Research Article

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CYTOPROTECTIVE EFFECT OF ANTIOXIDANT ON ENDOSULFAN INDUCED ARCHITECTURE OF SPERMATOZOA IN MICE: A TRANSMISSION ELECTRON MICROSCOPE (TEM) VIEW

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ABSTRACT
In the present investigation spermatozoa architecture after endosulfan treatment in mice have been evaluated. Similarly fruit extract of Amla (*Emblica officinalis*) as an antioxidant have been tested for probable restoration in sperm architecture exposed to endosulfan treatment. Endosulfan is a pesticide of organochlorine group. Government of Kerala (India) banned this pesticide due to its indiscriminate use in Kasargod district and hazardous effect on health among cashew nut workers. In the present investigation, the dose of 3 mg/Kg b.w of Endosulfan was continuously administered to male mice for 35 days after that aqueous extract of *Emblica officinalis* (Amla) fruit has been administered for 35 days at the dose of 150 mg/kg b.w. to evaluate ameliorating effect on sperm. The mice were sacrificed on 35th day of Amla treatment followed by 35 days of endosulfan treatment to observe the architecture of spermatozoa through Transmission Electron Microscope (TEM). Electron micrograph of spermatozoa of 3 mg/kg Endosulfan treated for 35 days showed degeneration in apical acrosome region with degenerative changes in plasma membrane and nuclear membrane of head region of spermatozoa. The degeneration were also observed in 9+2 arrangement of microtubule and mitochondrial sheath of mid piece of spermatozoa while principal piece showed without plasma membrane which denotes complete degeneration of plasma membrane. Outer dense fibers were fused with microtubules. The divergent arms were degenerated and not visible clearly. This way marked degenerative changes were observed in the architecture of spermatozoa (Head Piece, Mid Piece and Principal Piece) after exposure of endosulfan. *Emblica officinalis* at the dose of 150 mg/kg b.w. showing restoration of acrosomal material, better effect on condensation of chromatin material, plasma membrane and nuclear membrane is almost reformed, middle piece and principal piece showing restoration of intact plasma membrane. Thus from the above study it can be concluded that amla as an antioxidant has more potency to check male reproductive deformities.
Keywords: *Emblica officinalis*(Amla), Endosulfan, Spermatozoa, Mice, Transmission Electron Microscope (TEM).

INTRODUCTION

Neurological disorders (epilepsy, cerebral palsy and mental retardation), congenital malformations, reproductive disorders and cancers of various organs reported from Kasargod district of north Kerala (India) have been linked with endosulfan, an organochlorine pesticide. Altered spermatogenesis was also reported in male mice treated by gavages with 3 mg technical endosulfan/kg/day for 35 days[1]. A dose-related decrease in testicular testosterone, plasma testosterone, LH and FSH in groups of male Wistar rats administered endosulfan at 0, 7.5 or 10 mg/kg/day for 15 or 30 days[2]. To evaluate a ameliorating effect, if any, to mitigate the sperm toxicity in mice for which aqueous extract of *Emblica officinalis* (Amla) have been taken as curative measure. Antioxidant effects of aqueous extract of *Emblica officinalis* (fruits) were evaluated against several species of free radicals (reactive oxygen species, ROS; reactive nitrogen species, RNS; and other radical-centered species, e.g. S- and C-). Furthermore, in some studies, the extent of antioxidant actions and their mechanisms were determined by using a well defined extract of amla (EA) and also fractions of polyphenols; with or without emblicanin – A(1) and – B(2), the two most potent antioxidant agents of amla. Free radicals, e.g. ROS and RNS, abstract hydrogen (H) from hydrogen donors (e.g. phenols) and accept an electron from electron-rich species. Hence, they act, as oxidants and are responsible for oxidative stress in aerobic organisms[3]. Antioxidants of natural origin are commonly used to counter the deleterious actions of free radicals[4].

