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Impact of Environmental Contaminants on the Testes of *Oreochromis niloticus* with Special Reference to Ultrastructure of Spermatozoa in Lake Manzala (Egypt)

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Abstract

Background: Pollution of the aquatic environment by inorganic and organic chemicals is a major factor posing serious threat to the survival of aquatic organisms including fish. Lake Manzala (LM) is one of the Egypt's northern Delta Lakes situated on the Mediterranean Coast of the Delta. It is affected by drainage water polluted by different heavy metals that their concentrations exceed the maximum world permissible levels.

Aim: This study aims to study the testicular histopathological alterations of *Oreochromis niloticus* using light microscope and the mature sperm using TEM during spawning season in LM.

Materials and methods: Samples of water and 48 specimen of Nile tilapia, *Oreochromis niloticus* about 14-20 cm in length were collected from two areas; Demitta branch of river (RN) at Mansoura city as a control site and LM as a polluted area during the spawning season from March- June. The gonado-somatic index (GSI) was then calculated for each fish samples. Pieces of testes were then taken and prepared for light and electron microscopic studies. Cd and Pb were estimated in water samples during the spawning season from March to June.

Results: Examined Sections revealed that the testes of specimens of LM had degenerative germinal epithelium of the seminiferous tubules, vacuolization in proliferating germ cells; decline in spermatogenic cell and sperm density than those collected from RN. Ultrastructure study of mature sperm of LM revealed nuclear degeneration, vacuolization and shortage of bilateral fins of the flagellum comparing to mature sperm of RN.

Conclusion: This study giving an alarm that environmental hazard like increasing the levels of different heavy metals as Cd and Pb could alter the sperms structure and their ability to produce new offspring, and further studies may be needed to confirm these findings.

Keywords: Heavy metals; *Oreochromis niloticus*; Testis; Histology; Ultrastructure; Sperm

Introduction

The importance of Nile tilapia, *Oreochromis niloticus* in aqua cultures is due to its spreading in most countries of the world [1]. It breads in captivity and wide variety of water conditions [2]. Lake Manzalah (LM) is one of the most important lakes in Egypt that provides more than 70% of the total fishery of the country [3] and considered as a highly polluted with toxic heavy metals and pesticides due to progressive increase of industrial and agricultural drainage, as well as sewage out fall from the surrounding governorates [4]. The mean values of some heavy metals (Cd, Pb, Fe, Cr and Mn) recorded in LM were higher than the world permissible levels [5] and according to the Egyptian laws [6]. Such heavy metals are not lethal to fish, but concentrated in their flesh and create a hazard to the consumer [7,8]. Cd, Pb, Zn, and Cr are considered the main causes of pollution in aquatic ecosystem [9]. The serious effect of these metals is derived from their persistence, toxicity and bioaccumulation, and consequently exert dangerous problem to man [10]. Experimental studies on heavy metals proved that they could impair the respiratory functions of the gills; consequently death in fish is probably caused by tissue hypoxia [11]. Metals affect not only the fish morphology, but also all biological activities [12]. Among the heavy metals; Cd and Pb are the serious heavy metals that produce histopathological alterations including liver damage [13], respiratory dysfunctions [14], testicular and ovarian alterations [15-17]. These pathological alterations were primarily attributed to damage of cell membranes which allows higher uptake of Cd and Pb and thus the injury extends to more critical targets [18]. Many studies focused on bioaccumulation of heavy metals in fish and biological studies. Thus we aimed in this work to study the testicular histopathological alterations of *Oreochromis niloticus* using light microscope and the mature sperm using TEM.

Materials and Methods

Samples collection

Samples of water and 48 specimen of Nile tilapia, *Oreochromis niloticus* about 14-20 cm in length and 120-200 g in weight were collected from two areas; Demitta branch of RN at Mansoura city as a control site and LM as a polluted area during the spawning season from March- June and transported to the laboratory in tanks provided with oxygen pump. The fish were weighed, dissected and the testes were

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removed carefully wiped with filter paper and weighed. The gonado-somatic index (GSI) was then calculated for each fish according to the following formula:-

GSI=Gonad weight (g)/total body weight (g) ×100 [19].

Water analysis

According to the measurement of Saeed and Shaker and Elewa [20,21] (Tables 1,2) . The samples of water from the two studied areas were analyzed by using a flame atomic absorption spectrophotometer (Model 2380, Perkin-Elmer) at the chemistry department, Faculty of Sciences, Mansoura University, Egypt.

