Research Article

Abnormality in Microtubules Due to Effect of Endosulfan Toxicity Leads to Asthenozoospermia in SWISS Albino Mice.

Nath, Priyanka*, Aseem Kr. Anshu, Mona Hassan, Chandan Kumar Singh, Sachhidanand Behera and J.K. Singh.
S.S. Hospital and Research Institute, Kankarbagh, Patna-800020, India.
***Corresponding author's E-mail:** priyankapgbt@gmail.com

Accepted on: 30-08-2016; **Finalized on:** 31-10-2016.

ABSTRACT

Endosulfan is an endocrine disruptor and organochlorine pesticide, detrimental to human health. The present investigation was undertaken to observe the toxic effect of endosulfan in testis of Swiss albino mice and to find out its possible role in infertility. Endosulfan was administered as 3mg/kg body weight into Swiss albino mice for 2, 4, 6, 8, 10 and 12 weeks. After administration of endosulfan blood and tissue samples were collected and hormonal, biochemical, histopathological and electron microscopic assessments were done. Significant decreased levels of testosterone and sperm count were observed whereas MDA level was increased significantly in each successive group of endosulfan administered mice. Low sperm mortality was observed in 6 weeks, 8 weeks, 10 weeks and 12 weeks of endosulfan administered Swiss albino mice. Destruction of '9+2' arrangement of microtule of spermatozoa were observed under electron microscope (TEM) in 12 weeks of endosulfan administered Swiss albino mice. Therefore, on the basis of present study it can be concluded that endosulfan significantly reduces the testosterone level, sperm count and increases the MDA level in experimental Swiss albino mice. It also induces histopathological alterations of testicular structure at cellular and sub-cellular levels. Low sperm count, low sperm motility and destruction of '9+2' arrangement of microtubule of spermatozoa after administration of endosulfan confirms that its toxicity leads to asthenozoospermia resulting in infertility in Swiss albino mice.

Keywords: Endosulfan; Asthenozoospermia; MDA; Swiss albino mice.

INTRODUCTION

The presence of endocrine disruptor like endosulfan (ES;6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) in the environment affect targeted or non-targeted species. This organochlorine insecticide of cyclodiene group used mainly in agricultural field. Technical-grade endosulfan consists of two stereochemical isomers, α-endosulfan and β-endosulfan, in concentrations of approximately 70% and 30%, respectively[1]. Endosulfan converts into endosulfan sulfate by the process of oxidation and this metabolite is equally toxic as the parent molecule. In India, it is widely used in the Kerala where its detrimental effect on human population has been observed. Children were found with abnormalities like mental retardness, epilepsy, physical deformities, cerebral palsy etc. The public health hazard due to endosulfan also recorded in the other part of the world[2-3]. Due to its toxicity, the supreme court of India permanently banned the production, storage, sale and use of endosulfan in 2011. The growing concern of this hazardous pesticide has led to do more research on animal model. Previously the toxic effect of endosulfan has been reported on testis[4], ovary[5], liver[6], kidney[7] etc. in animal model study. Spermatogenesis and steroidogenesis are two important functions of the testes. Spermatogenesis involves conversion of undifferentiated germ cell (spermatogonia) to spermatozoa which is hormonally controlled by negative feedback loop consisting hypothalamus, pituitary and testis[8]. In mice testis, process of steroidogenesis occurs within Leydig cells. Leydig cells synthesize testosterone hormone necessary for the development of sperm within the testis. Some studies demonstrated that organochlorine pesticides play an important role in the impairment of mice testis[9-10].

The exact metabolism of endosulfan is not clear. There are two main metabolites of endosulfan exist, endosulfan sulfate and endosulfan diol, which can be further metabolites to endosulfan lactone, endosulfan hydroxyl ether and endosulfan ether. It has been reported that conversion of endosulfan sulfate from α- endosulfan mediated by the cytochromes CYP2B6 and CYP3A4 (Casabar et at., 2006). Studies based on endosulfan metabolism showed that in animal, endosulfan can be converted into other lipophilic compounds which further easily accumulated in tissue and exert toxic effects (ATSDR, 2015).

The high concentration of endosulfan has been reported in infertile men rather than the fertile men (Çok et al 2010). Endosulfan causes infertility by inhibiting testicular androgen biosynthesis etc. (Singh & Panday, 1990). Approximately 90% of the infertility among male occur due to azoospermia, oligozoospermia, asthenozoospermia and teratopemia (Huynh et al., 2002). The mid piece of spermatozoa normally contains nine outer dense fibres (ODFs) present outer side of nine doublets and forms 9+9+2 structure (Eddy, 2006).