MATERIALS AND METHODS

In the present investigation, experiments were performed on 10-12 weeks old healthy male Swiss albino mice, *Mus musculus*. For the optimal growth and reproduction, the mice were kept in ideal condition under a well regulated light and dark (12 h : 12 h) schedule at 23±1°C in the animal house, Mahavir Cancer Institute and Research centre, Patna, India (CPCSEA Regd. No. 1129/bc/07/CPCSEA, dated 13/02/2008) and study was duly approved by the IAEC. Animals were given food and water *ad libitum*. The oral LD_{50} value of endosulfan for mice was estimated by standard interpolation method, which was 7 mg/kg b.w. The standard data reference for LD_{50} of endosulfan for mice is 7.36 mg/b.w.[5]: Endosulfan manufactured by Excel Industries Mumbai (E.C-35 %) was dissolved in distilled water to prepare sublethal dose of 3 mg/kg b.w which was administered for 35 days by gavage method thereafter aqueous extract of amla was administered at the dose of 150 mg/kg b.w. again for 35 days. Fresh dried form of amla purchased from herbal store in Patna, India. Amla was identified by Dr. Ramakant Pandey (Botanist) Dept. of Biochemistry, Patna University, Patna, India. A vehicle of control group of mice was established and served with equal volume of distilled water by gavage method. The treated mice and control group were sacrificed on targeted day of treatment. Cauda epididymis were excised and fixed in 2.5 % glutaraldehyde for Transmission Electron Microscopy study.

Tissue Processing For Transmission Electron Microscopy

Small pieces of tissues were fixed in 2.5 % gluteraldehyde for overnight and washed with 0.I M phosphate buffer at 4^0C each. Post Fixation was done in 1 % Osmic acid (OsO_4) in 0.1 M in chilled phosphate buffer and again washed with 0.I M phosphate buffer at 4^0C. Tissues were dehydrated in graded series of alcohol. Clearing of tissues were done in toluene, infiltration of tissues were carried out in toluene plus araldite mixture. Then tissues were brought to pure araldite and tissues were embedded in plastic moulds in embedding medium and the blocks are withdrawn out of the moulds. Blocks were trimmed then its semi thin (of the order of 1-2 μ) and ultra thin sections (of silver gray colour) were cut on ultra microtome. The semi thin sections were stained and observed under light microscope for marking of section area. Then grids were prepared after final staining. The ultra thin sections were observed under Transmission Electron Microscope.

RESULTS

The control group of mice showed normal structure of spermatozoa (Head region). Head region showed normal acrosome (AC), head cap (HC) covering the acrosomal material, condensation of chromatin material (Cr) and plasma membrane (PM) and nuclear membrane (NM) were observed almost normal (Figure 1). Electron micrograph of spermatozoa of 3 mg/kg Endosulfan treated group for 35 days showed degeneration in apical acrosome (AC) region with degenerated head cap (HC). Degenerative changes were clearly observed in Plasma membrane (PM) and Nuclear membrane (NM) of head region of spermatozoa consequently reduced condensation of chromatin material was observed (Figure 2). Mid piece of spermatozoa of control mice showed intact plasma membrane (PM) with distinct mitochondrial sheath which contained mitochondria (M). Outer dense fibers (ODF) were clearly observed. 9+2 arrangement of microtubules (Mt) was present with normal structure (Figure 3). Electron micrograph of spermatozoa of 3 mg/kg Endosulfan treated for 35 days showed disorientation of middle piece (MP) from neck region, plasma membrane of middle piece got ruptured, mitochondrial sheath were degenerated and dissolved mitochondria signified the level of toxicity and degeneration in the cell, massive degeneration were also observed in the 9+2 arrangement of microtubule. Electron microphotograph of principal piece from control mice showed normal structure of plasma membrane intact over principal piece, fibrous sheath, 9+2 arrangement of microtubules and divergent arm were also observed normal (Figure 5), where as treated with Endosulfan at the dose of 3 mg/kg b.w. for 35 days showed principal piece without plasma membrane which denotes complete degeneration of plasma membrane. Outer dense fibers were fused with microtubules. The divergent arms were degenerated and not visible clearly (Figure 6). However, endosulfan-treated groups receiving supplementation of aqueous extract of amla had ameliorating effect on architecture of spermatozoa compared to that of the group treated with endosulfan alone (Figure 8). Amla treated group showed ameliorating condition of head piece of spermatozoa. After treatment with amla acrosome was covered with head cap, regeneration of plasma membrane can be seen and condensation of chromatin material was restored; while mid piece of spermatozoa showed distinct mitochondrial sheath which contained mitochondria (M). 9+2 arrangement of microtubules (Mt) was present with normal structure (Figure 9). Electron microphotograph of Principal piece showed normal structure of plasma membrane intact over principal piece, fibrous sheath, 9+2 arrangement of microtubules and divergent arm were also observed normal (Figure 10).