*PL: permissible limits according to USEPA (1986)

Light microscopy

The testes were cut into small pieces and fixed in Bouin's solution, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and mounted in molten parablast at 58-62°C. Specimens were sectioned 5 µm by microtome, stained with Hematoxylin & Eosin [22] and examined using light microscope.

	Fe	Zn	Cu	Mn	Cd	Pb
min	0.72	0.32	0.36	0.28	0.01	0.012
max	1.98	0.66	0.68	0.84	0.09	0.22
mean	1.416	0.464	0.513	0.513	0.044	0.099
± SD	± 0.08	± 0.02	± 0.02	± 0.03	± 0.005	± 0.012
PL	1.0	1.0	1.0	0.05	0.01	0.05

*PL: permissible limits according to USEPA (1986)

Table 1: Mean value of heavy metals concentration (mg/L) in water of Lake Manzala. According to the measurement of Saeed and Shaker (2008).

	Fe	Zn	Cu	Mn	Cd	Pb
min	0.116	0.005	0.001	0.027	0.001	0.005
max	0.750	0.060	0.005	0.142	0.003	0.017
mean	0.340	0.24	0.002	0.73	0.001	0.007
± SD	0.24	0.019	0.001	0.045	0.001	0.003
PL	1.0	1.0	1.0	0.05	0.01	0.05

*PL: permissible limits according to USEPA (1986)

Table 2: Mean value of heavy metals concentration (mg/L) in water of RN in Mansoura region according to Elewa 2010.

		March		April		May		June	
		RN	LM	RN	LM	RN	LM	RN	LM
Cadmium mg/L	Mean	0.003	0.068	0.004	0.096	0.005	0.140	0.005	0.146
	Min	0.002	0.050	0.003	0.091	0.003	0.140	0.002	0.140
	Max	0.004	0.090	0.005	0.099	0.007	0.140	0.008	0.150
	± SD	0.001	0.02	0.001	0.03	0.001	0.00	0.002	0.005
Lead									
mg/L	Mean	0.005	0.07	0.005	0.09	0.009	0.14	0.009	0.16
	Min	0.004	0.04	0.002	0.07	0.008	0.13	0.008	0.17
	Max	0.006	0.09	0.008	0.1	0.01	0.15	0.01	0.15
	± SD	0.001	0.02	0.002	0.012	0.001	0.01	0.001	0.01
GSI %	Mean	0.340	0.136	0.520	0.338	0.890	0.734	1.17	0.914
	Min	0.29	0.09	0.35	0.26	0.63	0.64	0.96	0.75
	Max	0.43	0.17	0.85	0.44	1.1	0.85	1.53	1.12
	± SD	0.05	0.03	0.19	0.07	0.22	0.08	0.26	0.14

± SD = standard variation

Table 3: Monthly variation of the mean concentrations of Cd & Pb in water and gonaosomatic index of male *O.niloticus* from RN & LM.

Electron microscopy

Testicular tissues prepared for TEM were cut into very small pieces and immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for two hours. After rinsing in 0.1 M cacodylate buffer, samples were post fixed in a buffered solution of 1% osmium tetroxide at 4°C for 1.5 hour. This was followed by dehydration in ascending grades of ethyl alcohol and embedded in epoxy-resin [23]. Ultrathin sections were obtained with a diamond knife on a LKB microtome and mounted on formvar-coated grids, stained with uranyl acetate and lead citrate for 30 mins and examined using a Joel 1200 EXII at the Electron Microscope unit in Alexandria University, Egypt. Then these sections were examined and photographed with an Olympus CX41 digital camera.

Results

Heavy metals in water samples

The water analysis of RN at Mansoura city showed slight pollution in comparison with that of LM. This study showed that the mean values of heavy metals especially cadmium (Cd) and lead (Pb) were higher than those of RN, whereas these heavy metals were at the permissible levels during spawning season (Table 3).

Gonadosomatic index

The mean values of male gonado-somatic index (GSI) were increased gradually from March -June and showed that the mean percentage of GSI was ranged between 0.34 and 1.17 for RN and between 0.14 and 0.91 for LM (Table 3). These results indicated that the minimum value of mean percentage of GSI in both RN and LM was observed in March, while maximum value was in June (Table 3).