In the year 2010, production of endosulfan in India was around 10,500 tonnes in the states Gujarat, Kerala and Maharashtra (Risk Management Evalution, 2010).

The purpose of the present study was to investigate the toxic effect of endosulan on testis of Swiss albino mice and to find out its possible role in infertility.

METHODOLOGY

In the present study, to observe male reproductive disorder; endosulfan has been chosen as a toxic agent. It was administered to Swiss albino mice by oral gavage method as 3.0 mg/kg body weight/day.

Animals

Swiss albino mice (Mus musculus) were reared in the animal house of S.S. Hospital and Research Institute, Patna. 12 weeks old mice weighed 30±2 grams were selected for experiments. The mice were kept in the polypropylene cages with paddy husk at room temperature 28±2ºC and humidity 50±5% in a controlled light (12 hrs light and 12 hrs dark). Animals were maintained in ideal conditions as per the ethical guidelines of the CPCSEA, (CPCSEA Regd. No. 1840/PO/ReBi/S/5/CPCSEA) Govt. of India and Institutional Animal Ethics Committee (IAEC).

Study Groups and Sample Collection

In the beginning of the present study, a total of 42 Swiss albino mice were selected and grouped in four as; Group A and Group B for control, and for endosulfan treatment. Further, Group B was subdivided into 6 groups on the basis of administration of endosulfan into Swiss albino mice for 2,4,6,8,10 and 12 weeks.

After endosulfan administrations, blood samples of Swiss albino mice were collected by orbital sinus puncture method. Blood samples of each mice group were incubated for 30 min. and then serum samples were separated by the process of centrifugation at 3000rpm for 15 minutes. These serum samples were kept at 40C for further biochemical and hormonal analysis. Testosterone level was assessed by ELISA method. The quantitative assessment of malondialdehyde (MDA) has also been done by U. V. Spectrophotometer.

After sacrificing mice at each successive month's cauda epididymis were collected and Sperm count at each interval were recorded.

Measurement of Testosterone Level

The quantitative measurement of testosterone level in the serum samples of all groups of endosulfan administered Swiss albino mice was determined by using testosterone ELISA kit of LILAC Medicare (P) ltd.

Assessment of Malondialdehyde (MDA) Level

The quantitative measurement of malondialdehyde (MDA) level in the serum samples of all groups of endosulfan and arsenic administered Swiss albino mice was determined by standard TBRs method with slight modification.

Epididymal Sperm Count

The epididymis was removed and washed in isotonic saline. Semen was collected by puncturing epididymis at several places in 1ml distilled water and then sperm count was performed with help of Neubauer's chamber and light microscope.

Assessment of Sperm Motility

Sperm motility was determined as per the protocol described by Huang et al (2003) and Benoff et al (2008).

Histopathological Study

For histopathological study by light microscope of endosulfan administered mice, tissue samples were processed and stained by routine procedure (H&E stain) and slides were observed under light microscope.

Electron Microscopic Study

For transmission electron, microscopic (TEM) study, tissue samples (6 weeks and 12 weeks) were fixed in 2.5% gluteraldehyde and were sent to All India Institute of Medical Sciences (AIIMS), New Delhi.

Statistical Analysis

Mean ± SD were calculated for the present study. p- value was calculated by using one way ANOVA.

RESULTS

Significant decrease in testosterone hormone ($p \leq 0.0001$) and sperm count ($p \leq 0.0001$) was observed in endosulfan administered male Swiss albino mice at 3.0 mg/kg b.wt. whereas increased level of MDA was observed after the administration of same pesticides as the dose of endosulfan administration duration increases. Table –I shows the testosterone, MDA level and sperm count of endosulfan administered Swiss albino mice for the duration of 2, 4, 6, 8, 10 and 12 weeks.

Sperm motility was found less than 12%, of the control group of mice than the 2 weeks 4 weeks, 6 weeks, 8 weeks and 10 weeks whereas it was found less than 50% in 12 weeks of endosulfan administered mice ($p \leq 0.05$).

