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Studies on the adverse effects of bleached sulphite pulp mill effluents on physiology of liver function in Heteropneustes fossilis

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Abstract: *The liver of Heteropneustes fossilis inhabiting the water bodies contaminated by bleached sulphite pulp mill effluents from the Hindustan Paper mill, Nogaon exhibited severe damage in the hepatocytes. The hepatocytes showed massive degenerative changes characterised by cellular swelling, cytoplasmic vacuolation and detachment of plasma membrane.In most of the hepatocytes the nuclei were pyknotic.Besides these, haemorrhages and haemosiderosis were observed in most of the cases. In pulp mill effluent- exposed fish, the nucleus was found to be disturbed with breakage and dissolution of the membrane at places. Some of the mitochondria were found to exhibit breakage and distortion of either inner or outer membrane or both. Vacuolization of different degree in the cytoplasm of hepatocytes was also evdient. Besides these, hyperchromatic nuclei were also observed in the hepatocytes. In some of the hepatocytes of effluent- exposed fish, endoplasmic reticulum were found to assume whorled appearanc.The significance of the aforementioned cellular and fine structural abnormalities of hepatocytes in response to physiological stress resulting from the exposure of the fish to bleached sulphite pulp mill effluents are discuussed in the light of available literature.*
Keywords: *Pollution;paper mill; effluents;TEM; Heteropneustes; Fine structure*

I. Introduction

The pulp and paper industry is recognized as one of the most polluting industries in India [1] (Bajpai and Bajpai 1997). The industry discharges large volumes of brownish effluent containing chlorinated hydrocarbons, lignin and polymerized tannins [2-5]. The pulping process generates a considerable amount of waste-water which is highly toxic. Depending upon its processes and capacity, a typical pulp and paper mill, uses 60,000-1, 00,000 gallons of water per day [6]. Almost the entire amount goes out as effluent. In most cases, mill effluent (treated or raw) is discharged into a river, stream or other water bodies resulting in negative environmental impacts.

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Now-a-days, in most of the Countries of the World modern Kraft pulp mills are being employed for the processing of pulp. However, in many developing Countries including India, the old generation sulphite pulp mills are still in use.

Studies have revealed that the level of Chlorinated organics and resin acids are generally lower in the effluents from modern Kraft pulp mills [7] than effluents from old generation sulphite pulp mills [8]. The pollutants of the pulp and paper mills include a wide variety of chemicals depending on the nature of the raw materials used and the manufacturing process adopted. In most of the small paper mills in India, the chemical recovery is not practiced due to economical reason. As such, the pollution potential of the wastes of smaller mills is higher than that of large mills [9]. The effluents from the industry cause slime growth, thermal impacts and scum formation. They also destroy the aesthetic beauty in the environment. Further, they increase the toxic substances in the water, causing death of zooplankton and fish. Several authors have reported different kinds of abnormalities in tissues of certain fish in response to paper mill effluents entering the water bodies [10-14]. Keeping these in view, the present work undertook a detail study on the effect of paper mill effluent from Nogaon Paper Mill, Jagiroad on a fish inhabiting the water body contaminated by the pulp mill effluents. Emphasis was laid on Water quality analysis, Histopathological studies, Fine-structural approaches and behavioral analysis. The fish selected for the study was *Heteropneustes fossilis,* a cat fish, with accessory respiratory organs, which enable it to survive in a polluted water body with relatively greater efficiency. The tissue selected for the study was Liver since it is the organ of detoxification and the major target for xenobiotics.

II. Materials And Methods

2.1. *Collection of water samples*

Effluent-contaminated and control water were collected from Taranga and Monoha beels respectively. Both effluent- contaminated and normal water samples were collected in sterilized plastic containers and were brought to the laboratory for the study of different physico-chemical parameters. and elemental analysis. For the study of dissolved oxygen and free carbon di-oxide glass- stopper bottles of 100 ml were used and analysis was done immediately. Collection of water was done according to standard method of APHA (1998) [15].

2.2. *Study of physico-chemical parameters of water*

The study of physico-chemical parameters of the fresh water body(control) and the water body contaminated with paper mill effluent(polluted), at Jagiroad was studied for three years (2009-2012), Study period was divided into four seasons: Winter (December – February), Pre- monsoon (March – May), Monsoon (June – August) and Post monsoon (September – November).

