide, ultrastructure, liver.

ABSTRACT

Adult sexually mature male specimens of the mosquito fish, *Gambusia affinis* (Baird & Giard) were exposed to a low concentration (0.2 mg/l) of the molluscicide Bayluscide for 15 days. Semithin and ultra-thin sections of the liver, of both control and treated specimens, were examined by light and electron microscopes. The livers of the control fish showed that the typical hepatocyte has hexagonal shape and a large single euchromatic nucleus. Its cytoplasm contains rough endoplasmic reticulum, which is concentrated mainly around the nucleus and subjacent to the cell membrane. Many round, oval or rod-shaped mitochondria, a considerable amount of glycogen granules, in addition to rounded prominent fat globules are observed in the hepatocyte cytoplasm. The blood sinusoids are lined by endothelial cells, whereas numerous microvilli extend from hepatocytes into the sub-endothelial space (space of Disse). The bile canaliculi are formed at the junction of the hepatocytes.

Liver cells of treated fish showed detectable changes from that of controls. The changes include reduction of rough endoplasmic reticulum (RER) and glycogen granules, as well as mitochondrial degeneration. The primary and secondary lysosomes increased in number and became variable in size and shape. The hepatocytes also showed vacuoles of variable sizes and shapes and prominent leached fat globules. In addition to the previous changes, the severely affected cells showed evidence of nucleus and cell membrane degeneration with large cytoplasmic vacuoles communicating with each other.

INTRODUCTION

Pesticides have been widely used in the recent years and their effects have been extensively investigated. However, the mode of

toxic action of most pesticides has not been elucidated. and the effect of these pesticides remain largely undefined (Ibrahim, 1987).The molluscicidal activity of Bayer 73, a molluscicide sold commercially as Bayluscide, was first recognized by Gonnert and Schraufstatter (1958). Since then, it had been used in Egypt and other countries for the control of snails, which are the intermediate hosts for schistosomiasis. Meanwhile, it is harmful and toxic to other aquatic organisms including fish (Gonnert, 1962; Webbe, 1963; Marking & Hogan, 1967; Marzouk & Bakeer, 1991; Talha, 1994 and Danasoury *et al.*, 1997).

The preliminary laboratory work, which was carried out by Shoman (1995), indicated that the continuous bath exposure of the mosquito fish, *Gambusia affinis* to low concentration of Bayluscide (0.2 mg/l) induced a degenerative effect in the liver.

Knowledge of normal features is necessary for the understanding of pathological alterations, since no ultrastructural studies on the liver of the ovoviviparous mosquito fish, *G. affinis* have been previously reported. So, the present work aims to describe the ultrastructure of the normal liver of this species, and the alterations associated with the exposure to a sublethal concentration of Bayluscide. Also, it aims to reveal the mechanism of action of this molluscicide and the reason of fish mortalities, where the accidental discharges of pollutants are suspected.

MATERIAL AND METHODS

A total of 100 adult male specimens of the mosquito fish, *Gambusia affinis* (Poecilliidae), with a total length ranging from 2.5 to 3 cm, were used in the present study. The fish were acclimatized for 2 weeks in 60 liters glass aquarium receiving dechlorinated tap water before the experiment. Then, they were divided into two equal groups. The first one "the control" was kept for 15 days in fresh water. The second group "the treated" was kept in fresh water containing a sublethal concentration (0.2 mg/l) of Bayer 73 for 15 days. Bayer 73 is a molluscicide available on the market under the commercial name of Bayluscide (2, 5-dichloro-4-nitrosalicylanilide).

At the end of the experiment, the fish were quickly dissected and their livers were removed, minced into small pieces (0.5 – 1 mm) and immediately fixed in cold 5 % glutraldhyde in 0.1 M sodium cacodylate buffer, and kept overnight at 4°C. After washing in the same buffer, post-fixed in 1% osmium tetra-oxide in the same buffer, and processed to obtain epon embedded material. Semithin sections

(1μ in thickness) were cut, picked on glass slides and stained with toluidine blue for light microscope examination. Ultra-thin sections were obtained, picked up on uncoated copper grades, stained with uranyl acetate and lead citrate, and examined by a JEOL 100 S transmission electron microscope.

