Fine-structural deformities in hepatocytes of a hill stream fish *Brachydanio rerio* due to toxic effects of environmental acid stress

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**Abstract**
The natural habitat of the ornamental fish, *Brachydanio rerio* in the North East Indian city of Shillong has been found to exhibit acidic pH, which is likely to affect the physiology, growth and survival of the fish adversely. The objective of the study was to address the issue through ultra structural approach. A transmission electron microscopic study revealed loss of cellularity in many hepatocyte cells, evagination and invagination of nuclear membrane, random peripheral distribution of chromatin, inner and outer membrane distortion in mitochondria and disruption of endoplasmic reticulum. However, detail biochemical studies will be needed to get additional information on the topic. The study assumes significance due to its relevance to conservation of biodiversity and socio-economic aspects related to hill stream ornamental fish.

**Citation:**

1. Introduction

1.1 Adverse effects of acidic pH on fish
Among the abiotic factors, pH plays a very important role in growth, survival and reproduction in fish (Barlaup et al, 1989). The acidic pH of water is known to cause distortion in the fish population dynamics by high rate of mortality during early as well as late stages in the life history (Peterson et al, 1982; Barlaup et al, 1989). Aquatic organisms are affected by pH, because most of their metabolic activities are pH dependent (Wang et al,2002).Optimal pH range for sustaining aquatic life is 6.5 - 8.2 (Murdoch et al,2001).It has been reported that acidic pH of water significantly affects the growth of fish. The stagnation or depression of growth in response to acidic pH has been described even at low population density and relatively good food availability (Peterson et al, 1982).

Low pH of water has also been reported to cause disturbances in ionic balance (Dey et al,2001) and structural damage in gills, muscles, skin and gonads of some fish (Kharbuli,2005).The structural damage in tissues did not occur when the fish *Danio aequipinnatus* was reared in aquarium where neutral pH was maintained (Kharbuli,2005).

1.2. Physiological response of fish to acidic pH
The disturbances in ionic balance are some of the important physiological responses shown by fish exposed to water having acidic pH (Muniz and Leivstad, 1980).It has been reported that acid stress was responsible for near extinction of some fish population in Ontario, Canada (Harvey and Jackson, 1995).

Primary cause of fish death in acidic water is loss of sodium (Na⁺) ions from the blood (Wilson et al, 1999). Also, the less availability of oxygen to the cells and tissues leads to anoxia and death as acid water increases the permeability of fish gills to water, affecting the gill functions (Brown and Stadler, 1989).

The ionic imbalance in fish may begin at pH 5.5 or higher, depending on the tolerance of fish species. Severe anoxia occurs at pH 4.2 and below (Potts and McWilliams, 1989). However, low pH that is
not directly lethal may also adversely affect the fish growth rate and reproducations (Kimmel, 1983). Besides these, the morphology of fish, volume of eggs as well as gonadosomatic index are reported to be influenced by the change in water temperature and pH (Joshi et al, 2007).

1.3. Different approaches for studying the effect of acid stress in fish
Studies on adverse effects of experimentally induced acid stress on fish have been amply highlighted in existing literature but very few studies on this have been carried out in relation to acidic pH of water in natural habitats. Further, although physiological, biochemical, ecological and histological studies have been carried out in detail on this aspect, very few reports on the same from ultra structural point of view are available in the existing literature. In this context it is worthwhile to mention that Electron microscopy has been used extensively for understanding surface micro structural and fine structural details of fish and other animals under experimental as well as natural conditions (Dey et al, 1999, 2009, 2015; Prasad et al, 2014). As far as the ultra structural approach to acid stress in fish is concerned, a recent report (Prasad et al, 2014) on the effect of acid stress on skin and muscle of the hill stream fish, Brachydanio rerio experiencing acidic pH of water in its natural habitat is worth mentioning. Brachydanio rerio, which is a zebra fish, is a hill stream ornamental fish.

1.4. Importance of zebra fish as an experimental model
The Zebra fish is important not only as an aquarium ornamental fish but also as a vertebrate model for biomedical research such as toxicity test, aging, genomic and neutrophilic respiratory diseases(Braunbeck et al,1992;Driever et al,1994; Alestrom et al,1995).

2. Objective of Research
The present study has been undertaken to have detail information on fine structural alterations in liver of the fish, Brachydanio rerio caused by acidic pH of water in its natural habitat

2.1 Definition of the problem
The problem regarding the streams and their inhabitants used in the current study is the acidic pH of water, which causes difficulties in growth, multiplication and survival of the fish. The goal is to clearly exhibit the fine structural and cellular abnormalities of a vital organ, liver of the fish Brachydanio rerio inhabiting the streams (with acidic pH of water) to understand the adverse impact of acid stress on the fish health.