2.2.1. *Dissolved oxygen*

Estimation of dissolve oxygen was done by the modified Winkler's method and the other parameters were recorded as per standard procedures [15, 16].

2.2.2. *Determination of P^H*

pH was determined with the help of Alico-LI 120 digital pH meter.

2.2.3. *Conductivity*

Conductivity was measured using conductivity meter DB-1046 (Decibel).

2.2.4. *Free carbon di oxide, total hardness and total alkalinity*

The free carbon di oxide, total hardness and total alkalinity were analyzed following the standard method [15].

2.2.5. *Collection of fish sample*

The samples of the fish *Heteropneustes fossilis* were collected from "Toronga" beel, where the paper mill effluents (PME) from the Nogaon Paper Mill (NPM) is released from the effluent treatment plant(ETP) . Control fishes were collected from "Monoha"beel located 15km away from the mill in North-West direction, where there is no chance of contamination with PME because of geographical reasons. After the collection, fishes were taken to the laboratory, washed thoroughly with double distilled water and were processed for histopathology and transmission electron microscopy. The number of control and effluent exposed fish used in different aspects of the study were 20 each in the quarterly collection during the year 2009-2012.

2.2.6. *Behavioral studies*

The control and effluent-exposed fish were reared in aquarium for making detailed analysis of their behavior in regard to swimming, surfacing, grasping of air, response to food etc.

2.2.7. *Histo-pathology*

The liver samples of the fish, *Heteropneustes fossilis* were processed for histo-pathological study following standard method [17].The tissues were fixed in Carnoy's fluid for 4-5 hours, washed briefly in tap water, dehydrated in ascending grades of ethanol for 15 minutes in each grade and were cleared in xylene. Impregnation of tissues was done in a mixture of melted paraffin and xylene followed by preparation of blocks in paper boats. The sections were cut with a rotary microtome at 5 µm thickness and were stained with Hematoxylin and Eosin. Observations were made in a BLS 107 optical microscope. Ten liver samples from 20 individual fish were examined to ensure reproducibility of results.

2.2.8. *Transmission electron microscopy*

Liver samples were cut into small pieces (1mmx1mm) and were fixed in modified Karnovsky's fixative[18] having the composition of 250 ml of 0.2M Sodium cacodylate buffer, 20g of Para-formaldehyde dissolved in it at 60^0c, bringing the volume to 480 ml by double distilled water. To this, 20 ml of 25% glutaraldehyde and 12.5g of anhydrous calcium chloride was added. After 4 hours in the above primary fixative, the samples were washed thoroughly in 0.1M sodium cacodylate buffer and post fixed in 1% osmium tetroxide (prepared in the same buffer) for 1hour at 4^0c. Samples were then dehydrated in ascending grades of acetone with two changes of 15 minutes each and were cleared of acetone by propylene oxide for 30 minutes. Infiltration was carried out gradually in different proportions of propylene oxide with liquid resin, hardener and plasticizer

[Araldite CY 212-10 ml, DDSA (Dodecenyl succinic anhydride) - 10 ml, DMP-30{Tri (di-methylaminomethyl phenol}-0.4ml, and di-butyl phthalate-1ml].

Embedding of tissue was carried out in the araldite embedding medium using beem capsules. The embedding blocks were kept at 50^0c in an embedding oven for 24 hours. The temperature was then raised to 60^0c and the embedded tissues were kept for 48 hours to complete polymerization. Ultra thin sections (600-800 A0) were cut in an RMC ultra-microtome, MT-X, with a diamond knife. The sections were collected on copper grids and stained with alcoholic solution of saturated Uranyl acetate for 10 minutes at room temperature in the dark, followed by lead nitrate for 5 minutes [19].

The stained sections were examined in a Jeol JEM-2100 transmission electron microscope at an accelerating voltage of 80 KV.

III. Results

3.1. *Dissolved oxygen*

The dissolved oxygen level in effluent- contaminated water was found to be very low ranging between 2.7 and 3.22mg/l. In contrast the dissolved oxygen level of the control water body ranged between 7.8 and 8.95 mg/l.

3.2. *pH*

The recorded pH value in the contaminated water body ranged between 7.6 and 8.5, being maximum in the monsoon and minimum in the winter. The pH value of the control water body on the other hand was found to remain constant through out the year ranging between 6.8 and 7.3.