RESULTS

Light microscopy:

The examination of toluidine blue stained semithin sections of the control liver showed the general histological and cytological features of the normal liver of mosquito fish.

The hepatocytes are grouped in masses separated by blood sinusoids. They are polygonal in shape with irregular ill-defined boundaries. Each hepatocyte has a large rounded vesicular nucleus with a prominent nucleolus. Its cytoplasm contains numerous prominent variable-sized rounded fat globules and few small vacuoles, in addition to some basophilic small granules, which may be the mitochondria (PLATE IA). The blood sinusoids are lined with flat endothelial cells with thin attenuated cytoplasm and bulging elongated flat nuclei (PLATE IB).

The semithin sections of treated liver of mosquito fish showed a degenerative effect of Bayluscide on liver of fish. In most hepatocytes, a marked increase in size of both nuclei and nucleoli, leaching of fat globules with a decrease of both number and size. An obvious increase in the density of the cytoplasmic small darkly stained basophilic granules was observed. Moreover, in some hepatocytes, this pesticide caused severe or even complete disruption of the cell boundary with the disappearance of the cytoplasm. This cytoplasm was replaced by vacuoles of different shapes and sizes, which sometimes adhered to each other to form large vacuoles (PLATE IC & D).

The ultrastructure of normal liver:

The electron microscopic examination of the ultra-thin sections of the normal liver of mosquito fish, *G. affinis* showed that most hepatocytes are of parenchymal type. Adjacent parenchymal cells usually are in close contact; only the vicinity of the sub-endothelial space (space of Disse) and the bile canaliculi show a pronounced intercellular space. (PLATE IIA).

A typical parenchymal hepatocyte is hexagonal in shape. It has a large single and more or less rounded nucleus, which contains little heterochromatin and lined by a double membrane nuclear envelope. Hepatocyte nucleus usually contains an electron dense nucleolus. The large numbers of prominent fat globules are dispersed in the cytoplasm (PLATE IIA). The nucleus of all hepatocytes is surrounded by a rough endoplasmic reticulum "RER", which usually extends in one or more directions from the perinuclear region toward the cell periphery. In some hepatocytes, the extensive rough endoplasmic reticulum forms whorls or concentric patterns (PLATE IIB). Round to elongate mitochondria with electron opaque matrix and inconspicuous cristae predominate in hepatocytes (PLATE IIC). They are usually scattered all over the cytoplasm and associated with the rough endoplasmic reticulum (PLATE IIB). The rosette-shaped glycogen granules and free ribosomes are numerous and scattered all over in hepatocyte cytoplasm. In addition, small bundles of collagen fibers are seen in the intercellular space. Pinocytotic vesicles are commonly occurred along the cell membranes at interhepatocytic borders (PLATE IIC).

The blood sinusoids are encircled by groups of adjacent parenchymal hepatocytes and are lined by endothelial cells (PLATE IIA). These endothelial cells have elongated nuclei with abundant clumped heterochromatin and attenuated thin sheet-like cytoplasm (PLATE IID). The greatly attenuated thin region characterizing these endothelial cells constitute most of the sinusoidal lining and commonly contain varying-sized fenestrations. Adjacent endothelial cells overlap and interdigitate (PLATE IIA&E).

The sub-endothelial space (space of Disse) between the sinusoidal endothelium and hepatocytes contains small cells with a markedly heterochromatic nucleus with a limited cytoplasmic area around the nucleus and has a fat vacuole (PLATE IID). Numerous microvillar processes from hepatocytes project into the space of Disse (PLATE IIE).

The bile canaliculi between hepatocytes typically lie in apical cell regions. They have no specialized lining and are lined directly by the surrounding hepatocytes that are joined together by desmosomal junctions. The bile canaliculi are usually formed by 3 or 4 hepatocytes. Intracanalicular microvilli are seen projecting from the surrounding hepatocytes (PLATE IIF).

The ultrastructure of treated liver:

The examination of ultra-thin sections of treated mosquito fish liver showed ultrastructural pathological changes as signs of hepatocyte degeneration. The main change was the presence of primary and secondary lysosomes that were not only markedly increased in number but also abnormally varied in size (PLATE IIIA). Electron translucent patches were present in hepatocyte cytoplasm. Aggregation of most cytoplasmic organelles was observed in the opaque cytoplasmic matrix (PLATE IIIA).