2.2. Proposed solution of the problem
Solution of the problem is to find out the causes of acidic pH of water in the streams under study and to take appropriate steps to bring the pH at least to the level of neutral value for better health of the ornamental fish inhabiting the streams. Besides this, culturing of the fish in aquarium or small ponds with alkaline pH of water may be tried to conserve the ornamental fish, which is at present under severe stress due to acidic pH of water in its natural habitat.

2.3. Justification of Research
The study is needed for having an in-depth knowledge on adverse effects of environmental acid stress on fine structure of vital organs in hill stream ornamental fish. The significance of the study is that it is a non-conventional and effective attempt in addressing the issue of environmental acid stress through Electron microscopy approach. The study is going to be milestone in its scientific domain because of the novel concept and involvement of sophisticated analytical equipments with higher precision than the conventional ones.

3. Materials and Methods
3.1. Work plan
To achieve the goal of this study, samples of the ornamental fish, Brachydanio rerio were collected from “Umbang” and “Wah Umkhrah” Streams located in and around Shillong, a North East Indian city. Fifteen adult fish samples each from the two hill streams, from a monthly collection for a period of one year were used for the study. Besides this, fingerlings collected from the streams were divided into two groups of five fish each. One of the groups was cultured in aquarium in water collected from the natural habitat of the fish having acidic pH. The other group was cultured in aquarium in water from the same source, but its pH was made alkaline by adding few drops of dilute sodium hydroxide solution (Peterson et al, 1982; Barlaup et al, 1989)]. The pH of water samples from the streams and also of the water in experimental aquaria was measured with the help of pH meter, DB 1046 (Decibel) during different seasons of the year. Liver samples of five fish each from acidic and alkaline pH groups maintained in the aquarium and also fifteen samples of fish collected from each of the two streams in different seasons were excised and were cut into small pieces of approximately 1mmX1mm in size. Standard procedure was followed for preparing the samples for transmission electron microscopy (Hayat, 2000).

3.2. Study area
The study areas of the present investigation were “Umbang” stream located at about 25 Km towards north from the city of Shillong and the
Figure 1: *Danio rerio* (*Brachydanio rerio*)

Figure 2: Normal hepatocyte in *B. rerio* grown in alkaline pH. N, Nucleus; ER, Endoplasmic reticulum

Figure 3: Disturbances in hepatocyte of *B. rerio* grown in acidic pH. N, Nucleus with loss of nuclear materials; M, Mitochondria with distorted membranes; ER, distorted Endoplasmic reticulum
**Figure 4:** Hepatocyte of *D. Rerio* exposed to acidic pH of water showing destruction and loss of many cellular features. N, Nucleus

**Figure 5:** Nucleus of hepatocyte in *B. Rerio* grown in alkaline pH showing normal features

**Figure 6:** Abnormal shape of nucleus and random peripheral distribution of chromatin in hepatocyte of *B. rerio* exposed to acidic pH
**Figure 7:** A different type of abnormal shape of nucleus and haphazard chromatin distribution in hepatocyte of *B. rerio* exposed to acidic pH of water.

**Figure 8:** Normal mitochondria and endoplasmic reticulum in hepatocyte of *B. rerio* grown in alkaline pH. M, Mitochondria

**Figure 9:** Membrane distortion of mitochondria in hepatocyte of *B. rerio* grown in acidic pH. M, Mitochondria
**Figure 10:** Distortion of inner and outer mitochondrial membrane and vacuolization of mitochondria in hepatocyte of *B.rerio* exposed to acidic pH. V, vacuolization

**Figure 11:** Normal Endoplasmic reticulum in *B.rerio* grown in alkaline pH

**Figure 12:** Membrane distortion (Arrows) in Endoplasmic reticulum (ER)
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**Figure 13:** Breakage of ER membrane and vacuolization in between the membranes (arrows) in *B. rerio* grown in acidic pH

“WahUmkhrah” stream located at about 19 km from Shillong towards north. Shillong is located in the state of Meghalaya in the North Eastern part of India. The state of Meghalaya lies between 20.1˚North and 26.5˚North latitude and between 85.49˚East and 92.52˚East longitude.

3.3. Measurement of pH
The pH of water samples from the streams during different seasons of the year and also of the water in experimental aquaria was measured with the help of pH meter, DB 1046 (Decibel).