Histological examination

Histological examination of the testes of *O. niloticus* from both RN and LM in the studied period showed that each testis is lobular in shape and formed of many seminiferous tubules. Each one contains the germ cells that arranged in clusters and occurs in several places along the length of each tubule. During the spawning period, all spermatogenic stages were observed. Depending upon the morphology and size of the nucleus these stages could be described.

In march: In the specimens of RN, the seminiferous tubules showed intensive clusters of spermatogenic cells of large spherical spermatogonia containing large rounded central nuclei with distinct nucleoli. The primary spermatocytes possessed a darkly stained nucleus, whereas that of secondary spermatocytes was smaller than the primary spermatocytes with clump chromatin material. The smaller deeply stained spermatids and reduced number of sperms were also observed (Figure 1). The test is collected from specimen of LM showed several histological alternations such as degenerative changes, vacuolation in some parts of seminiferous tubules and the number of different spermatogenic cells were fewer than those of RN at the same time of collection (Figure 2).

In april: The seminiferous tubules of specimens of RN showed obvious packed great number of spermatogenic cells especially spermatids (Figure 3). However, in comparison with those of LM there were a dispersion, degeneration and decrease in number of the spermatogenic cells of the seminiferous tubules, as well as, vacuolization in interstitial tissue (Figure 4-6).

In may and june: The seminiferous tubules of specimens from RN were packed with sperm masses but the tail portions were rarely visible

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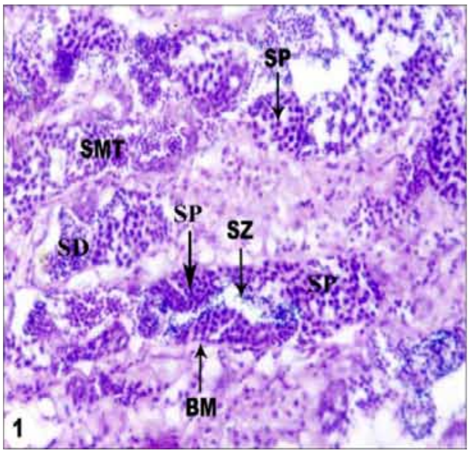

Figure 1: Photomicrograph of T.S. of testes of *Oreochromis niloticus* of RN in March showing intensive spermatogenic cells in the seminiferous tubules (SMT), spermatocytes (SP), and spermatids (SD) and reduced number of spermatozoa (SZ). (X 400).

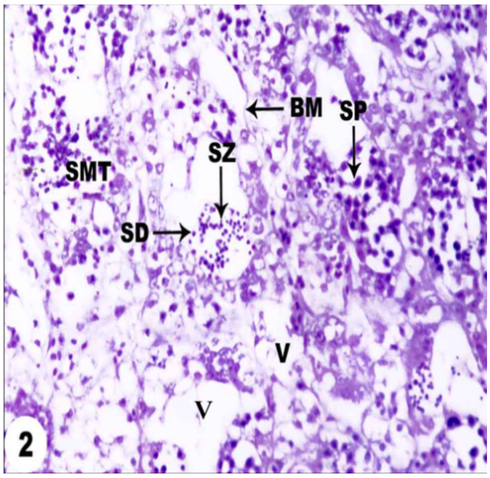

Figure 2: Photomicrograph of T.S. of testes of *O. niloticus* of LM in March showing reduced number of different stages of spermatogenic cells in seminiferous tubules (SMT) , spermatocytes (SP), spermatids (SD) and vacuolation (V). (X 400).

by light microscopic. While the specimens from LM showed sever decreases in the total number of sperm cells with the presence of some empty seminiferous tubules (Figures 7-13).

Ultrastructure observations

The TEM examination of specimens collected from RN showed late stages of spermatids and mature spermatozoa, with densely chromatin nuclei and extensive mitochondria (Figure 14). However, the specimens collected from LM reveals different early and late stages of spermatids and spermatozoa. The early stage of spermatids possesses spherical small round nuclei with electron dense & prominent chromatin patches interconnected by the cytoplasmic bridge (Figure 15). The middle stages of spermatids are characterized by electron dense nuclei with prominent batches of heterochromatin, appear the implantation fossa, both centrioles at the basal pole of the nucleus & the distal centriole starts to form the flagellum. Some mitochondria are located near the centrioles and aggregated irregularly on both sides of the mid piece around the flagellum (Figure 15). In late stage of spermatids, the nucleus becomes indented and a nuclear fossa is formed, the diplosome flagellar axis become perpendicular to the base of the nucleus and the proximal & distal centrioles are connected with electron dense filaments and located within the nuclear fossa, (Figure 16,17). Moreover, specimens of LM showed that, the cytoplasm of spermatid contains many vacuoles and few numbers of large sized mitochondria comparing to those from RN (Figure 16 and Figure 17).