The rough endoplasmic reticulum was severely reduced and had lost all normal architecture (PLATE IIIB). Dilation of RER was observed near the cell membrane with an absence of polysomes from the RER surface (PLATE IIIC). Degenerated mitochondria were seen either free in the opaque cytoplasmic matrix or within secondary lysosomes (PLATE IIIA). Numerous small vacuoles scattered in the cytoplasm, which communicated with each other giving the cytoplasm the foamy appearance (PLATE IIIA&C).

Also, the ultrastructural changes were observed in the bile canaliculi, where degeneration of hepatocyte microvilli took place. Reduction of the glycogen granules was observed in the hepatocyte around the bile canaliculus (PLATE IIID).

The severely affected hepatocytes had necrotic appearance. The nucleus had abnormal shape as an evidence of degeneration. Moreover, the cytoplasm showed disorganization, where it lost most of its organelles and occupied by large leached and translucent areas. The cell membrane was partially or completely degenerated (PLATE IIIE&F).

DISCUSSION

Intensive histological studies suggest that vertebrate livers are not tubular glands, but consist of a continuous mass of liver cells, tunneled by a three-dimensional network of cylindrical sinusoids. The hepatic plates or laminae hepatis form the "muralium", the walls of which separate the sinusoids, usually two cells in width (Geyer *et al.*, 1996).

Geyer *et al.* (1996) revised that Eurell & Haensly (1982) distinguished three types of arrangement of the parenchymal cells in the fish liver. The first type consists of hepatocytes situated radially around a central vein in double-layered laminae. These laminae are separated from each other by sinusoids, formed by reticular and

stellate reticulo-endothelial cells (Veinrab & Bilstad, 1955; Bucke, 1971; Anderson & Mitchum, 1974; El-Habback, 1995; Abd El-Fattah, 1999 and Konsowa & Abd El-Gawad, 2001). In this arrangement of liver parenchymal cells, the bile canaliculi lie between adjacent hepatocytes (Hinton *et al.*, 1972). In the second type, the hepatocytes lie in the form of tubes or tubules with a bile canaliculus running through the center of this structure (Mugnaini & Harboe, 1967; Hampton *et al.*, 1985 and Robertson & Bradley, 1992). Sinusoids form an extended network around these tubules. In the third type of arrangement, the hepatocytes lie in anastomosing laminae around a central vein, with the bile canaliculi situated intercellularly.

In the present study, the microscopical examination of the liver of mosquito fish, *Gambusia affinis* (Poecilliidae) showed a typical structural organization, comparable to the majority of teleosts. The present results are in accordance with the third type of parenchymal cell arrangement of Eurell & Haensly (1982). A similar hepatocyte arrangement was recorded by Chapman (1981) in the liver of the fingerling of rainbow trout, and Geyer *et al.* (1996) in the liver of tigerfish.

Ultrastructurally, the present study showed that the hepatocytes of *G. affinis* have oval to round nuclei with a very prominent nucleolus, which lie centrally or basally in the cell. Rough endoplasmic reticulum was found to be abundant in most of the hepatocyte cytoplasm and concentrated around the nucleus, in addition to free ribosomes, which commonly occur in the cytoplasm. Mitochondria of the hepatocytes were observed as round to elongate in shape and usually associated with the RER. These results are similar to those of the channel catfish, *Ictalurus punctatus* (Hinton & pool, 1976) the rainbow trout, *Salmo gairdneri* (Chapman, 1981) the tigerfish, *Hydrocynus forskalii* (Geyer *et al.*, 1996) the Nile catfish, *Chrysichthys auratus* (Yoakim & Khidr, 1985) and the African catfish, *Clarias lazera* (Konsowa & Abd El-Gawad, 2001)

The lipid appeared in the form of varying sized droplets or globules in the hepatocyte cytoplasm of *G. affinis*. Such result was observed by many investigators (Eurell & Haensly, 1982; Leatherland & Sonstegard, 1988 and Konsowa & Abd El-Gawad, 2001). But, the tigerfish, *Hydrocynus forskalii* lack these lipid droplets or globules within the hepatocyte cytoplasm.