3.3. *Conductivity*

The conductivity in the effluent-exposed water ranged between179 and 222 micro Siemens/cm in contrast to 93-112 micro Siemens/cm in control water body.

3.4. *Free carbon di oxide*

The free carbon di oxide level in the effluent-exposed water body ranged between 45 and 52mg/l, being maximum in the monsoon and minimum in the winter. In the control water body on the other hand the level of free carbon di oxide was recorded as 2-4.86mg/l, being minimum in the winter and maximum in the monsoon.

3.5. *Total hardness*

The total hardness of water of the contaminated water body was found to be the maximum(185mg/l) in winter and the minimum (164mg/l) in monsoon, which were much higher than the maximum value of 88.8mg/l (in winter) and the minimum value of45.9mg/l (in monsoon) of control water body.

3.6. *Total alkalinity*

The maximum value of total alkalinity in the effluent-contaminated water body (202mg/l) was recorded in the pre-monsoon season and the minimum value (179mg/l) was recorded in winter. In the control water body on the other hand, the value of total alkalinity was found to be the maximum (70.9mg/l) in winter and minimum (36.4mg/l) in monsoon.

3.7. Behavioral Studies

Fish reared in control water exhibited normal pattern of swimming and surfacing. When the fish were supplied with food pellets, they moved forward and consumed the food. Fish reared in effluent - contaminated water, on the other hand were found to be quite weak and they exhibited slow swimming pattern. They were found to move frequently towards water surface. Their response to food was also not normal. They were found to move towards the supplied food pellets but were reluctant to consume food.

3.8. *Histo-pathology*

The transverse section of liver of *H fossilis* from control water body exhibited normal polygonal shape of the hepatocytes. However,those from contaminated water exhibited severe damage in the hepatocytes. The hepatocytes showed massive degenerative changes characterised by cellular swelling, cytoplasmic vacuolation and detachment of plasma membrane (Fig.1a).In most of the hepatocytes, the nuclei were pyknotic (Figs.1a and b) Haemorrhages and haemosiderosis were invariably observed in many hepatocytes (Figs1c and d).

3.9.*Transmission Electron Microscopy*

The Trasmission electron microscopy of hepatocytes of control fish revealed normal structural features of nucleus with intact nuclear membrane. Mitochondria also showed normal shape with regular features of inner and outer membrane (Fig.2a). Endoplasmic reticulum appeared to be normal and it was found to maintain the membrane continuety (Fig.2b). In pulp mill effluent- exposed fish on the other hand, the nucleus was found to be disturbed with breakage and distotion of the membrane at places (Fig.2c). Some of the mitochondria exhibited breakage and distortion of either inner or outer membrane or both (Fig.2d). Vacuolization of different degree in the cytoplasm of hepatocytes was also evdient (Fig.2e). Beside this, hyperchromatic nucleus was also observed (Fig.2e).In some hepatocytes of effluent- exposed fish endoplasmic reticulum were found to assume whorled appearanc (Fig.2 f).

IV. Discussion

4.1. Water quality parameters

4.1.1 *Dissolved oxygen*

Dissolved oxygen is a vital parameter to aquatic life. Oxygen enters water body by diffusion from the atmosphere and through photosynthetic activity of aquatic plants. The dissolved oxygen level is indicative of the degree of pollution in an aquatic system. In general a high level of dissolved oxygen indicates a better water quality, whereas its low level adversely affects the growth and survival of aquatic organisms including fish [20]. On that consideration, detection of a remarkably low level of dissolved oxygen in effluent-contaminated water body as compared to control in our present study is a cause of concern for the aquatic organisms inhabiting the contaminated water body.

4.1.2. *pH*

The higher pH of effluent-contaminated water body as compared to control also appears to be alarming for the proper growth and survival of fish inhabiting it. Although, at present the pH value of the effluent-contaminated water body is within the optimal limit, its higher range (8.5) in certain season of the year suggests that there is a possibility of further increase in pH of the effluent-contaminated water body due to continued release of pulp mill effluents in it. In this context, it is to be noted that alkaline water with pH 9.5 is unproductive due to availability of less carbon di oxide in it. In this context it is worth mentioning that deviation of pH from the normal range in a water body can adversely affect the tissues of fish inhabiting it [21-23].

