



Testicular Dysfunction in Male Rat Following Endosulfan Exposure

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ABSTRACT

Endosulfan is a chlorinated hydrocarbon insecticide and used for insect control. The poison could enter the body through respiration, absorption via skin and ingestion in human beings, or grazing by farm animals. It became a highly controversial agricultural chemical due to its acute toxicity, potential for bioaccumulation, and role as an endocrine disruptor. Because of its threats to human health and the environment, a global ban on the manufacture and use of endosulfan was negotiated under the Stockholm Convention in April 2011. Proven fertile male rats were divided in three groups of 10 animals each. Control animals Group-I received only the vehicle (groundnut oil), by gavage whereas the animals of Groups II and III received endosulfan (dissolved in groundnut oil) 10 mg/kg b.wt/d for 30 and 60 days. Sperm motility, density, fertility, testicular cell population dynamics, and hormonal analysis was performed to see the adverse effect of endosulfan on testes in male rat. In the present study a significant reduction in the weights of testes and accessory sex organs were noticed. A severe impairment of sperm motility and density in testes and a sharp decline in fertility and testosterone hormone were also observed. The number per testes of non types Bsg spermatogonia and spermatocytes were similar to controls where as that of type Bcg spermatogonia increased by 59% and that of early round spermatids (Est) and late elongated spermatids decreased by 37% and 72% respectively. It seems that endosulfan has an undeniably damaging effect on the testis accompanied by its unfavorable effects on the reproductive system which may lead to infertility due to the changes in sex hormones concentration, sperm density and motility. Inhibition of testicular spermatogenesis by endosulfan treatment associated with decreased seminiferous tubular diameter, volume and accessory sex organ weight may indicate adverse effects of endosulfan on testes which again lead to infertility in male rat.

Keywords: Endosulfan, Testes, Sperm dynamics, cell population dynamics.

INTRODUCTION

Endosulfan is a pesticide belonging to the organochlorine group of pesticides, under the Cyclodiene subgroup. It was introduced in the 1950's and it emerged as a leading chemical used against a broad spectrum of insects and mites in agriculture and allied sectors. It is used in vegetables, fruits, paddy, cotton, cashew, tea, coffee, tobacco and timber crops. It is also used as a wood preservative and to control tse-tse flies and termites. This pesticide is classified as a Highly Hazardous chemical by the US Environmental Protection Agency (EPA) and the European Union, as a Persistent Toxic Substance by the United Nations Environmental Programme (UNEP), as a Category II - Moderately Hazardous chemical by the World Health Organization (WHO), and as Extremely Hazardous chemical by the Industrial Toxicological Research Centre (ITRC) in India¹⁻³.

However, India is the largest producer, consumer and exporter of Endosulfan. In rats and mice studies suggests its teratogenic and carcinogenic properties⁴. It directly affects the central nervous system, causes liver and kidney (chronic glomerulonephrosis) damage⁵. It also impairs the reproductive system⁶. Behavioural and neurological changes have also been noticed⁷. In humans also Congenital Birth defects, reproductive health problems, Cancers, loss of immunity, neurological and mental diseases were reported⁸⁻¹¹. Little information is

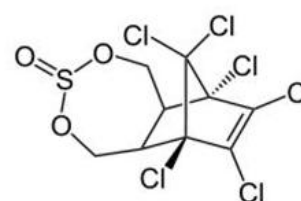
available on specific effects of endosulfan on germ cells of testes hence the present study is aimed to find out the mechanism and effect of endosulfan on sperm dynamics, testicular cell dynamics, sex hormone and fertility in mating trials.

MATERIALS AND METHODS

Animal Model

Healthy and fertile Albino rats (*Wistar*) weighing 150-200 gms, 100 days old were used for experimentation. The animals were housed in polypropylene cages, measuring 430 x 270 x 150 mm. The animals were fed on pelleted standard rat chow supplemented with soaked grams and wheat. Water provided *ad-libitum*. They were acclimatized for 7 days to the laboratory conditions at 22-24°C with provision 12 h light: 12h dark cycle. The "guidelines for the care and use of animals for scientific research" was strictly followed¹².

