An Electron Microscopic Analysis on the Ultra Structural Abnormalities in Sperm of the Common Carp *Cyprinus carpio L*. Inhabiting a Polluted Lake, Umiam (Meghalaya, India)

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KEY WORDS TEM; SEM; *Cyprinus*; lake; Umiam; sperm

ABSTRACT The present communication reports the ultra structural abnormalities in sperm of a fish species *Cyprinus carpio* inhabiting a polluted lake, Umiam in North-East India. Transmission electron microscopy (TEM) revealed absence of differentiation between head and midpiece (neck) of some sperm while scanning electron microscopy (SEM) showed some sperm tails with highly reduced length and some sperm with folded tail. Abnormal shape of some sperm head was also revealed by Scanning electron microscopy. Detachment of membrane from some parts of the sperm head and an outward expansion of the same was observed from Transmission electron micrographs of transverse section of sperm head. The well developed mitochondria surrounding the cytoplasmic channel in the sperm tail, as observed in control were found to be drastically disorganized in fish inhabiting the polluted lake. The study suggests that the fish *C. carpio* inhabiting the polluted lake Umiam is under severe stress as far as its male reproductive system is concerned. The study further suggests that Electron microscopic approach is extremely important in the assessment of adverse effects of environmental pollution on fish tissue. *Microsc. Res. Tech. 74:998–1005, 2011.*

INTRODUCTION

Umiam Lake, also known as Barapani Dam is the fourth largest reservoir in North-East India. It is located in Ri Bhoi District of Meghalaya, 15 kms from Shillong, the state capital. It lies at an altitude of about 900 m above the sea level. The lake was once a clean water body and was inhabited by a number of fish species. Besides being an important tourist spot, the lake is also socially and economically important water body for fishery. However, for the last few years, the population of fish in Umiam Lake has been showing a very high declining trend. The topography of the region makes all the rivers, streams and drains to flow directly into the Umiam lake. Toxic materials from metal-based paints, steel alloys, pesticides, waste batteries, fossil fuels, dumping of garbage, plastics, toilet wastes, car washing etc. enter the water body and make it highly polluted. It is a matter of great concern that no systematic study to assess the pollution level of the lake and also to ascertain the possible adverse impact on fish and other aquatic organisms has been carried out so far (The Shillong Times, Shillong, Tuesday, March 10, 2009, Umiam Lake under pollution threat; The Telegraph, Gauhati 30 September, 2009, Umiam, Shillong's "Wasted" Pride). It was therefore, thought that it will be logical to take up studies to understand the health status of some fish inhabiting the polluted lake and to assess the abnormal features exhibited by them due to the pollution load of the lake.

In the present study, the fish *Cyprinus carpio* was chosen because it is found to be the most dominant species inhabiting the lake, Umiam. As a part of our ongoing studies on the possible impact of the pollution load of the lake on different vital tissues, the present study was undertaken on the male reproductive structure i.e., the sperm of *C. carpio*. It is well known that normal structural features will ensure the proper functioning of the sperm which is directly related to the success in fertilization. The present study made an emphasis on electron microscopic investigation because it was thought that although histopathological studies involving optical microscope can reveal some of the abnormal features, many finer details are likely to be obscured due to the limitation in magnification and resolution of the optical microscope.

Scanning electron microscopy, due to its large depth of field and high resolving power appears to be important in studying surface ultra structural morphology and organization of the spermatozoa. Many authors could use this versatile tool with success in understanding the ultra structural morphology of sperm and other structures either in normal fish or in fish experiencing some kinds of stress (Dey et al., 2009a,b; Psenicka et al., 2007). The detail understanding on the cellular features of spermatozoa was possible through extensive studies involving TEM (Gusmao-Pompiani

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Received 31 July 2010; accepted in revised form 10 December 2010
DOI 10.1002/jemt.20986
Published online 20 April 2011 in Wiley Online Library (wileyonlinelibrary.com).

et al., 2009; Gwo et al., 1995; Matos et al., 1999; Pecio, 2003). Several authors have used electron microscopy for studying the ultra structure of spermatozoa from different view points. Jamieson (1991) studied fish spermatozoa ultrastructure with reference to systematics and evolution. Similar types of studies were carried out by Mattei (1991) who suggested that ultra structure of spermatozoa have implications in fish systematics. Mattei et al. (1995) reported unusual midpiece in the spermatozoon of the teleost fish *Citharinus* sp. Matos et al. (2002) studied biflagellate spermatozoon structure of the hermaphrodite fish *Satanoperca juripari*. It is to be noted in this context that despite a large number of published works in the existing literature about sperm ultra structure, very few reports exist on the same topic with reference to environmental stress and pollution (Dey et al., 2009a; Psenicka et al., 2008).