3.4 Fish sample
The fish used in the present study was *Brachydanio rerio*, a hill stream ornamental zebra fish (Fig. 1).

3.5. Collection of samples
The samples of the zebra fish, *Brachydanio rerio* were collected from the two streams, “Umbang” and “WahUmkhrah.” Fishes were sacrificed and the liver was excised. Liver was used in the study because it is well established that responses of the organ is quite high to any kind of stress [Braunbeck et al., 1982; Jayatilake et al., 2011; Sorour and Al-Harbey, 2013; Saenphet et al., 2015]. The excised liver samples were then processed for Transmission electron microscopy.

3.6. Transmission electron microscopy
The samples were fixed in modified Karnovsky’s fixative (David et al., 1973), having the composition of 250ml of 0.2M Sodium cacodylate buffer, 20gm of para-formaldehyde dissolved in it at 60˚C bringing the volume to 480 ml by double distilled water. To this, 20ml of 25% glutaraldehyde and 12.5g of hydrous calcium chloride were added. After fixing the samples for four hours in the aforementioned primary fixative, the samples were washed thoroughly in 0.1 M sodium cacodylate buffer for about an hour. Secondary fixation was done in 1% Osmium tetroxide (prepared in the same buffer) for one hour at 4˚C. The samples were dehydrated in ascending grades of acetone with two changes of 15 minutes each. The samples were then cleared off acetone by propylene oxide for 30 minutes (Hayat, 2000). Infiltration was carried out gradually in different proportion of propylene oxide and embedding mixture (Araldite CY 212, 10ml; dodecenyl succinic anhydride i.e. DDSA, 10ml; DMP-30 i.e. Dibutylphthalate, 1ml). Embedding of tissues was carried out in Araldite embedding medium using beem capsules. The embedded tissues were kept at 50˚C in an embedding oven for 24 hours. The temperature was then raised to 60˚C and the embedded tissues were kept for 48 hours for polymerization.

Ultra thin sections (600-800A˚) were cut in an RMC ultra microtome, MT-X with a diamond knife. The sections were collected on copper grids and were stained with 50% alcoholic solution of Uranyl acetate for 10 minutes at room temperature in the dark, followed by Lead nitrate for 5 minutes (Reynolds, 1963). The stained sections were examined in JEM 100CXII and JEM 2100 Transmission electron microscope at an accelerating voltage of 80 and 120KV respectively.

4. Results

4.1. pH of water
_Umbang stream:_ The pH of water samples of Umbang stream during the study period (2011-2012) ranged between 5.57 - 6.88 with a mean at 6.39 during spring season; 6.10 - 6.73 with a mean at 6.38 during summer season; 5.49 - 6.55 with a mean at 6.01 during autumn season and 5.06 - 6.49 with a mean at 5.76 during winter season respectively. The annual pH range recorded was 5.06 - 6.88.

_Wah Umkhrah Stream_

The pH of water samples of Wah Umkhrah stream during the study period (2011-2012) ranged between 5.86 - 6.76 with a mean at 6.41 during
spring season; 6.65 - 6.91 with a mean at 6.76 during summer season; 6.30 - 6.86 with a mean at 6.60 during autumn season and 6.23 - 6.70 with a mean at 6.40 during winter season. The annual pH range recorded was 5.86 - 6.91.

Experimental aquarium
Acidic pH
The pH of water in the aquaria with acidic pH was recorded as 5.9
Alkaline pH
The pH of water in the aquaria with alkaline pH was maintained at 8.5

4.2. Transmission electron microscopy

General features of hepatocytes
Alkaline pH group:
Transmission electron microscopy of liver showed prominent hepatocytes with all the normal cellular features including different cell organelles in the liver of fish grown in alkaline pH (Fig.2)

Acidic pH group:
The liver of fish collected from the natural habitat with water pH below 6.5 and also of the fish maintained in aquaria in water with acidic pH (5.9) exhibited loss of cellularity in many cells. The cells appear more or less empty, exhibiting destruction or loss of many of the cellular features including cell organelles (Figs.3, 4).The aforementioned abnormal features were recorded in 25-35% of the samples examined.

Nucleus
Alkaline pH group:
The nucleus of the hepatocyte was found to be round in shape with normal membrane integrity and central localization of chromatin (Fig. 5) in fish grown in water with alkaline pH.