4.1.3. *Conductivity*

Remarkably higher value of conductivity in effluent-contaminated water body as compared to control indicates the presence of higher amount of total dissolved solid in the aforementioned water body. This is likely to make the water body less conducive for growth, development and survival of the fish. Several authors have reported high conductivity level in water bodies contaminated by sewage & industrial effluents [24, 25] and different degrees of tissue damage in fish inhabiting these contaminated water bodies [26-29].

4.1.4. *Free carbon di oxide*

Significant rise of free carbon di oxide level in effluent-contaminated water body as compared to control indicates difficulties for proper growth, development and survival of the fish inhabiting it. It is known that when oxygen concentration in a water body falls, carbon di oxide concentration rises and the increasing concentration of free carbon di oxide makes it very difficult for fish to use the limited amount of oxygen present. It is obvious that fish must discharge the carbon di oxide present in their blood in order to take in fresh oxygen. However, this process slows down considerably when the free carbon di oxide level in water rises.

4.1.5. *Total hardness*

A remarkably higher water hardness in effluent-contaminated fish as compared to control in the current study assumes significance in view of some reports on adverse effects of high water hardness on some fish. Water hardness level of about 200mg/l in effluent contaminated water body in some seasons of the year, as observed in the present study is a cause of concern for fish inhabiting it since abnormalities in egg hatching and larval viability were reported in some fish at the aforementioned water hardness level [30].

4.2. Behavioral studies

General weakness of the fish reared in effluent-contaminated water as revealed from their slow swimming pattern appears to be due to their less food consumption. This is confirmed from their reluctance to take food as found from the behavioral analysis and also from the appearance of whorled endoplasmic reticulum in the hepatocytes, which reflects starvation.

4.3. *Histopathology*

Like other vertebrates, fish lliver also playes an important role in controlling many physiological functions such as carbohydrate , protein and lipid anabolism , detoxification, glycogenolysis etc. and act as store house, mainly of glycogen along with others[31-33]. Vacuolization in the hepatic cell cytoplasm as observed in the current study may be an indication of imbalance between synthetic activity and the rate of release of substances from the cell. Similar observation was reported by some authors in the liver of some fish exposed to certain pesticides [34]. Due to the detoxification ability of liver many of the pollutants are biotransformed into metabolites with the help of various enzyme system and as a result liver undergoes different levels of damages. The observations made in the present sudy on abnormal histological features of liver in response to bleached sulphite pulp mill effluent are in the same line with the reports of many earlier workers involving different toxic substances[35-37]. As liver is concerned with detoxification and biotransformation process and as it is having rich blood supply, different components of hepatic circulatory system are likely to be affected by contaminants in the water [38]. The focal area of necrosis and congestion in the blood sinusoids observed in the current study may be attributed to direct toxic effects of pollutants present in the paper mill effluents since liver detoxifies all types of toxins and chemicals. This observation is in agreement with the findings of other authors who did some bochemical and physiological investigation on monosex Tilapia following chronic exposure to pesticides [39]. Congestion in the liver sinusoids, as found in the present study implies that the flow of blood from the hepatic artery and vein gets hampered [40]. The consequence of this congestions is reflected in the occurance of hemorrhage and appearance of hemosiderin. Hemosiderin is especially abundant in situations following hemorrhage. Its formation is related to phagocytosis of red blood cells and abnormalities in hemoglobin. Hemosiderin can accumulate in liver and other organs in various pathological conditions or in the process of intoxication. Similar observations of sinusoidal congestion, pyknosis and karyorrhexis of hepatocyte nucleus were reported in case of severe intoxication with some pollutants [41].

Pyknosis is a clear indication of deterioration of the cellular environment, because it is the irreversible condensation of chromatin leading to necrosis which is followed by karyorrhexis. As these conditions along with cytoplasmic vacuolization and shrinkage of hepatocytes were observed in *H.fossilis* in the current study, it is implied that the physiology of normal liver functions of the fish exposed to paper mill effluents were highly disturbed.