Keeping these in view, an ultra structural analysis have been taken up on the spermatozoa of the fish species *C. carpio* to understand the possible impact of pollution load of the lake sustaining the fish. The study appears to be relevant in understanding the pollutant effect on the fish in view of the fact that abnormal structural feature of sperm is bound to cause disturbances in the functional physiology leading to impairment of reproductive success. In this context, it is to be noted that, normal sperm length is reported to be extremely important for sperm motility because sperm length parameter is correlated positively with ATP, energy charge and fertilization success (Vladic et al., 2002). The movement of spermatozoa in most of the fish is controlled by energetic and cytoplasmic ionic conditions which are responsible for marked changes in cell morphology (Dreanno et al., 1999). Water quality and pollution of aquatic body is likely to have adverse impact on fish sperm as revealed from some earlier studies. It is worthwhile to mention here that sperm motility in some fish was reported to be very low in acidic pH of water (Kime and Tvieten, 2002). Similarly, a recent study has reported a number of abnormalities in sperm surface ultra structural morphology in some fish exposed to acidic pH of water (Dey et al., 2009a). Fine structural abnormalities have also been reported in some fish species inhabiting a polluted lake in Egypt (Abdelmeguid et al., 2007). The objective of the present study therefore was to find out the possible impact of pollution load of the lake Umiam on spermatozoa of the fish, *C. carpio*.

MATERIALS AND METHODS

Fish Samples

Health Condition of Evaluated Fish. In the absence of any published data on the health condition of fish from the polluted lake, Umiam, the present study relied on the interaction with the fishermen regarding the same. The interaction revealed that the number of fingerlings during postbreeding season had been showing a very high declining trend during the last few years suggesting poor reproductive health of the fish, *C. carpio* inhabiting the polluted lake. In contrast, the control water body (RRTC) has been recording a reasonably high, stable population trend of fingerlings during the same period.

Collection of Sample. Male common carps (*C. carpio*) were collected during breeding season (May–July, 2009 and 2010) from Umiam Lake (Polluted water) and from a control water body, Rural Resource and Training Centre (RRTC). The testis of fish from both control and polluted water body were dissected out and processed for TEM. A total of 20 samples with five replications each from control as well as polluted water body were used for the study. Milt from the fish was used for studying the morphology of individual sperm and also for Sperm motility and viability tests. Samples from both the polluted and control water bodies were collected at the same time and were processed following same methods under the same condition.

Scanning Electron Microscopy.

Air-Drying Method. For studying the sperm from milt samples under Scanning electron microscope, a few drops of milt were fixed in 0.1 M 2% glutaraldehyde (prepared in 0.1 M sodium cacodylate buffer) for 30 min. The fixed samples were centrifuged at 1500 rpm for 5 min and supernatant was decanted. The residue was washed in 0.1 M sodium cacodylate buffer solution and was centrifuged at 1500 rpm for 5 min. The supernatant was then decanted and the residue was washed in doubled distilled water and was centrifuged for 5 min at 1500 rpm. The sample was then re-suspended in double distilled water and a thin film was applied on a cover slip and air-dried (Van der Horst and Cross, 1978).The air drying method was used in the study because despite the fact that many people use dehydration and critical point drying, the air drying is being used successfully for SEM of sperm even now, as revealed from some recent publications (Jaroensastraraks and Damrongphol, 1999; Verma et al., 2009). However, critical point drying method was also used for comparison.

Critical Point Drying Method. A few drops of milt were fixed in 0.1 M 2% glutaraldehyde (prepared in 0.1 M sodium cacodylate buffer) for 4 h at 4°C, dehydrated through increasing grades of acetone and dried in a critical point dryer(SAMDRI-PVT-3, TOUSIMIS).

Coating. The dried samples (prepared by critical point drying) were placed on the cover slip with the help of poly-l-lysine. The cover slips (containing air dried as well as critical point dried samples) were secured to brass stub with double coated adhesive tape connected via a patch of silver paint to ensure charge conduction.

A conductive coating of gold was applied to the sample using JFC-1100 (Jeol) Ion-Sputter Coater by establishing a low vacuum (10^{-3} Torr) in the sputtering chamber. The coated samples were examined in JSM-6360 (Jeol) scanning electron microscope at an accelerating voltage of 20 kV. The length of the sperm tail was measured in 20 samples from different individual fish using the scale bar of the scanning electron micrograph.

TEM. The testis were cut into small pieces of ~1 mm × 1 mm in size and were fixed in modified Karnovsky's fixative having the composition of 250 mL of 0.2 M sodium cacodylate buffer, 20 g of para-formaldehyde dissolved in it at 60°C, bringing the volume to 480 mL by