The mature spermatozoon of RN is simple elongated cell composed of a head, short mid piece and a tail or flagellum. The head has no acrosome and consists of nucleus that has very dense homogenous chromatin. The head becomes completely surrounded by the cytoplasm, with dispersed large population of mitochondria (Figure 18). The neck region shows well developed proximal and distal centrioles in the implantation fossa (Figure 18,19). The proximal centriole, showed a

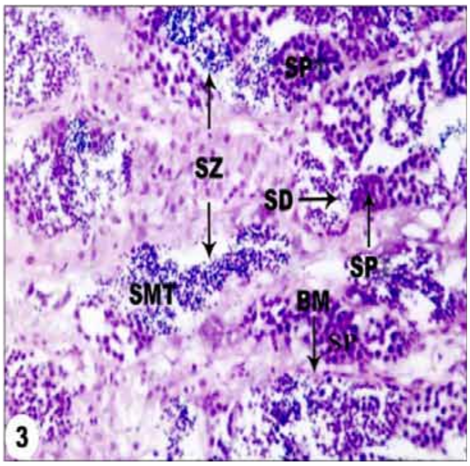

Figure 3: Photomicrograph of T.S. of testes of *O. niloticus* of RN in April showing intensive number of spermatocytes (SP), spermatids (SD) and presence of spermatozoa in the lumen of seminiferous tubules (SZ). (X 400).

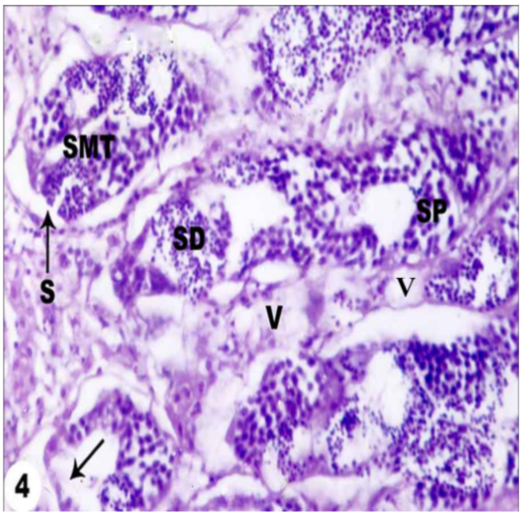

Figure 4: Photomicrograph of T.S. of testes of *O. niloticus* of LM in April showing separation between the boundary membrane of seminiferous tubules (SMT) (S), degeneration in some area of the seminiferous tubules (arrow) and large numbers of vacuoles (V). (X 400).

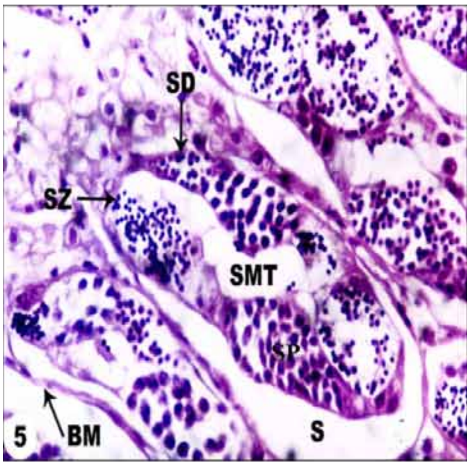

Figure 5: Photomicrograph of T.S. of testes of *O. niloticus* of LM April showing the reduction in the amount spermatocytes (SP), spermatids (SD) & the spermatozoa (SZ) cells in the seminiferous tubules (SMT), obvious separation (S) of the boundary membrane of seminiferous tubules (BM) and presence of many vacuoles in interstitial cells and between spermatogenic cells (V). (X 400).