DISCUSSION

Amla Supplements are very rich sources of Vitamin C and act as natural anti oxidants that help manage/prevent the damage done by these free radicals in the body. Acting as natural 'free radical scavengers', these supplements help to locate the free radicals in the body and inhibit the actions of the latter to a great extent. Amla Supplements contain Emblicanin, a pro-oxidation free cascading antioxidant that can be found only in these supplements. The presence of Emblicanin in them helps Amla supplements to effectively manage/prevent tissues arising out of oxidative damage in the body. Accordingly, Emblicanin prolongs its antioxidant capabilities by adapting a multilevel cascade of antioxidant compounds unlike its counterparts which just go from active to inactive state once inside the body. Emblicanin undergoes several transformations (to other antioxidants) until it is finally converted into Emblicanin oligomers. This way, the multiple antioxidants formed in the process help to fight off more free radicals from the body than a normal supplement would do. Exposure to pesticides could cause male infertility by causing a significant decrease in sperm quality and quantity[6]. Anomalies in sperm and hormonal imbalance (Testosterone) of Mus musculus due to Endosulfan exposure were studied in detailed[7]. Endosulfan was classified by the WHO in the category of technical products that are moderately hazardous and it has been shown that endosulfan has estrogenic property[8] and male rats are more sensitive to the effect of endosulfan than female rats[9]. It has been observed that after endosulfan exposure changes were hazardous and especially claims of abnormality in spermatozoa. Endosulfan is readily absorbed by the stomach, by the lungs and through skin hence all routes of exposure can pose a hazard. As a result of its world wide use, it can be a potential environmental contaminant and may cause a public health hazard[10]. Khan and Sinha reported that the endosulfan, phosphamidon and maneozeb induced various types of structural alterations in chromosomes, pairing impairment among homologous chromosome and division disruptive changes in the primary spermatocytes of mice[1]. Low level of endosulfan can inhibit the human sperm acrosome reaction initiated by progesterone and lycine[11]. Similar observation was in the present investigation that endosulfan treatment to mice at the dose of 3 mg/kg b.w. for 35 days causes leakage at the tip of acrosome and acrosome became flattered. Blunt shape of acrosome is found because of loss of acrosomal hook.

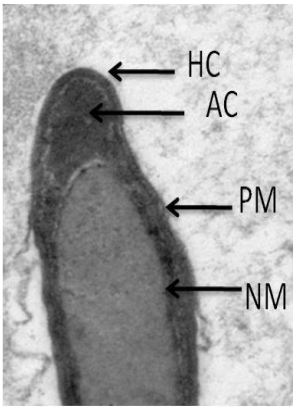

Figure 1: 36,000 X
(Electron micrograph of spermatozoa (Head region) from control mice