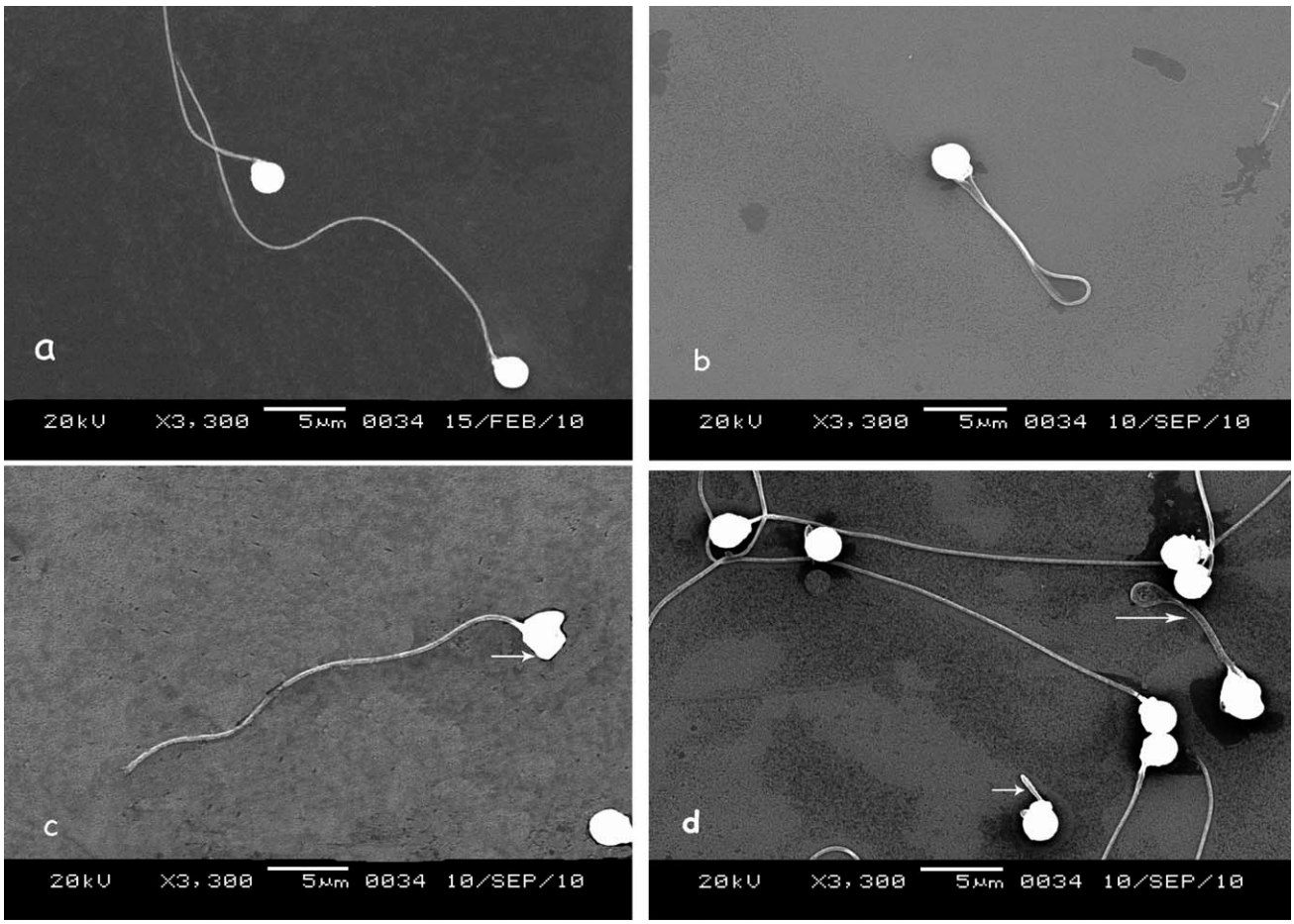

Fig. 1. Scanning electron micrographs of sperms of *C. carpio*. **a**. Control showing normal sperm tail length and elliptical shape of sperm head, ×3,300. **b**. Folded sperm tail in pollution-affected fish. **c**. Deformed sperm head (arrow) in pollution-affected fish. **d**. Highly reduced sperm tail length (short arrow), folded sperm tail (long arrow) in pollution-affected fish.

double distilled water. To this 20 mL of 25% glutaraldehyde and 12.5 g of anhydrous calcium chloride was added. After 4 h in the above primary fixative, the samples were washed thoroughly in 0.1 M sodium cacodylate buffer. Postfixation was carried out in 1% osmium tetroxide in the same buffer for 1 h at 4°C. Specimens were dehydrated in ascending grades of acetone (30, 50, 70, 80, 90, 95, 100% and dry acetone) with two changes of 15 min each. Dehydrated samples were then cleared off acetone by propylene oxide for 30 min. Infiltration was carried out gradually in different proportions of propylene oxide with embedding medium [Araldite CY212—10 mL, DDSA (dodecenyl succinic anhydride)—10 mL, DMP-30 {Tri-(di-methylaminomethyl) phenol}—0.4 mL, and dibutyl phthalate—1 mL]. Embedding of tissue was carried out in the araldite embedding medium using beem-capsules. The embedding blocks were kept at 50°C in an embedding oven for 24 h. The temperature was then raised to 60°C and the embedded tissues were kept for 48 h to complete polymerization. Ultra-thin sections (600–800 Å) were cut in an RMC Ultra-microtome, MT-X, with a diamond knife. The sections were collected on copper grids and stained with alcoholic saturated solution of Uranyl Acetate for 10 min at room temperature in the dark, followed by lead nitrate for 5 min (Reynolds, 1963). The stained sections were examined in a Jeol JEM 100CX II Transmission electron microscope at an accelerating voltage of 80 kV. The size of the sperm head was measured in 20 samples each from individuals of control and pollution-affected fish by dividing the size of the sperm head (in contact print) with the magnification factor. The measurement of the sperm head size was not made from SEM because change in size of the main body of sperm head due to membrane detachment (as revealed from TEM) could have not been measured correctly from scanning electron micrograph.

Sperm Motility and Viability Tests. For sperm motility test, 0.5 mL of the milt was added to 100 mL of buffer (a mixture of 20 mM Tris-HCl and 125 mM NaCl buffer), the dilution ratio of milt and buffer being 1:500. One or two drops of the diluted sample were placed in a glass slide, put under a cover slip and were observed with a CARL ZEISS-426126 Optical Microscope at a magnification of 100×. Hundred sperms with five replicates were examined to determine the number of sperms with rapid progressive movement,

sluggish movement and no movement (Vladic et al., 2002).