4.4. ***Transmission electron microscopy***

It is amply highlighted in existing literature that Industrial pollutants can adversely affect certain physiological and biochemical activities of different tissues when they enter the organ systems of fish [42]. Pertinent here is to mention that fine structures of hepatocytes are adversely affected by different environmental pollutants including city garbage and municipal wastes [43]. Liver is the main detoxification organ of the body and has an inherent capability to adapt and compensate many of the changes [44]. In this context, nuclear and mitochondrial membrane abnormalities, the development of whorled endoplasmic reticulum and other intra cellular abnormalities exhibited by *H.fossilis* exposed to paper mill effluents revealed through transmission electron microscopy in the present study merits elucidation. It has been suggested that the aforementioned changes are likely to represent the cellular response of fish to the disturbances in the aquatic environment [45].
Nuclei are considered as a major intoxication site of cell [46] (Yang and Chen, 2003) and hence, the presence of many nuclei with distorted membrane in the hepatocytes of the fish inhabiting the pulp mill effluent- exposed water body suggests that the effluent contains certain cytotoxic substances. The observed nuclear membrane abnormalities in the hepatocytes may be related to defects in nuclear membrane components and lipid synthesis induced by the toxicants. The interaction of nuclear membrane and lamina proteins with cytoskeletal elements & chromatin, and modifications in lipid biosynthesis might have also been involved. Condensation and marginalization of the heterochromatin as well as an irregular nuclear envelope, observed in the hepatic nuclei in the present study were interpreted by Braunbeck (1994)[47] as progressive inactivation of the nuclear components.

Among the cytoplasmic organelles, mitochondria are known to respond sensitively to environmental contaminant exposure. The observed membrane distortion of mitochondria in hepatocyte of effluent exposed *H.fossilis* suggests serious problems in energy generation in the cell, which can cause adverse affects on liver functioning, ultimately leading to the failure of the whole organ [48]. It has been reported that mitochondrial degeneration is accountable for the impaired oxidative capability of the cells [48]. Vacuolization observed in the mitochondrial matrix of the hepatocytes is significant because of the fact that autophagic cell death is morphologically characterized by an accumulation of vacuoles [49]. Further, the role of abnormalities in mitochondria in both necrotic and apoptotic cell death is amply highlighted in existing literature [50].

In the present study, intense vacuolization observed in the cytoplasm of the hepatocytes of *H.fossilis* inhabiting the effluent-contaminated water body supports the finding of earlier authors [45], who observed large vacuoles in the hepatocytes after exposure to some pollutants. In this context, it is to be noted that according to some studies [51] the increase in the number of vacuoles indicates the degree of degeneration of the exposed cells. The intense vacuolization in hepatocytes of fish inhabiting the effluent-contaminated water body, as observed in the current study is highly significant in view of the fact that autophagic cell death or apoptosis is morphologically characterized by an accumulation of vacuoles [49].

The progressive loss of structural integrity of the rough endoplasmic reticulum in the hepatocytes of H.fossilis exposed to pulp mill effluent observed in the present study is similar to the reports of other authors [45, 48]. Rough endoplasmic reticulum is studded with ribosome that is actively associated with protein synthesis. Hence, de-granulation, dilation, and fragmentation of the RER system have been classified as typical but non-specific lesions induced by noxious agents that reflect disturbance in protein synthesis [52], which is detrimental for the cells and the organisms as a whole. The whorled endoplasmic reticulum observed in the hepatocytes of effluent-exposed *H.fossilis* suggests that the fish inhabiting the pulp mill effluent-contaminated water body undergo starvation, since whorled endoplasmic reticulum are reported to be associated with starvation of the organism exhibiting the abnormal endoplasmic reticulum[53,54]. The fact that the effluent-exposed fish undergo starvation is supported by behavioral analysis also.

V. Conclusion

The present study involving water quality analysis, behavioral study of effluent-exposed fish, histopathology and Electron microscopy of liver of *Heteropneustes fossilis*, exposed to bleached sulphite pulp mill effluent in its natural habitat exhibits a number of abnormal features indicating the harmful effects of bleached sulphite pulp mill effluents from Nogaon paper mill of Assam, India on the adjacent water body and its inhabitants. The disturbances in the water quality parameters caused by pulp mill effluents are reflected in the adverse effects on fish health, as revealed from histo-pathology and Electron microscopy of liver tissue. The abnormal shape of nucleus, distortion of inner & outer mitochondrial membrane, appearance of whorled endoplasmic reticulum in hepatocyte cells etc, indicate the cytotoxic nature of certain components of pulp mill effluents released from the Nogaon pulp mill, Assam, India. The study suggests immediate steps to be taken to ensure proper treatment of the effluents so that the release of toxic substances to the near-by water bodies is minimized. Further, the present study suggests that immediate steps are to be taken for replacement of the old generation bleached sulphite pulp mills by the modern Kraft mills to reduce the toxic substances released due to pulp processing.