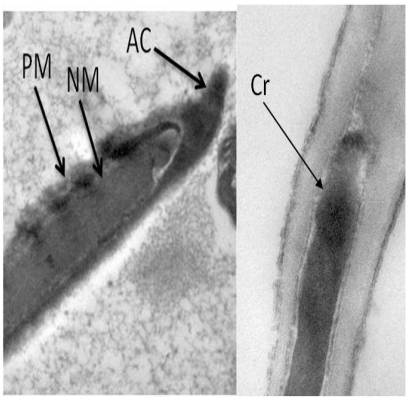

Figure 2: 28,000X Figure 3: 26,000 X
Electron micrograph of spermatozoa (Head region) treated with Endosulfan at the dose of 3 mg/kg b.w. for 35 day.

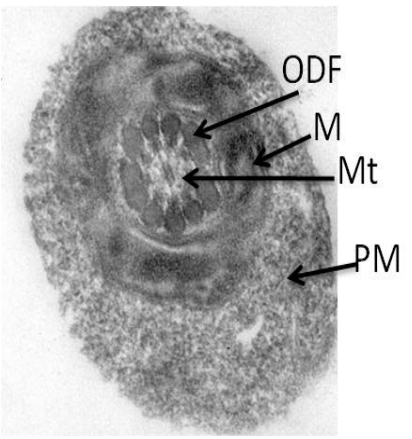

Figure 4: 54,000 X.
Electron micrograph of spermatozoa (Mid Piece) from a control mice

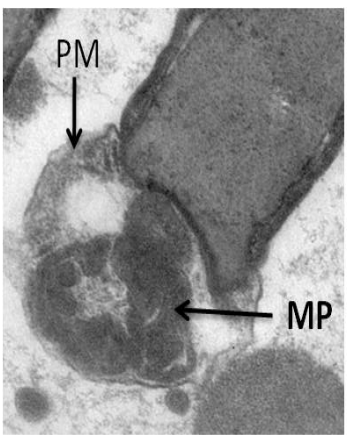

Figure 5: 40,000 X
Electron micrograph of spermatozoa (Mid Piece) treated with Endosulfan

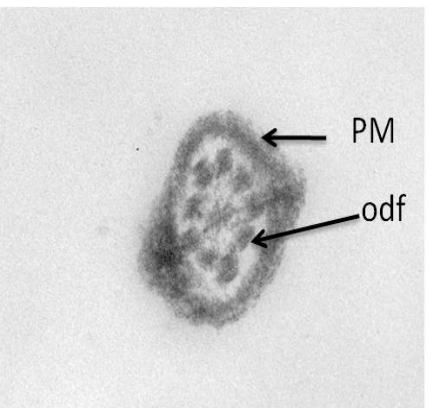

Figure 6: 58,000 X
Electron micrograph of spermatozoa (Principal Piece) from control mice at the dose of 3 mg/kg b.w.for 35 days

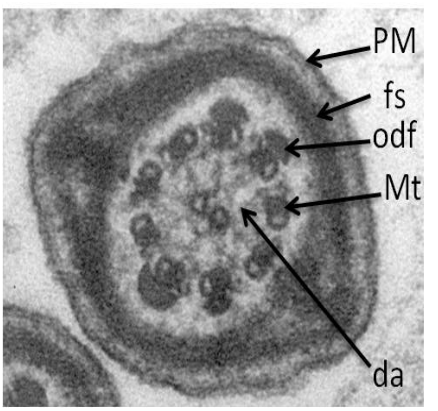

Figure 7: 42,000 X
Electron micrograph of spermatozoa (Principal Piece) treated with Endosulfan at the dose of 3 mg/kg b.w. for 35 days