For sperm viability test, two drops of diluted milt samples (diluted in a mixture of 20 mM Tris-Hcl and 125 mM Nacl buffer) were put in a glass slide and were stained with a few drops of Nigrosin-Eosin stain (Mortimer, 1985, 1994). A thin smear was then made and observed under CARL ZEISS-426126 optical microscope at a magnification of 100×.

Statistical Analysis. Students' *t* test was used to note the significant differences between control and pollution-affected fish in sperm-tail length; sperm-head size; percentage of rapid progressive, sluggish and immotile sperms; percentage of live and dead sperms. The data is presented as average with standard deviation along with number of measurements and level of significance.

RESULTS

Scanning Electron Microscopy

Scanning electron micrographs have shown a considerably large number of sperm samples with short tail (Fig. 1d, Table 1) and some with folded tail (Figs. 1b and 1d) from fish inhabiting the polluted lake Umiam. In contrast, most of the sperm from control fish exhibit longer tail (Fig. 1a; Table 1), and no sperm tail is found to be folded. Similarly, some sperm head samples show irregular shape in fish from the polluted lake (Fig. 1c) unlike the normal elliptical shape in control (Fig. 1a). The two drying methods, air drying and critical point drying have been found to show similar sperm morphology at the desired magnification used in the current study.

TABLE 1. Sperm tail length (μ) and sperm head size (μ) in control and pollution-affected C. carpio

Sl. no.	Parameters	Control	Pollution-affected
1	Sperm tail length (mean ± S.D)	38.453 ± 8.4 [20]	20 ± 7.5 [20]
2	Sperm head diameter (mean ± S.D)	2.38 ± 0.1 [20]	1.61 ± 0.1 [20]

Figures in parenthesis indicate number of samples. Differences of values between control and pollution-affected samples are significant at 0.05.

TEM

Sperm Head and Midpiece (Neck). TEM reveals well differentiated head and neck in sperm of control fish (Fig. 2a), whereas, in fish grown in polluted lake, the absence of differentiation of head with neck is evident (Fig. 2b). Enlarged view of the sperm head in control reveals the crenate arrangement of the membrane (Fig. 3a), which is found to be in intimate contact with the sperm head cell. In fish inhabiting the polluted lake on the other hand, a clear dislocation of the membrane from some parts of head and its outward extension is evident. Besides these, intense vacuolization is also observed in the sperm head cell adjacent to the dislocated and extended membrane (Fig. 3b). Further, the size of the sperm-head (1.8 μm) is found to be much less than that of the control (2.3 μm) (Table 1). The longitudinal section of the neck shows the presence of well developed mitochondria in the lateral lobes of the neck of sperm from control fish (Figs. 4a and 4b). In the sperm of fish from the polluted lake, on the other hand, the lateral lobes are irregular in shape and some of the mitochondria are totally distorted (Figs. 4c and 4d).

Sperm Tail. The tail of the spermatozoon in control is found to be characterized by long, well developed mitochondria surrounding the cytoplasmic channel (Figs. 5a and 5b). In contrast, the mitochondria surrounding the cytoplasmic channel are found to be distorted to a remarkable extent in fish exposed to polluted water of the Lake Umiam (Figs. 5c and 5d). Besides these, regularly arranged vacuoles surrounding micro-tubular assembly in control (Figs. 5a and 5b) are found to be absent in pollution-affected fish sperm (Figs. 5c and 5d).

Longitudinal section of the sperm-tail reveals dilation of the membrane at places in pollution-affected sperm and vacuolization in the area. (Figs. 4c and 4d). The control samples, however, do not exhibit any such abnormality (Figs. 4a and 4b). The transverse section of the sperm-tail exhibit normal features in micro-tubular assembly and plasma membrane in control (Fig. 6a), while in fish collected from the polluted lake, the plasma membrane of the sperm-tail show distortion and breakage at places (Fig. 6b) along with some disturbances in micro tubular assembly.

About 70% of the samples examined from fish inhabiting the polluted lake are found to exhibit the afore-