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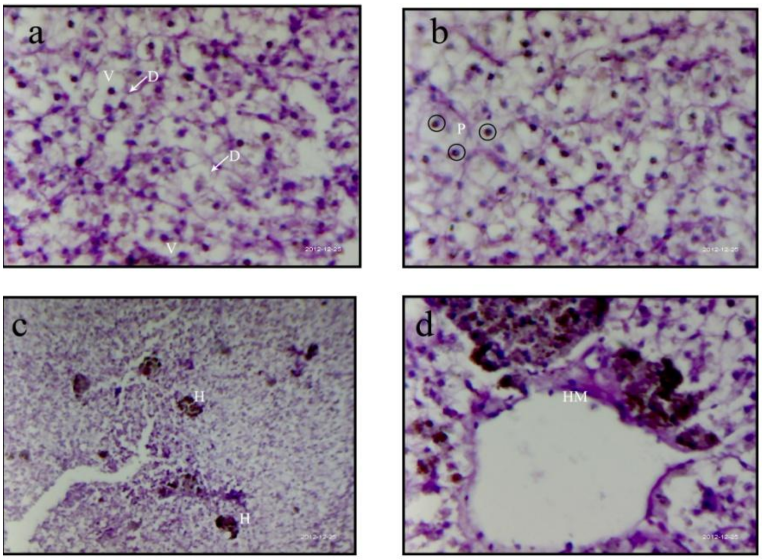
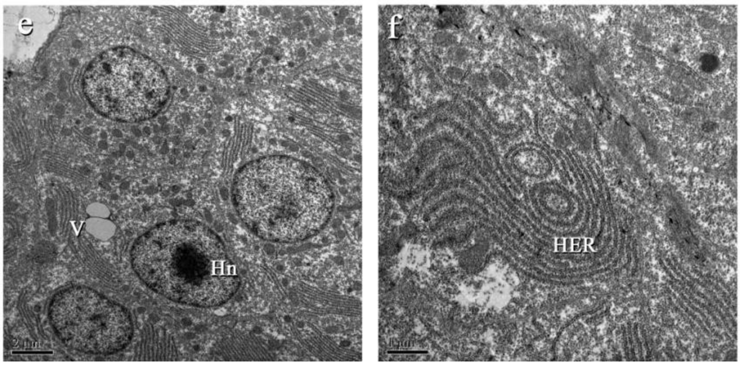
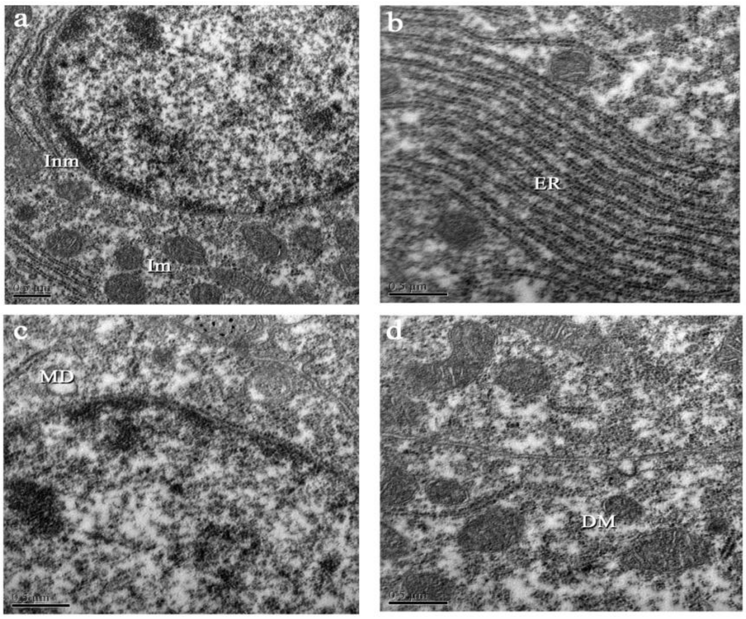


Fig-1



Figs.2.e,f



Figs.2a-d