Histopathological observation of testis of control Swiss albino mice shows normal process of sperm formation (spermatogenesis) (Plate – I, Figure: 3A). After 2 weeks of endosulfan administration, increased intracellular spaces between seminiferous tubules has been

observed at some places and normal spermatogenesis was observed. Architecture of seminiferous tubules was almost near to the normal (Plate – I, Figure: 3B). Deformed architecture of seminiferous tubules was observed after 4 weeks of endosulfan administered Swiss albino mice (Plate – I, Figure: 3C). Thin wall of seminiferous tubules were observed and reduced number of sperms were also observed in 6 weeks endosulfan administered mice (Plate – I, Figure: 3D). In seminiferous tubules of testes of Swiss albino mice, process of sperm production i.e. spermatogenesis occurs. This process of

spermatogenesis was arrested and diminished in most of the seminiferous tubules after 8, 10 and 12 weeks of endosulfan administered mice (Plate – I, Figure: 3E, F and G respectively).

Plate II shows the electron microscopic study of tail portion of spermatozoa of normal, 6 weeks and 12 weeks of endosulfan administered Swiss albino mice. The normal structure of spermatozoa shows intact plasma membrane and nine sets of doublet microtubule forming a ring like structure, 'central pair' of single microtubule occur at its centre ('9 + 2' arrangement) (Plate II, Fig: H).

Plate II fig I shows the cross section of mid piece of spermatozoa showing intact plasma membrane although thickening of outer circle of microtubules was observed after 6 weeks' administration of endosulfan. All flagella show a normal 9+2 axoneme structure. Among them, one flagellum shows 9+2 axoneme, all complement of outer dense fibers (ODFs) and very few mitochondria. Dilated intraspace in of mitochondria have been observed. These mitochondria were not organized regularly in the mid piece of flagella. The magnified view of fig E, plate II shows the enlargement of space between ODFs and ribs of Fibrous Sheath (Plate II, fig. J).

Table 1: Testosterone, MDA level and sperm count of endosulfan administered Swiss albino mice for the different durations.

	Testosterone level (ng/ml) (a)	MDA Level (nMol/ml) (b)	Sperm Count (Million/µl) (c)
	Mean± SD	Mean± SD	Mean± SD
Group A (Control) (n= 6)	4.91 ± 1.033	1.81 ± 0.176	3.66±0.150
2 weeks, (n= 6)	4.85 ± 0.306	21.16 ± 1.956	3.35±0.271
4 weeks, (n= 6)	3.95 ± 0.123	39.48 ±5.688	3.66± 0.167
6 weeks, (n= 6)	2.75 ± 0.117	47.46± 2.656	2.94 ±0.116
8 weeks, (n= 6)	2.61 ± 0.191	53.22± 4.071	2.45±0.195
10 weeks, (n= 6)	1.99 ±0.237	58.69 ± 2.303	1.98±0.753
12 weeks, (n= 6)	1.89 ± 0.141	69.49±2.406	1.75±0.453

p-Value for (a), (b) and (c) = P ≤ 0.0001

DISCUSSION

In present study, toxic effects of endosulfan on testis of Swiss albino mice were observed. After the administration of endosulfan, level of testosteron and sperm count have been found significantly decreased where as high MDA level was observed with each increased duration of endosulfan administration. Histopathological alteratios in testicular tissues were observed in endosulfan administered mice. It was previously reported by Nath et al., (2007). Histopathological alteration in testicular tissue has also neen reported by Nath et al (2015) after the administration of endosulfan into Swiss albino mice.

Interestingly, at the sub-cellular level, mitochondrial disorganization and intraspace formation in the mid piece of spermatozoa have been observed which indicates endosulfan toxicity. The defects observed in tail portion of spermatozoa by electron microscopic study may lead to asthenozoospermia, a prominent cause of male infertility in Swiss albino mice. Tektins proteins in the mid piece of tail portion of spermatozoa have been found as normal whereas some researchers indicate abnormal tektins protins can cause infertility (Tanaka et al., 2004). Genotoxicity caused by endosulfan has been reported by some researchers which include chromosomal breakage, sister chromatid exchange, sex-linked recessive mutations, etc. (Dulout et al, 1985; Naqvi and Vaishnavi, 1993 & Pandey et al, 1990) Taken together, administration of endosulfan in Swiss albino mice induces low testosterone level, low sperm count, high MDA level and pathophysiological changes, which may lead to infertility in male mice. Recently, similar results have been observed by Sebastian and Raghavan (2015). Infertility due to endosulfan toxicity has been reported in fish, birds and mammals also (Leatherland, 1992; and Reijnders, 1986).