Test Material



Endosulfan

Chemical Name: (6,7,8,9,10,10- hexa chloro-1,5,5a,6,9,9a- hexahydro – 6,9- methano- 2,4,3- benzodioxathiepine-3-oxide)

Chemical Formula : C₉H₆Cl₆O₃S

Chemical Structure: Endosulfan

Trade name: Agrosulfan, Banagesulfan, Cyclodan, Endocel, Endoson, Endonit, Endomil, Endosol, Endostar, Endodaf, Endosulfer, E-sulfan, Endorifan, Hildan, Redsun, Seosulfan, and Thiodan.

Technical endosulfan (α and β isomers in the ratio of 70:30) obtained from Hoechst, Mumbai (India) was used for the experimentation. It is brownish crystals with slight odour of sulphur dioxide and endosulfan sulfate.

Median lethal dose (LD₅₀) of Endosulfan

The LD₅₀ is statistically derived single dose of a substance that can be expected to cause death in 50% of the animals. In this investigation various calculated doses (mg/kg.b.wt.) of insecticide was given orally. Ten animals were tested for each dose level. Poisoning symptoms and mortality was observed daily for three days following the treatment. Results of the toxicity was analyzed statistically¹³ for the determination of LD₅₀ of the endosulfan.

Experimental Procedure

Proven fertile male rats were divided in three groups of 10 animals each. Control animals Group-I received only the vehicle (ground nut oil), by gavage whereas the animals of Groups II and III received endosulfan (dissolved in groundnut oil) 10 mg/kg b.wt/d for 30 and 60 days. The animals were weighed and autopsied under light ether anesthesia. Blood was collected from heart in preheparinized tubes. The plasma was separated from the blood by centrifugation at 3000 rpm and stored at – 20oC. Testosterone concentration was measured by radioimmunoassay.

Two parallel slices perpendicular to the testicular long axis were cut from each testis and a half or a quarter of each slices was cut of embedding After dehydration in absolute ethanol and butanol the tissue blocks (Slices) were embedded in hydroxyl ethyl methacrylate resin. According to manufactures instruction One 25 micro um thick section were cut from each block (Average section area : $24 \pm 2 \text{ mm}^2$ for testicular section) and stained with periodic acid schiffs reagent and hematoxylin.

Spermdynamics

(i) Sperm motility: Sperm motility was assayed by the method of¹⁴. The epididymis was removed immediately after anaesthesia and known weight of cauda epididymis was gently teased in a specific volume of physiological saline (0.9% NaCl) to release the spermatozoa from the tubules. The sperm suspension was examined within five minutes after their isolation from epididymis. The results were determined by counting both motile and immotile sperms in at least ten separate and randomly selected

fields. The results were finally expressed as percent motility.

(ii) Sperm density: Sperm density was assayed by the previous published method¹⁴. Briefly total number of sperms were counted using haematocytometer after further diluted the sperm suspension from cauda epididymis and testes. The sperm density was calculated in million/ ml as per the dilution.

(iii) Fertility Test: The mating exposure test of all the animals was performed. They were cohabited with proestrous females in the ratio of 1: 3. The vaginal plug and presence of sperms in the vaginal smear was checked for positive mating. The mated females were separated to note the implantation sites on day 16th of pregnancy.

(iv) Cell number tubule volume: The number of all types of nuclei in the testes were estimated with optical dissector. Briefly the section was observed using X 100 oil lens on computer monitor. The numerical density (no. per volume) of the nuclei in the organ was estimated by dividing the total number of nuclei counted by the total volume of "dissectors" (3D counting spaces inside section) and then the total number of per organ was calculated by multiplying the density by the volume of the organ. The volume fraction of the seminiferous tubule in the testes was conventionally estimated by point counting during the nuclear counting process and the total tubule volume per organ was calculated by multiply the fraction by the volume of the organ.