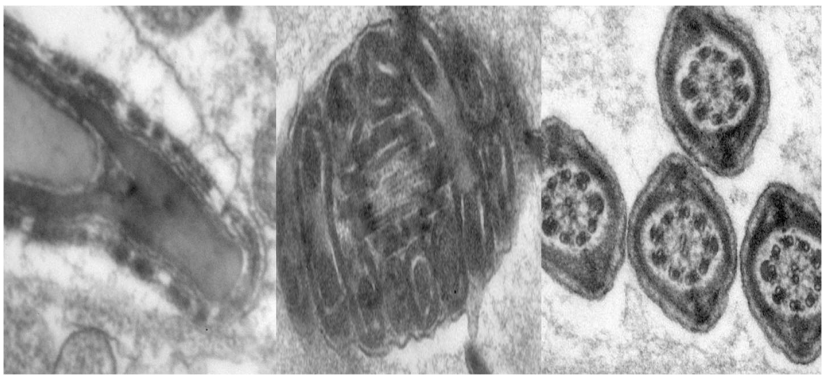

Figure 8: 36000 X Figure 9: 52,000 X Figure 10: 44, 000 X
Electron micrograph of spermatozoa (head piece, mid piece, principal piece) treated with *Emblica officinalis* (Amla) after treatment with Endosulfan

Degenerative changes were clearly observed in Plasma membrane (PM) and Nuclear membrane (NM) of head region of spermatozoa consequently reduced condensation of chromatin material was observed which is in support of work of Auger[12] that Sperm chromatin condensation is another valuable index of sperm quality that is essential for the capacity of the sperm to fertilize the ovum.

This suggests that a negative effect on chromatin condensation of the sperm could be another mechanism by which endosulfan exposure leads to reproductive toxicity. It has been reported that endosulfan causes degradation of testicular cells in laboratory animals[1,13]. Endosulfan has shown a negative effect on reproductive health in men residing in the Kasargod district of Kerala, India[14]. Endosulfan causes spermatozoa degeneration observed by Nath A. and R. Kumar[15]. Altered testicular enzyme activities, indicating spermatogenesis, were reported in mature rats treated by gavage with 2.5 mg technical endosulfan/kg/day for 70 days[16]. Similar results had been observed in my investigation that mid piece dissociated from neck region, mitochondrial sheath was degenerated and plasma membrane of middle piece get ruptured, 9+2 arrangement of microtubules were not visible. Principal piece showed without plasma membrane which denotes complete degeneration of plasma membrane. Outer dense fibers were fused with microtubules. The divergent arms were degenerated and not visible clearly. This showed the non motility of sperm activity, hence sperm cells are unable to reach to the ovum for fertilization. Similar observation has been observed by Nath and Chand[17] in fish and amphibia. Thus in the present investigation it has been observed that plasma membrane degenerated from head piece, middle piece and principal piece of sperm cells. This shows that plasma membrane of sperm cells are highly sensitive with oxidative stress produced by toxicants like endosulfan, which are in agreement with the work of Lenzi[18] that Plasma membranes of the sperms have a high content of polyunsaturated fatty acid; hence, they are highly sensitive to oxidative stress and lipid peroxidation. In the present study, Amla fruit extracts when administered to endosulfan treated mice showed very good protective effect. Use of Amla on fertility is also exclusively new work added in the field of toxicology. *Emblica officinalis* at the dose of 150 mg/kg b.w. showed restoration of acrosomal material, better effect on condensation of nuclear material; plasma membrane and nuclear membrane were almost reformed. Treatment also showed better effect on middle piece and restoration of intact plasma membrane over principal piece. Fibrous sheath was almost normal in appearance, 9+2 arrangement of microtubules were distinct, divergent arms were also distinct to some extent. Central fibrils were regenerated.

CONCLUSION

After 35 days administration of endosulfan causes severe effect not only on architecture of spermatozoa but also chromatin material in the nucleus were reduced, leaving only residue of chromatin material in the middle of nucleus. Loss of plasma membrane from entire region of sperm cells was also observed. Thus from the entire study it was observed that *Emblica officinalis* have capability to restore cellular integrity and architecture of sperm cells, therefore use of medicinal plants in the field of reproductive biology will definitely support the treatment for infertility.

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