Some researchers indicated that oxidative stress is associated with significant DNA damage including sperm DNA fragmentation (Subramani and Chaudhary, 2010).

The mechanism behind male infertility caused by endosulfan toxicity suggest that it is an androgen receptor antagonist (Vishwanath, 2010) and able to bind the ligand binding site of androgen receptor and reduces the androgen receptor positive Sertoli cells (Sebastian and Raghavan 2015).

Endosulfan has been classified as a potent endocrine-disrupting chemical. Male reproductive system and function may be susceptible to this endocrine disruptor. Although some countries banned few endocrine disruptors, their residues or derivatives have widely been detected in the environmental and biological samples. Endosulfan has been shown to produce ROS (Reactive

Oxygen Species), due to which imbalance between ROS and total antioxidant capacity (TAC) occur. This imbalance produces high oxidative stress which affects male reproductive system. Therefore, present study indicated the adverse effect of endosulfan on testis of Swiss albino mice which induces asthenozoospermia probably by reducing the sperm motility and destructing the '9+2' arrangement of microtubules of spermatozoa.

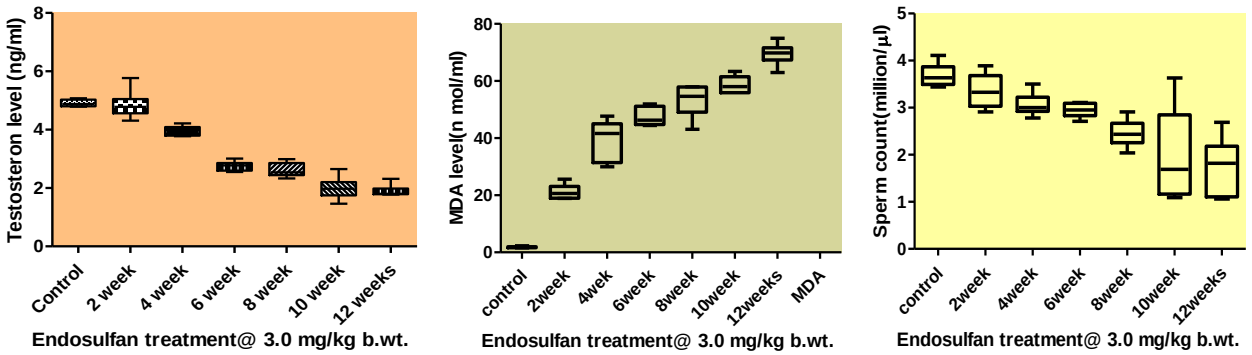


Figure1: Showing testosterone level (A), MDA level (B)and sperm count (C) in control and endosulfan treated male mice for 2, 4, 6, 8, 10, and 12 weeks.