In general, the hepatocytes of *Ictalurus punctatus*, *Salmo gairdneri*, *Serranus cabrilla* and *Clarias lazera* contain dense aggregates of glycogen in the form of rosettes or glycogen pools that

lie close to the sinusoids and bile canaliculi (Hinton & Pool. 1976; Chapman, 1981; Gonzalez *et al.*, 1993 and Konsowa & Abd El-Gawad, 2001). This disagreed with the present observations on the hepatocytes of *G. affinis*, where the glycogen granules are uniformly distributed in the hepatocyte cytoplasm.

In the light of the ultrastructural similarity of fat-storing cells recorded in fishes (Sakano & Fujita, 1982) and mammals (Kent *et al.*, 1977), cells observed in sub-endothelial space (space of Disse) can be described as fat-storing cells or Ito cells.

Uptake of organic compounds is influenced by the hydrophobicity (lipophilicity) and the molecular size of the chemical. A toxic chemical must pass through cell membrane barriers to reach target organs or tissues. Biomembranes consist of a bimolecular layer of phospholipids, with integral and peripheral proteins. Because lipids are relatively non-polar, those chemicals, which are more hydrophobic, will pass readily through them. However, there is evidence that large molecules (e.g. highly chlorinated PCBs and dioxins) cannot pass readily through membranes, even though they are very lipophilic (Bruggeman *et al.*, 1984).

Since most toxic organic chemicals are synthetic compounds that are foreign to biota (i.e. xenobiotics), it is surprising that there are biochemical mechanisms in fish to metabolize these compounds. Phase I mechanisms of biotransformation include oxidation, reduction or hydrolysis reactions in the cell. These reactions may activate, inactivate or leave the toxic activity of xenobiotic substrates unchanged, but they usually increase the polarity of the substrate, making the compound easier to excrete from fish. The most important Phase I biotransformation reactions in vertebrates are oxidations carried out in the endoplasmic reticulum of cells by mixed function oxidases (MFOs), or properly termed as cytochrome P450. In fishes, as in most vertebrates, the highest activity of MFOs occurs in liver tissue (Stegeman & Hahn, 1993). Phase II biotransformation reactions include a range of conjugation reactions where toxic substrates are bound to biomolecules, such as glucuronic acid and glutathione (Nimmo, 1987).

Damage to the liver is the most frequently reported histopathological response to organic compounds. The importance of the liver as a marker for pathological change reflects the central role of teleost hepatic tissues in nutrition, lipid and carbohydrate storage, synthesis of protein and enzymes, fatty acid metabolism, and

biotransformation and elimination of xenobiotics (Metcalfe, 1998). Gingerich (1982) in a review of fish hepatic toxicology, pointed out that the teleost liver might be susceptible to chemical damage because of the relatively slow hepatic blood flow in fish relative to humans. Poor hepatic perfusion may lead to stasis of chemical compounds and their metabolites in liver. This poor hepatic perfusion in fish can be attributed to the relatively simple microstructure of teleost liver.

In the present study, the obtained results of the treated liver clearly show that Bayluscide caused degenerative injurious effects on the liver ultrastructure; mainly on rough endoplasmic reticulum, mitochondria, fat globules and glycogen granules. There was a marked reduction in RER elements, fat globules were electron translucent and glycogen granules were markedly decreased. Some hepatocytes showed patches of degeneration and vaculation of the cytoplasm with fragmentation of the cell organelles. In severely affecting hepatocytes, there was parenchymal loss, the vacuoles occupied most of the cytoplasm and intercellular membranes were either broken or became indistinguishable. Similar alterations were observed in the liver of *Ictalurus punctatus* (Afifi & Mac Millan, 1992) and *Clarias lazera* (Yacoub, 1999).

The deformed hepatocytes with degenerated cytoplasmic organelles might be due to the cytotoxic effect of Bayluscide as a chemical irritant to cell membrane or may be due to the direct toxic effect of Bayluscide or its metabolites within the hepatocytes. The prominent leached fat globules showed evidence of mobilization and utilization of stored lipid in liver of *Gambusia affinis*, which may have been resulted from hepatotoxicity induced by Bayluscide causing abnormal metabolic situation, by MFOs in RER of hepatocytes (Stegeman & Hahn, 1993).