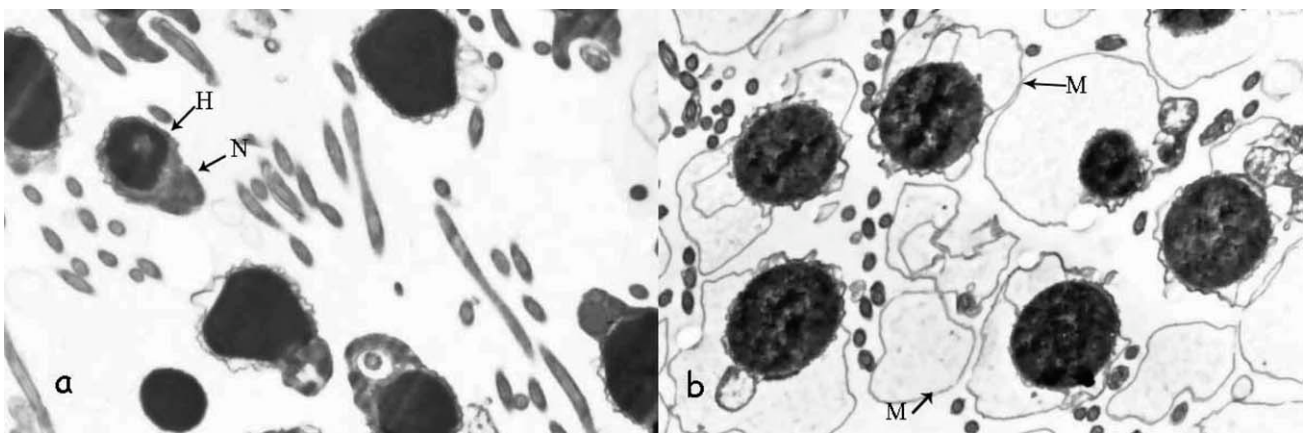

Fig. 2. Transmission electron micrographs of sperm heads of *C. carpio*. **a**. Control, showing differentiation between head (H) and neck (N), ×5,000. **b**. Effect of pollution showing absence of differentiation between head and neck. ×5,000.

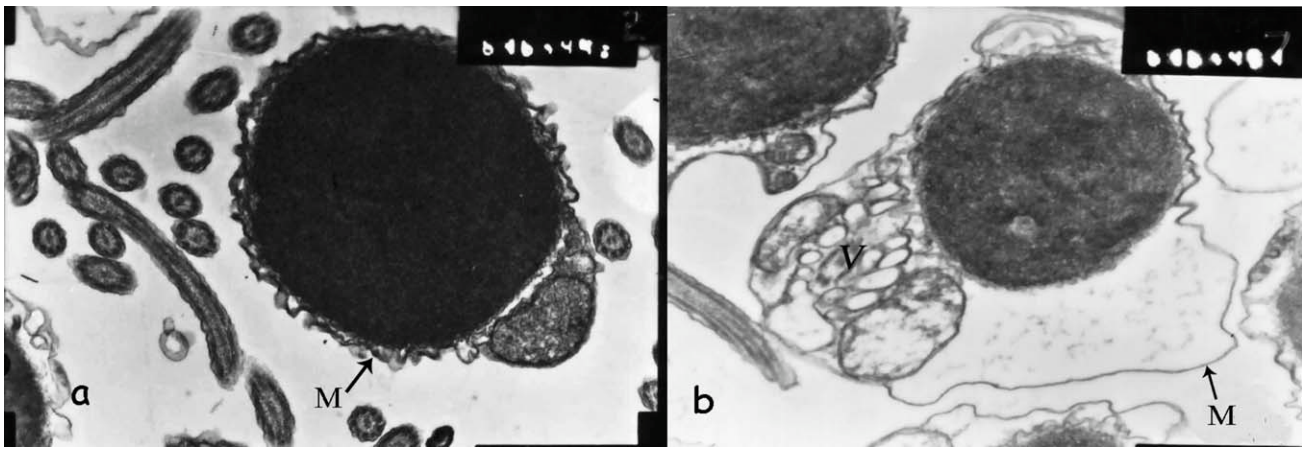

Fig. 3. Transmission electron micrographs of sperm heads of *C. carpio*. **a**. Control, showing normal plasma membrane (M), ×20,000. **b** Effect of pollution showing detachment of plasma membrane (M) from some parts of the sperm head and outward expansion of the membrane. Intense vacuolization (V) is evident, ×20,000.