Plate- I

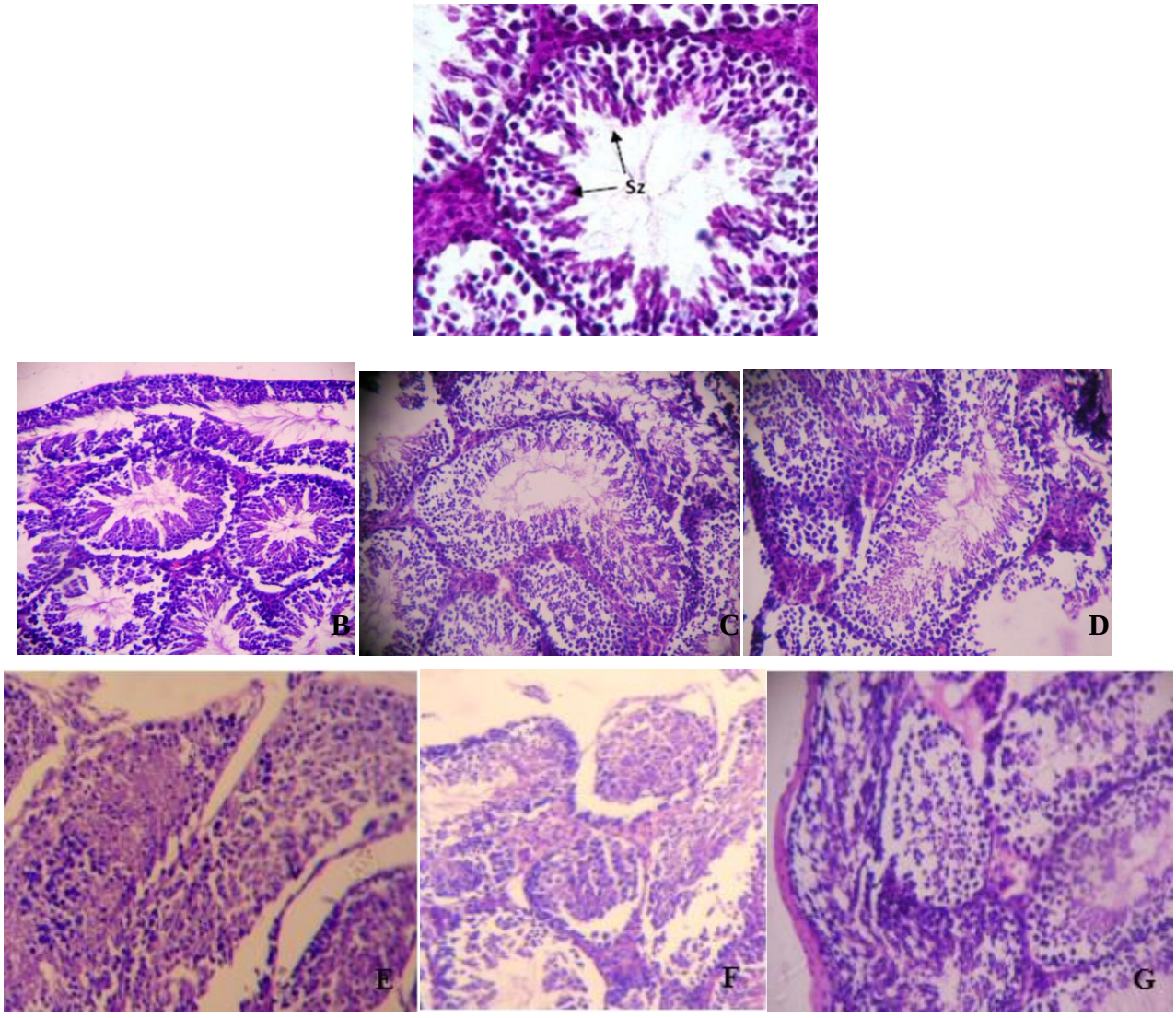
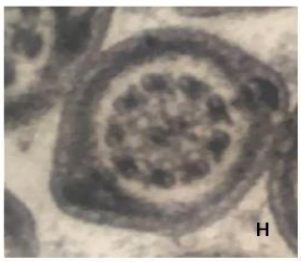
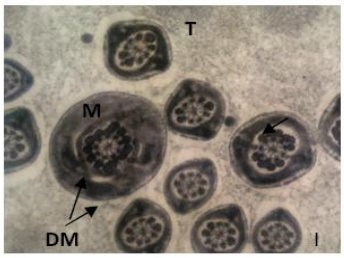


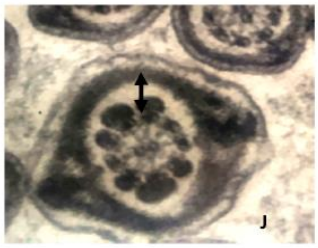
Figure 2: Showing control (fig: A, ×500) and endosulfan administered Swiss albino mice for 2 weeks, (fig: B, ×150), 4 weeks, (fig:C, ×200), 6 weeks, (D, ×200), 8 weeks, (fig:E, ×200), 10 weeks, (fig:F, ×150), and 12 weeks, (fig:G, ×200) weeks.



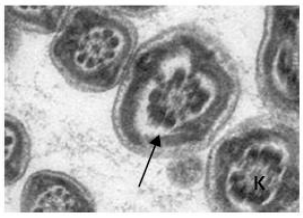
Cross Section (C.S.) of spermatozoa of control mice showing '9+2' arrangement of microtubule. X 42,000



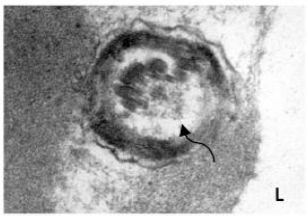
C.S. through Middle piece (M) & Tail piece (T) of spermatozoa (sz) after administration of endosulfan for 6 weeks. Note thickening of the outer circle of few microtubules (↑) and dilated mitochondria (DM) X 32,000