Hormonal analysis

Serum Testosterone was performed¹⁵ by radioimmunoassay in which monoclonal/Antibody, Isotope IgG2 was used. The commercially available kit was purchased from My Bio Source Llc San Diego,USA. Digital computer was used for the calculation

Statistical analysis

Data were tested for normal distribution and then analyzed by analysis of variance (ANOVA) and the significance of difference was set up at ($P \leq 0.001$).

RESULTS

The present observations obtained after oral administration of endosulfan showed significant reduction in the weight and volume of testes and sex accessory organs. The spermatozoal motility in cauda epididymis and spermatozoal density in cauda epididymis and testes were significantly decreased. A sharp decline in fertility (80% negative) and serum testosterone was also observed. See graph 1-4

Testicular volume was decreased by approximately 20% and 34%. Similarly tubule volume per testis was reduced significantly by approximately 15% and 30%. (Table-1) At 30 days treatment not marked changes were observed but at 60 days treatment more obvious changes observed included (i) looser arrangement of spermatogenic cells (ii) fewer elongated spermatids in the seminiferous

epithelium (iii) more detached (sloughed) round spermatids and spermatocytes in the tubule lumen and (iv) severe spermatogenic damage in which few spermatid were left in Seminiferous epithelium and many pachytene spermatocytes were detached and increased in type B spermatogonia (table-2). There was no significant effect on the number of sertoli cells or non Type-B spermatogonia were observed. The number of probable leydig cells at day 30 and 60 after endosulfan treatment was less than 8% and 10% of the leydig cell number in control.

DISCUSSION

Spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cells, a site of steroid biosynthesis may contribute to the decline of testes weight^{16, 17}. The reduction in weight of accessory sex organs may be due to reduced bio-availability of estrogenic and/or antiandrogenic activities of endosulfan.¹⁸ Sperm motility is considered one of the most important parameters evaluating the sperm fertilizing ability. Different chemical agents also compromised the mobile capacity of sperm and reduced fertility of animals^{19, 20}.

The decreased motility of sperm in cauda epididymis indicates less ability of sperm to interact with the oocyte

plasma membrane. Low caudal epididymal sperm density may be due to alteration in androgen metabolism. The 80% negative fertility test attributed to lack of forward progression and reduction in density of spermatozoa and altered biochemical milieu of cauda epididymis²¹. Testosterone is the principal androgen of the testes and it is essential for sperm production and maintenance. There may be two mechanisms by which insecticides could reduce circulating levels of testosterone; first by enhancing its degradation, excretion or tissue uptake or second by depressing circulating LH levels and thereby reducing LH dependent testicular steroidogenesis²². It is well established that organochlorine pesticides reduce acetylcholinesterase activity and block nerve impulses²³. This effect may alter the release of pituitary hormones, namely FSH and LH, leading to the reduction of sperm production in the testes^{24, 25}.

The morphometric data revealed that appreciable damage to spermatogenesis in the testis occurred by endosulfan treatment which was primarily spermiation failure i.e. retaining of late elongated spermatids in the seminiferous epithelium and sloughing of spermatogenic cells into the epididymal duct^{26, 27}.

Table 1: Volumes and morphometric results of the seminiferous tubules (Mean \pm SEM)