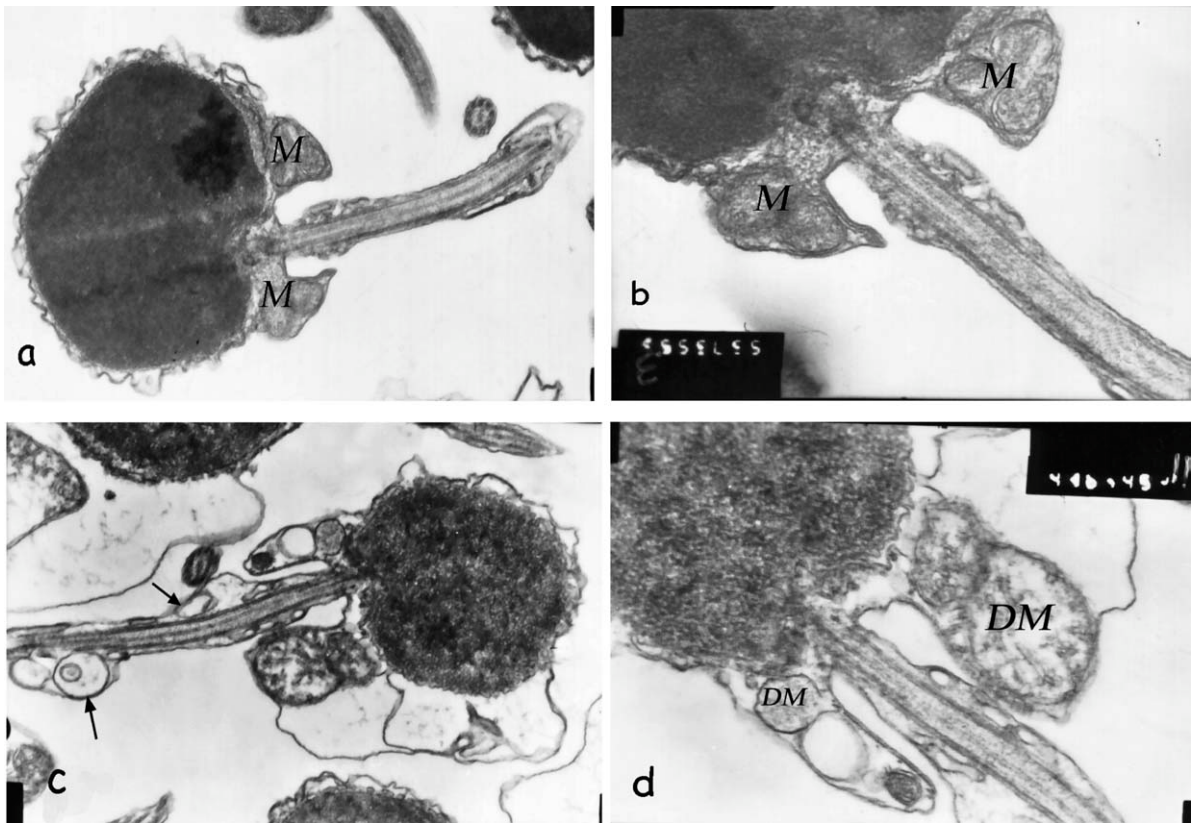

Fig. 4. Transmission electron micrographs of sperm mid piece and tail of *C. carpio*. **a**. Longitudinal section of sperm (control) showing the presence of well developed mitochondria (M) on right and left lobes of the mid piece, ×20,000. **b**. Enlarged view of Figure 4a, ×40,000. **c**. Longitudinal section of sperm (pollution affected) showing dilation of plasma membrane of the tail and occurrence of vacuoles in the dilated region (arrow), ×20,000. **d**. Longitudinal section of sperm (pollution affected) showing distorted Mitochondria (DM) at the two lobes of the sperm mid piece, ×40,000.

mentioned abnormalities. However, there have been some variations in the extent of the deformities and abnormal features.

Sperm Motility and Viability. Sperm motility test reveals 93–95% rapid progressive, 3–4% sluggish, and 1–2% immotile sperm in control fish. In contrast, fish inhabiting the polluted water body are found to exhibit 50% rapid progressive, 30% sluggish, and 20% immotile sperm (Table 2).

In sperm viability test with Nigrosin-Eosin stain, the dead sperm are found to take the stain while the live sperm have not taken any stain and show some glow