C.S. of magnified portion of a part of fig (D) after administration of endosulfan for 6 weeks X 42,000



C.S. through tail piece (T) of spermatozoa (sz) after administration of endosulfan for 12 weeks. Note the irregular shape of few microtubules (↑) X 40,000



C.S. through tail piece (T) of spermatozoa (sz) after administration of endosulfan for 12 weeks. Note the destruction of '9+2' arrangement of microtubule. (↑) X 40,000

Plate- II: Transmission Electron Microphotograph of Spermatozoa

CONCLUSION

It is evident from the present study that endosulfan significantly reduces the testosterone level, sperm count and significantly increases the MDA level in Swiss albino mice. It also induces histopathological alterations of testicular tissue at cellular and sub-cellular levels.

Low sperm count, low sperm motility and destruction of '9+2' arrangement of microtubule of spermatozoa after administration of endosulfan confirms that its toxicity leads to asthenozoospermia resulting in infertility in Swiss albino mice.

Acknowledgement: Authors are grateful to ICMR, New Delhi for the financial support provided for this research work. We are also thankful to all members and technical staffs of S.S.Hospital and Research Institute who helped to compile this work.

We would like to acknowledge Prof M. Rajalakshmi for the contribution towards interpretation of Electron Microphotographs. We also extend our thanks to all staff members of AIIMS, New Delhi, India for EM facility.

REFERENCES

1. Saiyed H, Dewan A, Bhatnagar V, Shenoy U, Shenoy R, Rajmohan H, Patel K, Kashyap R, Kulkarni P, Rajan B, Lakkad, B. Effect of Endosulfan on Male Reproductive Development. Environmental Health Perspectives, 111 (16), 2003, 1958-62.
2. Jia H, Li YF, Wang D, Cai D, Yang M, Ma J, Hu J. Endosulfan in China 1—gridded usage inventories. Environ Sci Pollut Res, 16, 2009, 295–301.
3. Siddique T, Okeke BC, Arshad M, Frankenberger WT. Biodegradation kinetics of endosulfan by Fusarium ventricosum and a Pandoraea species, J Agric Food Chem, 51, 2009, 8015–9
4. Singh JK, Nath A, Kumar A, Md. Ali, Kumar R. Study on the Effect of Endosulfan on Testosterone Level and Seminiferous Tubule of Testis of Mice. World Journal of Environmental Pollution, 1 (1), 2011, 01-04.
5. Hiremath MB, Kaliwa BB. Effect of endosulfan on ovarian compensatory hypertrophy in hemicastrated albino mice. Reproductive Toxicology, 16, 2011, 783–790.
6. Haloi K, Kalita M, Nath R, Borkotoki A, A Study on Histopathological Impact of Endosulfan on Liver of Channa punctatus Bloch., International Journal of Research in Zoology, 4(2), 2014,19-23.
7. Khan S, Study of histopathological changes induced by endosulfan in kidney of albino rats, Journal of Drug Delivery & Therapeutics; 4(3), 2014, 125-128.Çok I, Durmaz TC, Durmaz E, Satıroglu MH, Kabukcu C. Determination of organochlorine pesticide and polychlorinated biphenyl levels in adipose tissue of infertile men. Environ Monit Assess,162, 2010, 301–309.

8. Sujatha R, Chitra KC, Latchoumycandane C, Mathur PP. Effect of lindane on testicular antioxidant system and steroidogenic enzymes in adult rats. Asian J Androl,3, 2001, 135–8.

9. Chitra KC, Sujatha R, Latchoumycandane C, Mathur PP. Effect of lindane on antioxidant enzymes in epididymis and epididymal sperm of adult rats. Asian J Androl, 3, 2001, 205–8.

10. Casabar RC, Wallace AD, Hodgson E, Rose RL (2006) Metabolism of endosulfan-alpha by human liver microsomes and its utility as a simultaneous in vitro probe for CYP2B6 and CYP3A4. Drug Metab Dispos 34, 1779–1785

11. Cok I, Durmaz TC, Durmaz E, et al. 2010. Determination of organochlorine pesticide and polychlorinated biphenyl levels in adipose tissue of infertile men. Environ Monit Assess 162(1-4), 301 309.