Acidic pH group:
The nucleus of hepatocyte of the fish grown in water with acidic pH (below 6.5) exhibited a number of abnormalities in its shape. These include evagination or outward extension of nuclear membrane at certain locations and invagination of the membrane at other locations .The chromatin distributions were also disturbed and they were not localized in the centre. They were distributed randomly at different locations in the periphery (Figs. 6, 7). In some of the nuclei, the invagination of nuclear membrane made the nucleus a more or less dumbbell shaped structure. In this case also the distributions of chromatin were haphazard and they were localized in some of the peripheral parts (Fig. 7). The aforementioned abnormalities in the nucleus were observed in about 30% of the samples studied.

Mitochondria
Alkaline pH group:
Mitochondria of hepatocyte of the fish grown in water with alkaline pH were found to be well developed with intact outer and inner membrane (Fig. 8).

Acidic pH group:
In fish grown in water with acidic pH (below 6.5), some of the mitochondria (15-20%) were found to exhibit distortion of inner or outer membranes or both the membranes (Figs. 9,10).Swelling was also evident in some mitochondria (10-15% of the samples examined).

Endoplasmic Reticulum
Alkaline pH group:
In fish grown in alkaline pH, the endoplasmic reticulum was found to be intact and was studded with ribosome. The endoplasmic reticulum was found to be continuous without any breakage (Fig. 11).

Acidic pH group:
In contrast to the normal features in alkaline pH-group of fish, the membrane disruptions of the endoplasmic reticulum was prominent in the hepatocytes of the fish grown in water with acidic pH (below 6.5).This abnormality was recorded in 25-30% of the samples studied. In some cases (3-5% of the samples examined), the endoplasmic reticulum was distorted and broken to such an extent that the organelle appeared like a clumsy mass beyond recognition (Fig. 12). In some cases (10-15% of the samples examined), breakage of the membrane of endoplasmic reticulum and vacuolization in between the membranes were evident (Fig. 13).

5. Discussion

5.1. Changes in liver in response to environmental stress
Most of the changes in liver in response to environmental stress are considered to be of adaptive or compensatory nature (Braunbeck et al, 1992). Ultra-structural changes such as nuclear deformation, fragmentation and degradation of the rough endoplasmic reticulum (RER) & mitochondrial membrane distortion; swelling and cytoplasmic vacuolization seen in the hepatocytes of *Brachydanio rerio* inhabiting the hill streams with acidic pH of water appear to be due to a set of apparently unspecific reactions. Thophon *et al.* (2004) reported similar changes in the liver of White sea bass (*L. calcifer*) after cadmium exposure. Khangarot (1992) also observed similar
changes in the liver of snake-head fish (*Channa punctatus*) after copper exposure. However, since the concentration of heavy metals such as cadmium, copper, nickel, lead, selenium and iron were relatively low and were within the permissible limit in the streams taken up in the present study (Prasad, 2013), the structural abnormalities observed in hepatocyte of *B.rerio* appears to be due to the acidic pH of water and not due to any heavy metal. Never the less, these changes apparently represent the cellular responses in fish and disturbances in very basic cellular functions.

5.2. Present findings

Cell nuclei are recorded as a major intoxication site (Yang and Chen, 2003) and therefore, the presence of many deformed nuclei in the hepatocytes of the fish *Brachydanio rerio* experiencing acidic pH of water suggests that acid stress causes cytotoxic effects involving cell nucleus. An irregular nuclear envelope observed in the hepatic nuclei in *Brachydanio rerio* in the present study was also reported and interpreted by Braunbeck (1992) as progressive inactivation of nuclear components. Less regular nuclear shape could be interpreted to represent unspecific sign of stress (Braunbeck et al, 1992).

Abnormality in nuclear shape is related to the breakage - fusion-bridge type mitotic disturbances or chromosomal instability (Gisselson et al, 2001). An essential feature of every cell is the presence of a membrane that defines the cell boundary and various internal components of cells. The double - membrane nuclear envelope perforated with nuclear pore mediates two - way transport of materials between the nucleoplasm and the cytosol. Nuclear pore contains an elaborate protein structure called nuclear pore complex that controls the inward movement of protein around the nucleus and outward movement of RNA and ribosomal subunits (Becker et al, 2004). In this regard, the deformed, dilated, invaginated and evaginated nuclear membrane at places as observed in the hepatocytes of *Brachydanio rerio* in the present study appear to be related to adverse effects on the functioning of the liver of the fish. Among the cytoplasmic organelles, mitochondria respond sensitively to environmental stress. Damage to mitochondria has been reported to be a common response of fish hepatocytes to certain pollutants (Bowler and Duncan, 1970). Distortion of inner and outer mitochondrial membrane in the hepatic cell of *Brachydanio rerio* exposed to acidic pH of water accounts for the impaired oxidative capabilities of hepatocytes (Abdel-Moneim and Abdel-Mohsen, 2010).