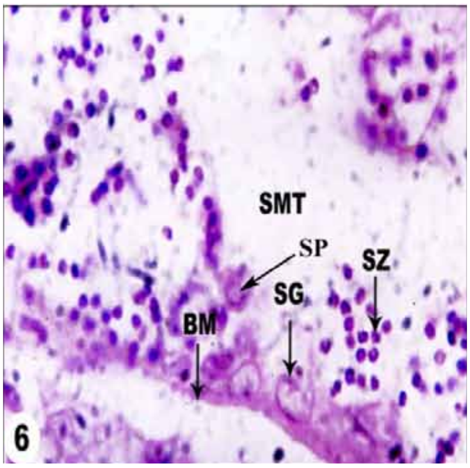

Figure 6: Amplified portion of T.S. of testes of *O. niloticus* of LM in April showing some spermatogonia (SG), sever reduction in spermatocytes (SP), and spermatozoa (SZ) and other spermatogenic cells of the seminiferous tubules (SMT) (X1000).

conventional 9+0 microtubular triplet's constructions, and fits in the articular fossa. The flagellum contains a typical axoneme which has conventional 9+2 microtubular doublet constructions (Figure 20,21). The plasmalemma of midpiece and anterior region of the flagellum exhibits 2 lateral or side fins. A very important point is the presence of one pair of side fins at the base of flagellum of the spermatozoa (Figure 20,21).

Spermatozoa of specimens of LM showed the same main features of those of RN, however, the nucleus was more or less irregular in shape with irregularly dense chromatin clumps of electron-dense material and numerous electron-lucent areas (Figure 19). The two lateral fins of sperms of LM were shorter, swelling and degraded than those of RN. There are also degenerative changes in the plasmalema of the lateral fins and the microtubule construction (Figure 21).

Discussion

Heavy metals, especially Cd and Pb are considered the most serious pollutants; and if present in high concentrations could have a negative effect not only on the river system, but also on fish population [24]. The values of heavy metals concentrations at RN were fewer than that for LM and differ from month to another. The heavy metals uptake by fish depends upon exposure time, concentration, temperature and diet. Among heavy metals Cd and Pb promote an early oxidative stress and lead to the development of serious pathological conditions because of their long retention in several tissues [18,25,26]. The dangerous effect

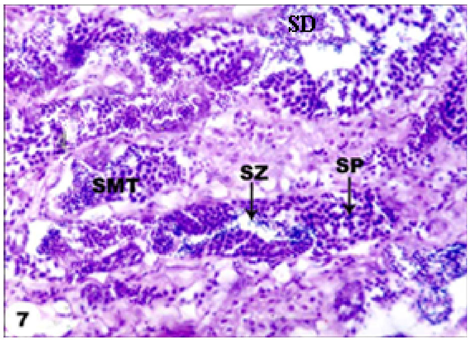

Figure 7: Photomicrograph of T.S. of testes of *O. niloticus* of RN in May showing the seminiferous tubules (SMT) containing increased number of spermatocytes (SP), spermatids (SD) and spermatozoa (SZ).

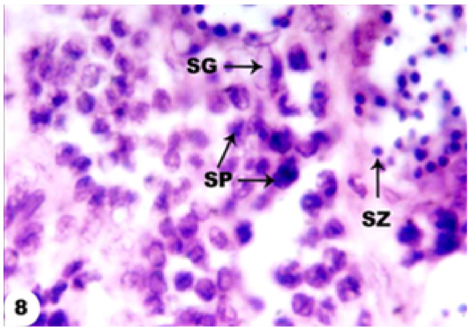

Figure 8: Amplified portion of T.S. of testes of *O. niloticus* of RN in May showing spermatogonia (SG), spermatocytes (SP), and spermatozoa (SZ) (X 1000).

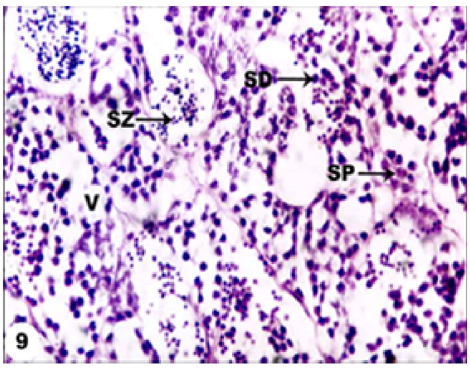

Figure 9: Photomicrograph of T.S. of testes of *O. niloticus* of LM in May showing sever decrease in the number of spermatogenic cells especially spermatozoa (SZ) and obvious vacuolation (V). (X 400).