12. Singh SK and Pandey RS. Effect of sub-chronic endosulfan exposures on plasma gonadotrophins, testosterone, testicular testosterone and enzymes of androgen biosynthesis in rat. Indian Journal of Experimental Biology, 28(10), 1990, 953-956.

13. Huynh T, Mollard R, Trounson A. Selected genetic factors associated with male infertility. Hum Reprod Update,8, 2002,183–198.

14. Eddy E M, The spermatozoon. In *Knobil and Neill's Physiology of Reproduction* (Neill, J. D., ed) vol. 1, 2006, pp. 3–54, Elsevier Academic, New York, New York, USA. Mathur P Pand D'Cruz SC, The effect of environmental contaminants on testicular function, Asian Journal of Andrology,13, 2011, 585–591.

15. Risk Management Evaluation Endosulfan, Long version – UNECE Context, Prepared by BiPRO GmbH, Germany, Mai 2010.

16. Huang HFS, Ming-Tang L, Wang S, Wang G, Ottenweller JE. Spinal cord contusion impairs sperm motility in the rat without disrupting spermatogenesis. J Androl, 24, 2003, 371-380.

17. Benoff S, Auborn K, Marmar JE, Hurley R, link between low-dose environmentally relevant cadmium exposure and asthenozoospermia in a rat model, Fertl Steril, 89 (2 Suppl), 2008, e73-e79. Doi: 10.1016/j.fertnstert.12, 2007,35.

18. Nath. A. Effect of sublethal dose of Endosulfan induced degenerative changes in *Musmusculus*with antioxidants as ameliorating agents. J. Ecophysiol. Occup. Hlth, 7, 2007,69-77.

19. Nath A, Priyanka, Kumari K, Anshu AK, Singh A, Kumar S, Anand P and Sinha P, Effect of Arsenic Toxicity in Testis of Swiss albino mice leading to low testosterone and high MDA level. Int. J. Pharm. Sci. Rev. Res., 30(2), 2015, 69-73.

20. Tanaka H, Iguchi N, Toyama Y, Kitamura K, Takahashi T, Kaseda K, Maekawa M, Nishimune Y. Mice deficient in the axonemal protein Tektin-t exhibit male infertility and immotile-cilium syndrome due to impaired inner arm dynein function. Mol Cell Biol,24, 2004, 7958–7964.

21. Dulout FN, Pastori MC, Olivero OA, González Cid M, Loria D, et al., Sister-chromatid exchanges and chromosomal aberrations in a population exposed to pesticides. Mutat Res, 143, 1985, 237-244.

22. Naqvi SM, Vaishnavi C, Bioaccumulative potential and toxicity of endosulfan insecticide to non-target animals, Comp Biochem Physiol C, 105, 1993 347-361

23. Pandey N, Gundevia F, Prem AS, Ray PK, Studies on the genotoxicity of endosulfan, an organochlorine insecticide, in mammalian germ cells. Mutat Res, 242, 1990 1-7.

24. Sebastian R, Raghavan SC. Endosulfan induces male infertility, News and Commentary. Cell Death and Disease,6, 2015; e2022; doi:10.1038/cddis.2015.368;

25. Leather l and J. Endocrine and reproductive function in Great Lakes Salmon In: Chemically induced alterations in sexual and functional development: the wildlife/human connection (Colborn T, Clement C, eds). Princeton, NJ:Princeton Scientific Publishing, 1992,129-145.

26. Reijnders PJH. Reproductive failure in common seals feeding on fish from polluted coastal waters. Nature, 324, 1986, 456-457.

27. Subramani E, Chaudhary K. Detrimental Effects of Oxidative Stress on Sperm Membrane and DNS Integrity in Normozoospermic Infertile Men. Proceeding of 2010 International Conference on Systems in Medicines and Biology, IIT Kharagpur, India, 2010, 198-202.

28. Viswanath G, Chatterjee S, Dabral S, Nanguneri SR, Divya G, Roy P. Anti-androgenic endocrine disrupting activities of chloropyrofos and piperophos, J Steroid Biochem Mol Biol, 122, 2010, 22.

29. Sebastian R, Raghavan SC. Exposure of endosulfan can result in male infertility due to testicular atrophy and reduced sperm count. Cell Death Discovery, 1, 2015, 1-10

30. Toxicological profile for endosulfan, U.S. Department of health and human services Public Health Service. Agency for Toxic Substances and Disease Registry, 2015.

Source of Support: Nil, **Conflict of Interest:** None.