Besides these, the mitochondria behave as osmometer, and, thus the swelling that develops in it in response to acidic pH of water reflects the entry of solutes and water into the matrix (Ghadially, 1988; Cheville, 1994). Since mitochondria are the sources of energy for the cells, mitochondrial swelling and membrane distortion is likely to lead to disturbances in energy release required for metabolic activities of the hepatocytes, ultimately resulting in failure of the whole organ.

The large number of vacuoles observed in the different organelles of hepatocytes of fish in the current study has also been reported elsewhere in some fishes exposed to some environmental contaminants (Gill et al, 1988). The intense vacuolization in different organelles in the hepatocytes of fish observed in the present study is relevant in view of the fact that autophagic cell death or apoptosis is morphologically characterized by an accumulation of vacuoles (Gonzalez-Polo et al, 2005).

The progressive loss of structural integrity of rough endoplasmic reticulum as observed in the hepatocytes in the current study is similar to the results reported by different authors in relation to certain environmental contaminants (Abdel-Moneim and Abdel-Mohsen, 2010; Gill et al, 1988).

Rough endoplasmic reticulum studded with ribosome is known to be involved in protein synthesis. Degranulation, dilation and fragmentation of rough endoplasmic reticulum as observed in the present study have been classified as typical but non specific lesions induced by some stressors which reflect disturbances in protein synthesis (Rez, 1986).

5.3. Advancement of present report as compared to earlier attempts

The earlier studies on effects of acid stress on fish from natural water bodies were confined to conventional approaches such as histo-pathology, eco-physiology and general biology. The present study on the other hand, has taken up a more sophisticated analytical approach such as electron microscopy which has provided us with relevant information about the tissue damage with more precision and scientific authenticity.

Research Highlights

The significant findings of the present study are the notable deformities in the nucleus, mitochondria, endoplasmic reticulum etc. in the hepatocyte of *Brachydanio rerio* experiencing acidic pH in its natural habitat. The study has been able to bring
some new information on adverse effects of acidic pH on hill stream fish inhabiting some water bodies with low pH.

Limitations

The limitations of the study are that only one species of fish was used and hence generalized conclusion on fine structural deformities related to environmental acid stress in fish cannot be drawn.

Recommendations

The aforementioned limitations can be overcome by conducting the study in a large variety of fish species experiencing the similar type of acid stress in their natural habitats.

Funding and policy aspects

Government, Private and public funding is welcome for initiating research studies on the topic. The current policy of the Government should be amended and relevant law should be enforced to minimize anthropogenic causes leading to acidic conditions in water bodies sustaining aquatic biota.

Authors’ contribution and competing interests

The first, second and third authors’ contributions to the study are field works & experimentations, conceptualization of the study as well as interpretation of data and Transmission electron microscopic analysis respectively.

Conclusion

Transmission electron microscopic studies on abnormal fine structural features of hepatocytes of the hill stream ornamental fish, *Brachydanio rerio* experiencing acidic pH (less than 6.5) of water in its natural habitat and the absence of the abnormalities in fish grown in alkaline pH in the laboratory suggests that the fish is under severe stress due to acidic pH of water in its natural habitat. The abnormal nuclear shape is indicative of mitotic disturbances or chromosomal instability (Gisselson et al., 2001). Distortion of mitochondrial membrane in the hepatic cells accounts for the impaired oxidative capabilities of hepatocytes (Abdel-Moneim and Abdel-Mohsen, 2010) in the hill stream fish. This is likely to lead to disturbances in energy release required for metabolic activities of the hepatocytes. The vacuolization in different organelles in the hepatocytes of the fish indicates the possibility of the occurrence of apoptosis (Gonzalez-Polo et al, 2005). Degranulation, dilation and fragmentation of rough endoplasmic reticulum reflect disturbances in protein synthesis (Rez, 1986). The present study supports the earlier reports on growth impairment, disturbances in metabolic activities, physiological disorders etc. in fish exposed to acidic pH of water. The present investigation further suggests that electron microscopic studies on fine structures of different organs and organelles are important in understanding the adverse effects of acidic pH of water on fish in detail and it can provide additional input to the earlier studies carried out with different conventional approaches. Further, the current study appears to be important for conservation of fish in general and hill stream fish in particular in view of the earlier reports on extinction of some fish exposed to acidic pH of water (Harvey and Jackson, 1995).

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