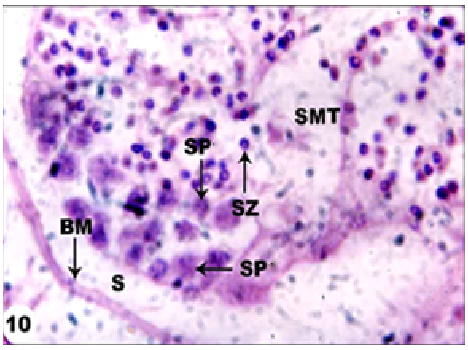

Figure 10: Photomicrograph of T.S. of testes of *O. niloticus* of LM in May showing decrease in the number of spermatozoa (SZ) and separation (S) between boundary membrane (BM) and seminiferous tubules (SMT) components. (X1000).

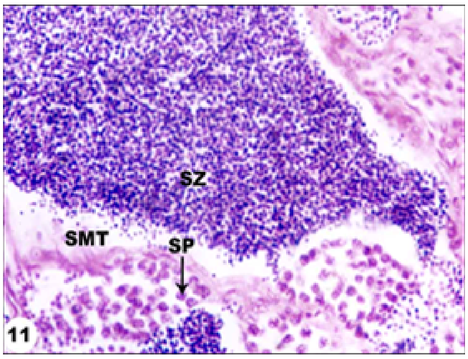

Figure 11: Photomicrograph of T.S. of testes of *O. niloticus* of RN in June showing that seminiferous lobules were packed with sperm masses (SZ) but the tail portions were rarely visible by light microscopy. (X 400).

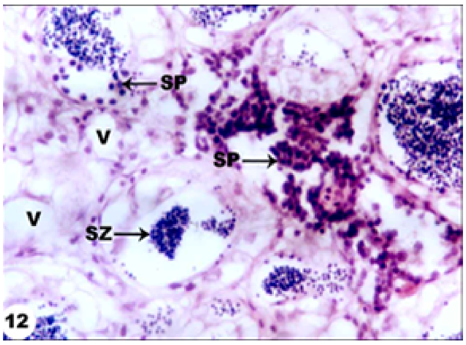

Figure 12: Photomicrograph of T.S. of testes of *O. niloticus* of from LM in June showing reduction in the different spermatogenic cells and clear vaculation (V).

from the presence of heavy metals, especially Pb and Cd in the aquatic environment derive, not only, from their persistence and toxicity, but also from the remarkable degree of bioaccumulation, and consequently exert serious problems to man [27].

The gonado-somatic index is generally used to determine the maturity stage of fish and subsequently degree of gonadal development. The increase of gonadosomatic index is related with the percentages of ripe females and males towards the spawning season [28,29]. In the present investigation, the data obtained showed that the values of male gonado-somatic index of *Oreochromis niloticus* were higher at RN than that in LM giving an idea about the exert effect of high level of pollution in LM. Such effect was reflected by the decrease of sperm account in the ripe testes during the spawning season as a result of high heavy metal concentration [30].

Many studies showed that the spermatid and sperm increase in number toward the end of spawning season [31,32]. The specimens of LM showed decrease in spermatogenesis, disruption of spermatogenic cells and reduction in the spermatids and spermatozoa. This implies the heavy metals bad effects that can disrupt and impair reproduction in

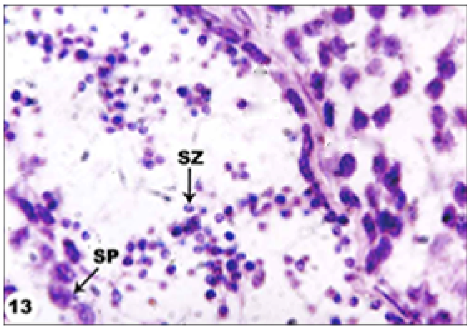

Figure 13: Photomicrograph of T.S. of testes of *O. niloticus* of from LM in June showing reduction in the spermatogenic masses. (X 1000).

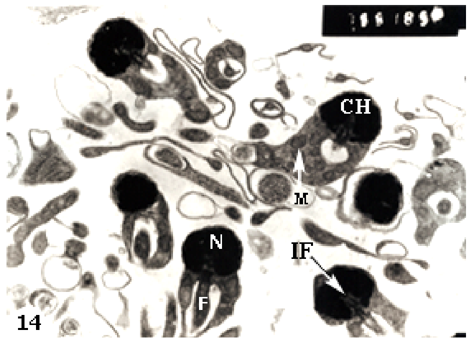

Figure 14: Transmission electron micrograph (TEM) of L.S. in spermatids of RN showing the nucleus (N) containing dense chromatin (arrow), completely formed implantation fossa(IF),the flagellum (F).the cytoplasm contain large numbers of mitochondria (M). (X7000).