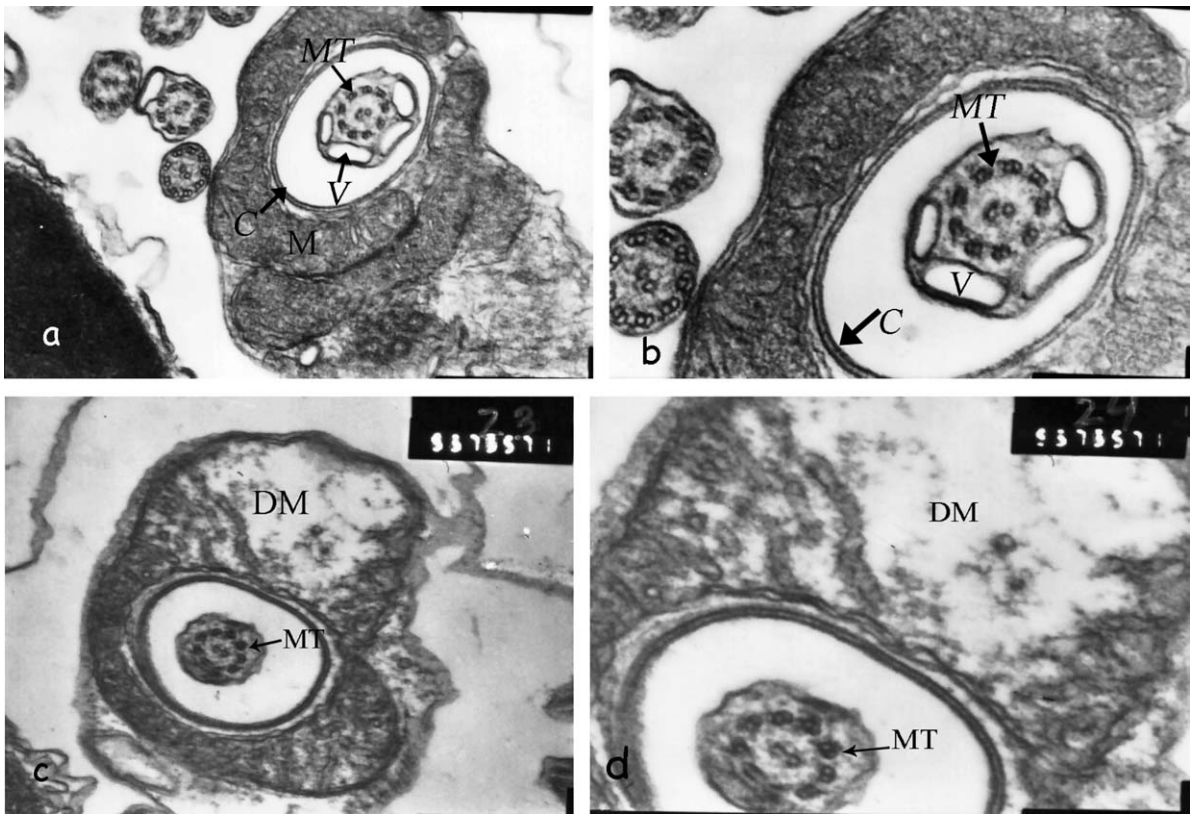

Fig. 5. Transmission electron micrographs of sperm tail of *C. carpio*. **a**. Control, showing long well developed mitochondria (M) surrounding the cytoplasmic channel (C), 9+2 arrangement of microtubules (MT) and vacuoles (V) surrounding the micro tubular assembly, ×40,000. **b**. Enlarged view of Figure 4a, ×80,000. **c**. Effect of pollution showing distortion of mitochondria (DM) and absence of vacuoles surrounding the micro tubular assembly, ×40,000. **d**. Enlarged view of Figure 4c, ×80,000.

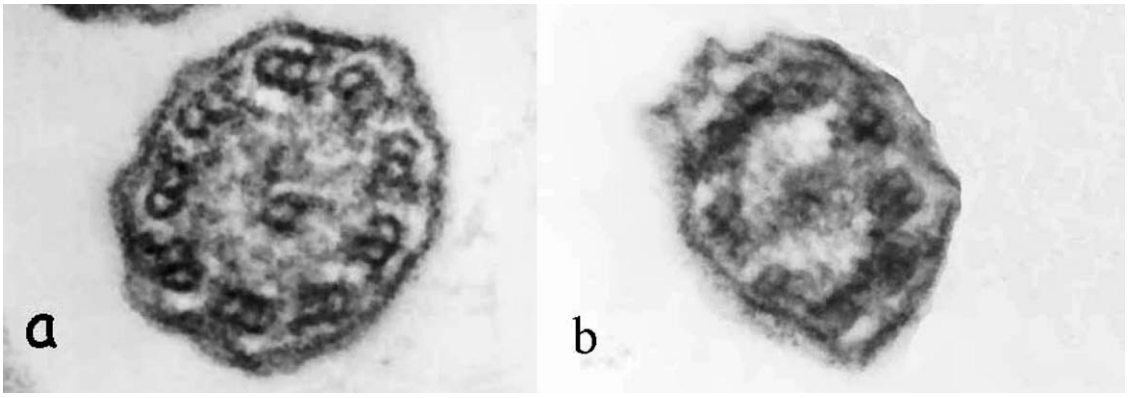

Fig. 6. Transmission electron micrographs of sperm tail of *C. carpio*. **a**. Transverse section of sperm tail (control), showing the typical 9+2 arrangements of microtubules, ×100,000. **b**. Effect of pollution showing distorted membrane at places and disturbed arrangement of the microtubules, ×100,000.

(Fig. 7). Percentage of live sperm in control is found to be 92–93%, while that of the pollution-affected fish is about 60% (Table 2).

DISCUSSION

The basic task of the spermatozoon head is to transfer genetic material localized in the nucleoplasma to the egg. Hence, an optimal shape and size of sperm head is a prerequisite for proper penetration of spermatozoon through the micropyle of egg (Psenicka et al., 2007). In this context, the membrane dislocation in some parts of the sperm-head and its outward expansion in fish inhabiting the polluted lake in the current study have definitely caused deformity in shape of the spermatozoon head which is likely to have adverse effect on the primary function of the sperm-head. Fur-

TABLE 2. Sperm motility and viability (%) of pollution-affected and control fish

Sl. no.	Parameters	Control	Pollution-affected
1	Sperm motility (%) (mean ± S.D)		
	a. Rapid progressive	94.4 ± 2.8 [5]	49.6 ± 1.3 [5]
	b. Sluggish	3.4 ± 0.3 [5]	30 ± 0.5 [5]
	c. Immotile	1.4 ± 0.3 [5]	20 ± 0.5 [5]
2	Sperm viability (%) (mean ± S.D]	92.4 ± 0.3 [5]	60 ± 0.5 [5]

The difference of values between control and pollution-affected samples are significant at 0.05. Figures in parenthesis indicate number of measurements.

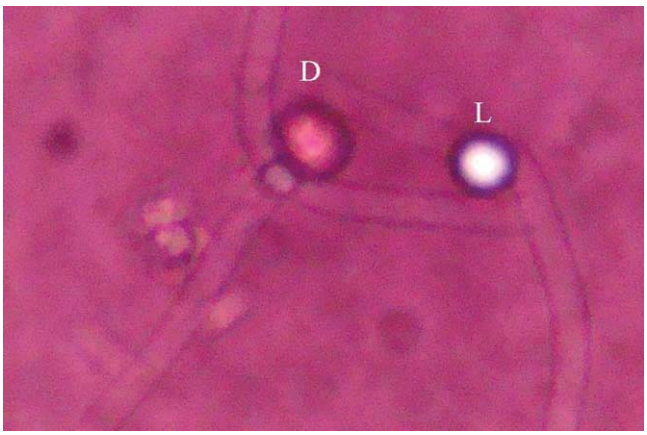

Fig. 7. Sperm viability test showing dead sperm (D) and live sperm (L). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ther, an essential feature of every cell is the presence of membranes that define the cell boundary and various internal components of cells. Cell membrane serves as a locus of specific functions and possesses transport proteins that facilitate and regulate the movement of substances into and out of the cell and its compartments. On that consideration, the dislocation of sperm-head membrane, and breakage of sperm tail membrane at places as observed in our study are likely to cause adverse effects on functioning of the sperm of the fish inhabiting the polluted lake. Pertinent here, is to mention that sperm plasma membrane is reported to play a very active role in sperm fertilization capacity and in spermatozoon-oocyte cross-talk (Lenzi et al., 1996).