This is in agreement with Afifi & Mac Millan (1992) who suggested that direct solvent action (according to Berger *et al.*, 1987) was not responsible for the changes observed. They recorded that the ultrastructural changes, observed in parenchymal hepatocyte mitochondria and other organelles, indicated that initial alterations occurred to the RER, and concluded that the lipid peroxidation accounted for the ultrastructural alterations that were detected (according to Comporti, 1985 and Costa & Trudell, 1989).

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LEGEND OF PLATES

PLATE I: A photomicrograph of a toluidine blue-stained semithin section of:

(A): Normal liver showing groups of hepatocytes "**H**" separated by blood sinusoids "**BS**", which contain nucleated red blood cells "**RBC**"; the hepatocyte contains a rounded nucleus "**N**" with a prominent nucleolus and having a large number of variable sized fat droplets "**F**". (X 1000)

(B): Normal liver showing blood sinusoidal pole "**BS**" lined with endothelial cells "**EC**" which are flat elongated and almost completely filled with elongated nuclei "**N**". (X 1000)

(C): Treated liver showing that some hepatocytes are severely damaged and lost their cellular architecture or disorganized, while in other hepatocytes the fat droplets became more or less leached "**F**" giving the liver a foamy appearance. (X 200)

(D): Treated liver showing the decreasing of fat droplets "**F**" in number and size and becoming leached; fragmenting of cellular organelles which aggregated in opaque matrix around nucleus "**N**"; small darkly stained basophilic granules "**arrow**". (X 1000)

PLATE II: Electron micrographs of ultra-thin sections of normal liver showing:

(A): A group of adjacent hepatocytes "**H**" which contain prominent fat droplets "**F**", rounded euchromatic nucleus "**N**" with electron dense nucleolus "**Nu**" and scattered mitochondria "**M**" with a relative dense matrix. The endothelial cells "**EC**" with flat nuclei "**N**" are noticed. (X 2000)

(B): A hepatocyte with a rounded euchromatic nucleus "**N**", a double nuclear membrane "**arrow**" and the rough endoplasmic reticulum "**RER**" is concentrated mainly close to and around the nucleus and subjacent to cell membrane. Its cytoplasm contains free ribosomes, rosette-shape glycogen granules and scattered mitochondria "**M**". (X 10000)

(C): Some hepatocytes at the intercellular space "**IS**" having rounded and elongated mitochondria "**M**" with electron dense matrix, rosette-shape glycogen granules and pinocytotic vesicles "**PV**". Notice the bundle of collagen fibers "**CF**" in the space. (X 10000)

(D): The endothelial cell "**EC**" which lining blood sinusoid "**BS**" has a heterochromatic nucleus "**N**", the sub-endothelial space between sinusoidal endothelium and hepatocytes "**H**" contains a small fat-

storing cell or Ito cell "*" with a markedly heterochromatic nucleus "N" and surrounded by a thin cytoplasmic layer. (X 3000)

(E): Part of a wide blood sinusoid "BS" containing red blood cells "RBC" and the microvilli "Mv" projecting from hepatocytes "H" in the space of Disse "D". (X 3000)

(F): A bile canaliculus "BC" formed at the junction of adjacent hepatocytes "**arrowhead**" and the microvilli "Mv" extend from hepatocytes into the canalicular lumen. Notice rosette-shape of glycogen granules "**arrows**". (X 10000)

PLATE III: Electron micrographs of ultra-thin sections of treated liver showing:

(A): Group of affected hepatocytes having aggregation of mitochondria in an opaque matrix, leached fat droplets "F", primary and secondary lysosomes "Ls", numerous small vacuoles scattered in the cytoplasm and reduction of rough endoplasmic reticulum. (X 5000)

(B): A group of affected hepatocytes having a reduction of rough endoplasmic reticulum elements around the nucleus "N" and beside the cell membrane, signs of mitochondrial degeneration "M", electron translucent parts of the cytoplasm and leached fat droplets "F". (X 5000)