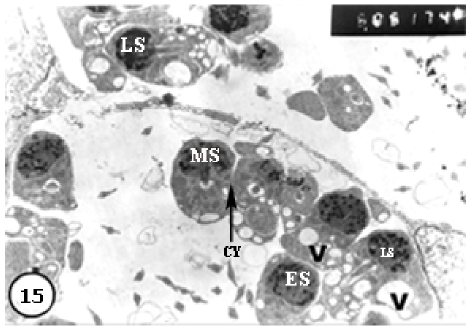

Figure 15: TEM of L.S. in seminiferous tubules showing different stages of spermatids of *O. niloticus* of LM showing early stage of spermatid (ES), middle stage of spermatid (MS) and late stage of spermatid (LS), all the mentioned stages have excessive number of vacuoles (V) in their cytoplasm and remain attached by the sytoplasmic bridge (CY). (X 5000).

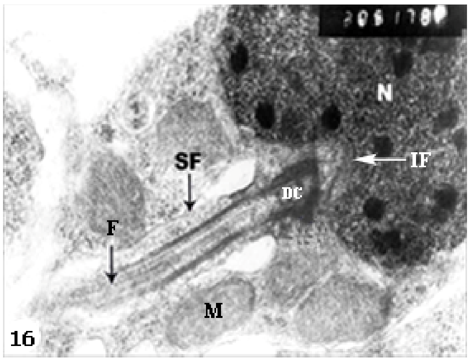

Figure 16: TEM of L.S. in late stage spermatid of *O. niloticus* of RN showing granular and condensed chromatin in the nucleus (N), mitochondria (M), the implantation fossa (arrow),distal centriole (DC) ,the flagellum (F) and one pair of side fins at the base of the flagellum (SF). (X 30000).

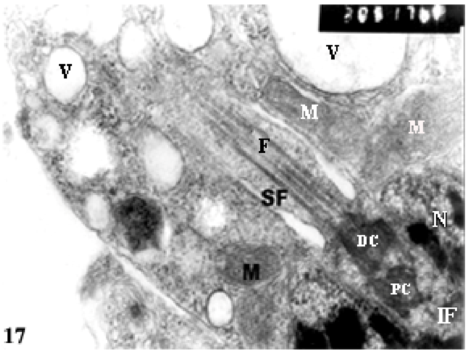

Figure 17: TEM of L.S. *O. niloticus* of late stage spermatid of LM showing the nucleus (N) contains granular and less condensed chromatin, the mitochondria (M) being less in number and presence of large sized mitochondria. The centriolar complex,proximal centriole (PC) and (DC) are housed in nuclear depression called implantation fossa (IF). Notice the large number of vacuoles (V). (X 30000).

fish [17,33]. It was noticed that increasing histopathological alterations is associated with increasing the concentration of Cd and Pb pollution that causing severe testicular atrophy with arrested spermatogenesis, necrotic spermatogenic cells, and vacuolization in the interstitial tissue [34]. Also, animals that exposed to high doses of Cd and Pb exhibits severe testicular atrophy [35,36] as well as biochemical processes [37,38]. The marked inhibition in the process of spermatogenesis due to Cd and Pb pollution simulates with that described by Mousa and Mousa [24] who found a decline in the gonadal activity as reflected by decreasing of sperm amount in the ripe testis. The high levels of heavy metals may interfere with the release of hormones and disturb the feedback mechanisms of gonadal cycle) [39].

The effects of pollution on spermiogenesis of *O. niloticus* were reflected on the modifications in nucleus, flagellum, spermatids, distribution and organization of the cytoplasmic organelles and implantation fossa of spermatids [40]. Ultrastructural study of mature sperm of *O. niloticus* inhabited RN showed the normal structure of spermatozoa that consists of head with no acrosomes, an ovoid nucleus, short middle piece and bifin (lateral fins) flagellum with a classical axoneme pattern (9+2 arrangement), these results coincide with many authors [41-44]. The absence of acrosomes in sperms is compensated by presence of micropyle in the oocytes that allows the sperm penetration and has a role in the prevention of polyspermy [45]. On the other hand, the ultrastructural morphology of sperm of *O. niloticus* collected from LM displays some alternations including nuclear distortion changes in head morphology, incomplete chromatin condensation, and shortage of bilateral fins of the flagellum, wavy and ruptured plasma membrane. As well as, the presence of some vacuoles and the relative decreases in the density of chromatin condensation might be associated with chromosomal abnormalities, which is associated with decrease fertility potential [46,47]. Moreover, LM fish's spermatozoa revealed the presence of few numbers and large sized mitochondria in front of the proximal centriole toward the lateral aspects of the nucleus. The enlarged size of the mitochondria appeared to be a considerable hypertrophy and a sign of acclimatization of fish in these samples rather than RN [48,49]. The bilateral fins of the spermatozoa that formed by the plasma membrane extended from the flagellum is very important for perfect motility and exhibits higher fertilization capacity [40]. The present results indicated that the samples of RN exhibited more differentiation and development of the flagellum than that of LM samples. The deformities change of the sperm's flagellum of *O. niloticus* of LM may lead to decreasing the capacity of sperm motility and reducing its fertilization capacity, and consequently lead to failure of reproduction [50,51]. Most probably these alterations in sperms may result from environmental hazards increasing the levels of Cd and Pb. The changes in sperms structure as a result of environmental hazards