Poor differentiation of head and neck of the sperm, shortening of sperm tails and abnormalities in the shape of sperm head in some of the fish collected from polluted lake, as revealed in the current study suggests that the pollutants contaminating the lake has serious adverse effects on the male reproductive unit of the fish. In this context, it is to be noted that high motility of sperm is a prerequisite for fertilization and it correlates strongly with fertilization success (Rurangwa et al., 2004). Since the sperm tail is primarily responsible for motility of sperm, our present observation on shortening of the sperm tail as well as its abnormal structural features in some of the sperm appears to be relevant to the poor sperm motility and consequent failure in fertilization. The sperm motility test revealing 50% rapid/progressive, 30% sluggish, and 20% immotile sperm in fish inhabiting the polluted lake supports our observation on abnormalities in sperm ultrastructure. The sperm viability test indicating 40% dead sperm in fish inhabiting the polluted lake in contrast to only 7–8% dead sperm in control further supports our Electron microscopical observation on sperm abnormalities.

The distortion of mitochondria in the lateral lobes of neck in many of the sperm of fish inhabiting the polluted lake suggests disturbances in energy release required for sperm movement of the fish.

The intense vacuolization in sperm-head in the region enclosed by the dislocated and extended membrane of fish inhabiting the polluted lake is highly significant in view of the fact that a close relation between vacuolization and apoptosis has been reported by some authors (Gonzalez-Polo et al., 2005).

An entirely different situation of the presence of well organized vacuoles surrounding the micro-tubular assembly near the cytoplasmic channel in sperm tail of control fish and their complete absence in the pollution—exposed fish appears to be interesting. In animal cells, vacuoles are frequently used for temporary storage or transport (Becker et al., 2004). This structure functions as a primary site of protein and metabolite degradation and recycling. Vacuoles are also defined as terminal product of secretory pathway (Becker et al., 2004). The secretory pathway functions to transport protein and metabolite-containing membrane vesicles from sites of synthesis or uptake to vacuoles (Becker et al., 2004). On that consideration, the complete absence of vacuoles in the sperm tail of fish from the polluted lake suggests some kind of functional problems. Besides these, interactions between microtubules and vacuoles such as role of microtubules in maintaining the distribution of vacuoles, depolarization of microtubules affecting the vacuoles and their movement (Oda et al., 2009), dynamic organization of vacuolar and microtubule structures during cell cycle progression (Kutsuna and Hasezawa, 2002) have been reported. Although the relation between microtubules and vacuole has been reported only in plant cell, the present observation on the association of vacuoles with microtubules in specific location of sperm tail in control fish and the complete absence of vacuoles in the same location in pollution affected fish, suggests the possibilities of some interaction between the two in animal cells as well.

The present observation thus suggests that the fine structural components of the sperm of *C. carpio* have been adversely affected due to the pollutants present in the lake Umiam. These fine structural abnormalities are bound to cause physiological and functional disturbances in the sperm, leading to reproductive inefficiency. Although the present study did not make an attempt to identify the specific pollutant(s) responsible for the observed fine structural defects, certain possibilities can be suggested in the light of relevant studies by earlier authors.

It has been suggested that the increasing incidence of male reproductive anomalies in fish and other animals may be the result of environmental pollution by toxic chemicals (Toppari et al., 1995). Heavy metals in particular, are known to be associated with altered steroid levels and hindered gonadal development in a

variety of fish species (Joy and Kirubagaran, 1989; Wester and Canton, 1992). Mercury is reported to damage sperm resulting in decreased sperm motility probably by interfering with flagella function (Mottet and Landolt, 1987). A significant decrease in motility of cat fish sperm exposed to cadmium and zinc has also been reported (Kime et al., 1996). Besides this, endocrine-disrupting chemicals in some polluted water bodies have been reported to cause abnormalities in sperm head and tail (Gill et al., 2002).

Despite the fact that studies on the effect of environmental pollution on fish sperm have been carried out with a number of different approaches, ultrastructural aspect of the same which explains the effect with better precision are lacking in the existing literature except a few (Abdelmeguid et al., 2007). The present study is thus relevant in understanding the adverse effect of environmental pollution on fish tissue ultrastructure, which explains the possible functional problems. The observations made in the current study suggests that scanning and TEM should be included as essential components in studies on environmental pollution affecting vital tissues of fish, so that the deleterious effects on the functional physiology can be understood. Besides this, information on ultra-structure can help the investigators to use other approaches to address the problem.

ACKNOWLEDGMENTS

The authors are thankful to the Head, Sophisticated Analytical Instrument Facility, North Eastern Hill University, Shillong and the Principal, St. Anthony's College, Shillong, for their encouragement and kind permission to carry out the work. Thanks are also due to Dr. R. K. Bordoloi, Principal Scientist and Dr. M. H. Khan, Scientist, of Animal Production Division, ICAR Research Complex, Umiam, Meghalaya for their expertise help in sperm motility and viability tests.

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