(C): Reduction of RER around the nucleus "N" and its dilation with absence of polysomes adjacent the cell membrane, numerous vacuoles "V" with different size which sometimes communicated with each other giving the cytoplasm foamy appearance and some of these vacuoles containing remainants of degenerated mitochondria "M". (X 5000)

(D): Signs of hepatocyte degeneration as glycogen reduction "**arrowhead**", dilation of RER and degeneration of mitochondria "M" as well as degeneration of hepatocyte microvilli inside the bile canaliculi "BC". (X 7500)

(E): Hepatocyte having huge secondary lysosomes "Ls" and abnormal shape of the nucleus "N". (X 5000)

(F): One of severe affected hepatocyte with evidence of nucleus "N" degeneration in the form of loss of nuclear envelope and irregular boundary, the great loss of cytoplasmic mass which occupied by translucent spaces, severe reduction in RER elements and partial degeneration of cell membrane. (X 3000)

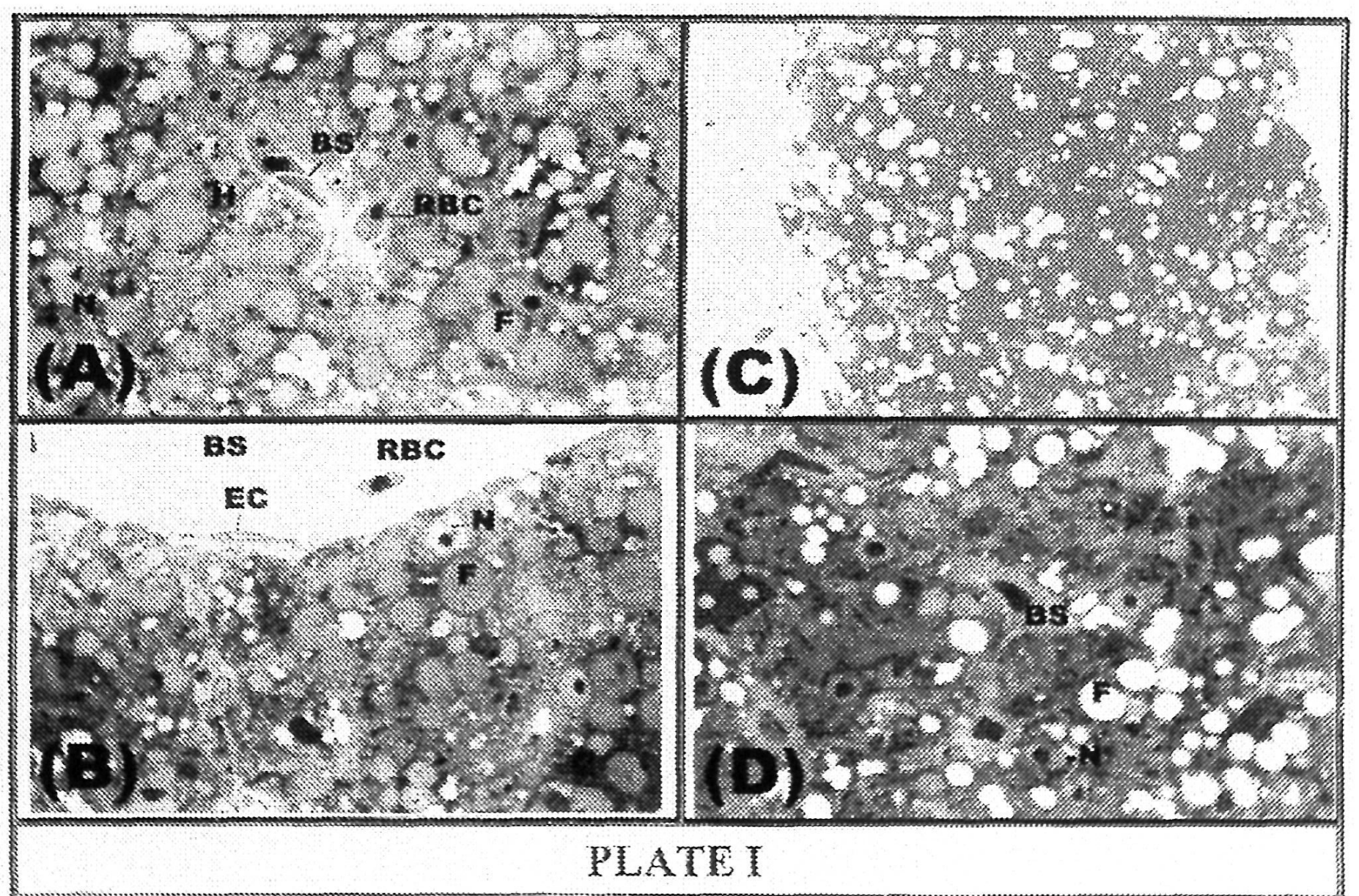

PLATE I

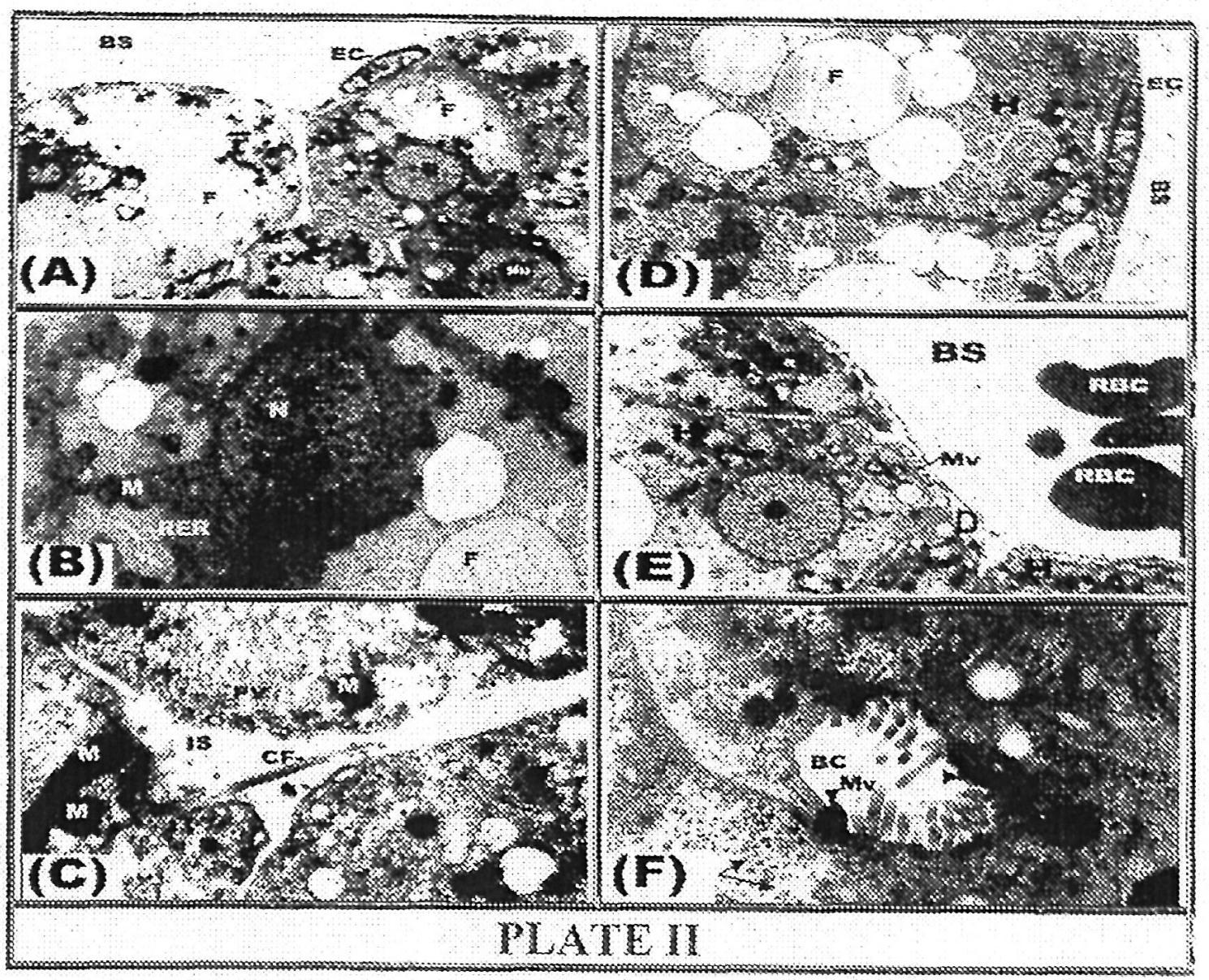

PLATE II

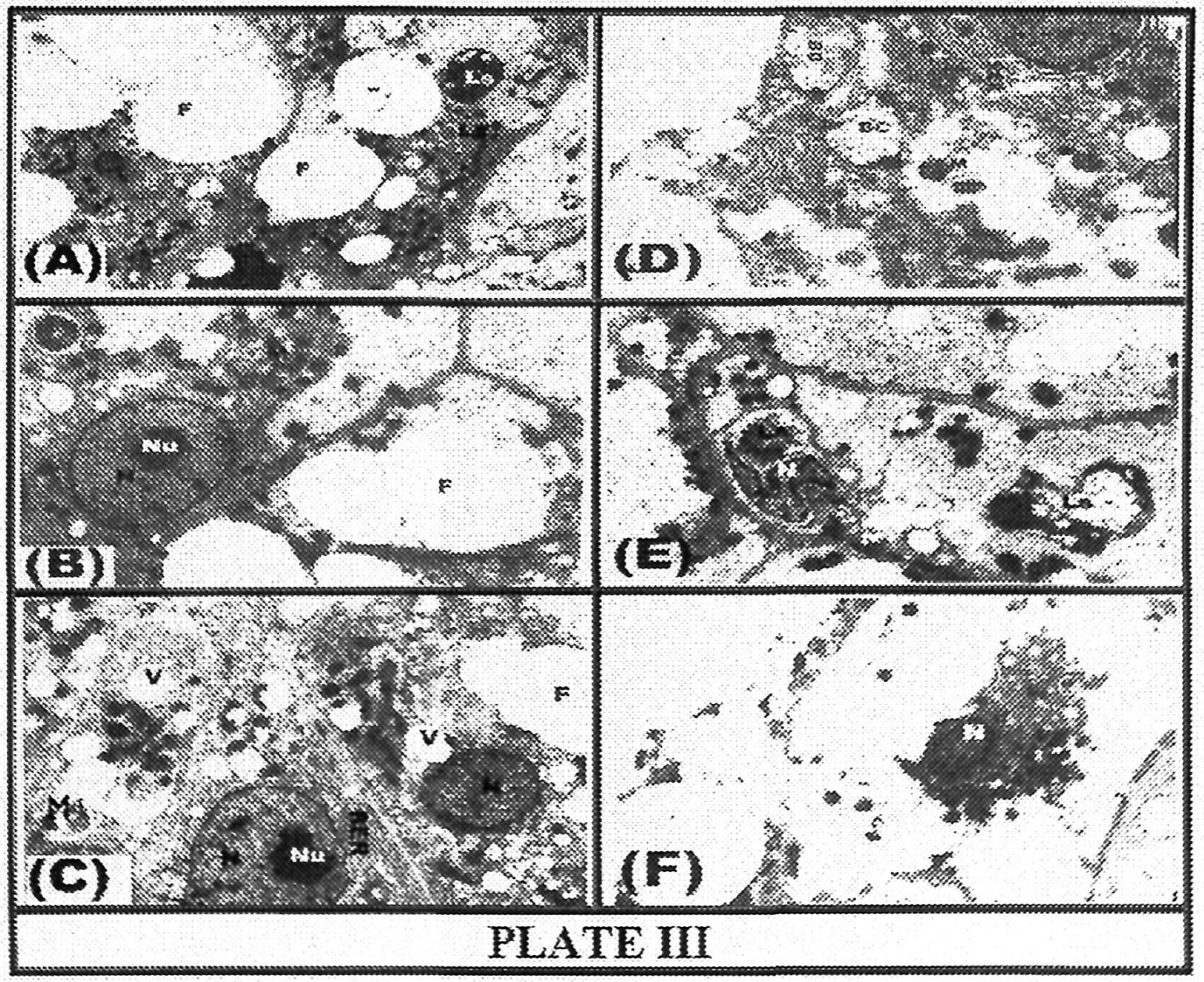

PLATE III