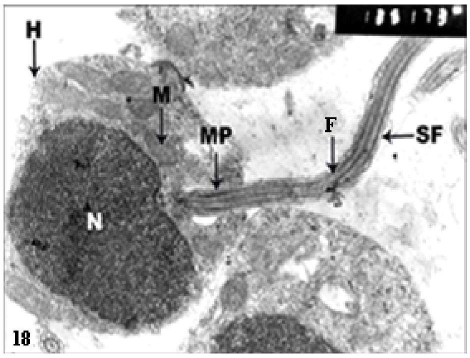

Figure 18: TEM of L.S. in spermatozoon of *O. niloticus* of RN showing ovoidal head (H), nucleus (N) with condensed chromatin, middle piece (MP) and a flagellum (F), mitochondria (M) and the side fins (SF). Densely grouping of mitochondria were found. (X 13500).

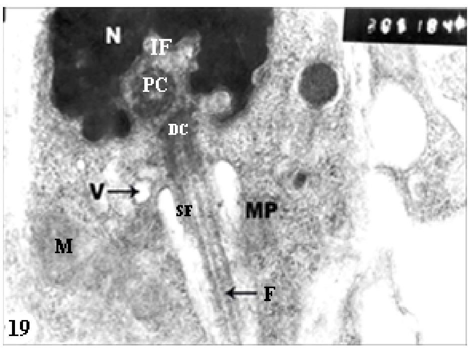

Figure 19: TEM of L.S. of spermatozoon of *O. niloticus* of LM showing a change in the chromatin shape of the nucleus (N), Flagellum (F) and side fins (SF), Notice the presence of vacuoles (V), the implantation fossa (IF) containing the proximal centriole (PC), distal centriole and the basal segment of the flagellum. (X 30000).

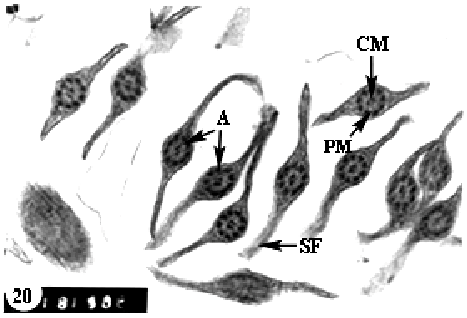

Figure 20: TEM of T.S. of flagellum of *O. niloticus* spermatozoon of RN showing the side fins (SF), the normal structure of axoneme (A) ring of nine doublets, peripheral microtubules (PM) and central pair of microtubules (CM). (X30000).

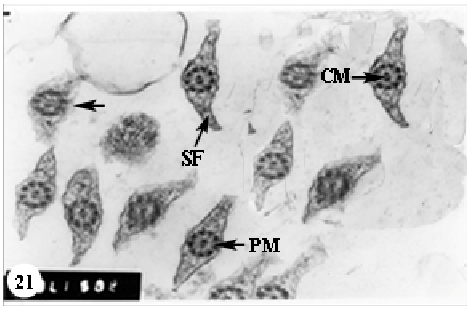

Figure 21: TEM of T.S. of flagellum of *O. niloticus* of LM showing the shortage in length of side fins (SF), the ring of nine doublets peripheral microtubules (PM) and central pair of microtubules (CM). Notice disruption& irregular wall of the side fins (SF) (arrow). (X 30000).

giving an alarm into how the toxic heavy metals could alter the natural biology of feral fish and their ability to produce new offspring.

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