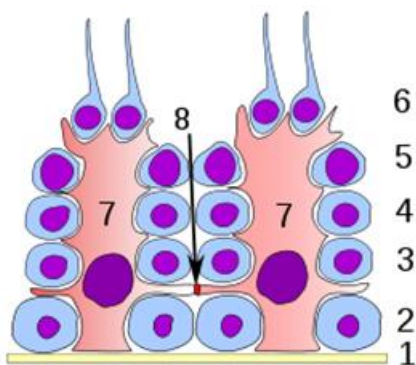
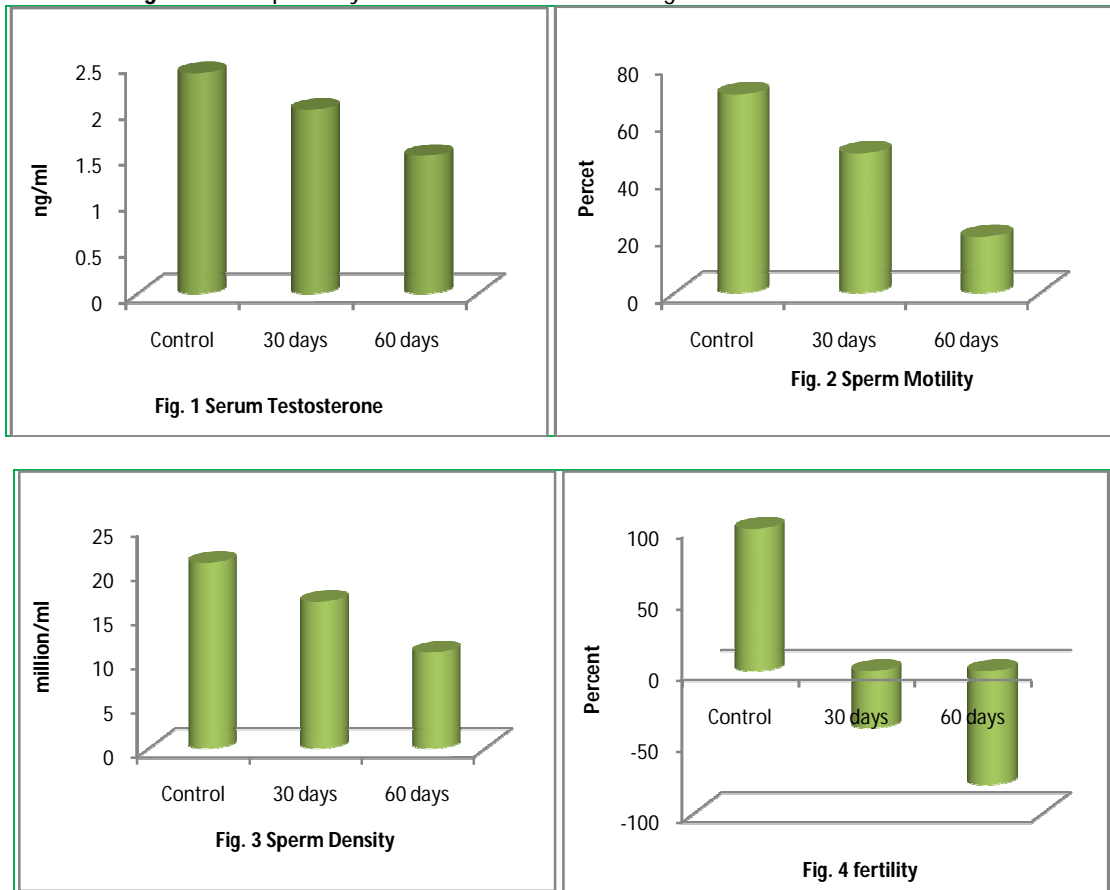
Parameters	Control	Endosulfan 10mg/kgb.wt./day (30 days)	Endosulfan 10mg/kgb/wt/day (60 days)
Volume Cm ³	1.29 (\pm 0.04)	1.12 (\pm 0.06)	0.80 (\pm 0.08)
Volume fraction of duct (%)	80.4 (\pm 0.9)	84.9 (\pm 0.7)	8.60 (\pm 0.7)
Total tubule volume Cm ³	1.01 (\pm 0.03)	0.95 (\pm 0.05)	0.62 (\pm 0.07)
Tubule diameter (μ m)	305 (\pm 5.0)	286 (\pm 6.0)	254 (\pm 7.0)
Total tubule length (μ m)	14.5 (\pm 0.6)	14.1 (\pm 0.08)	11.6 (\pm 1.00)

Table 2: Testicular cell population dynamics in the rats following endosulfan exposure. Number (10^6) of the cells (nucluei) count per Testis (Mean \pm SEM)

	Control	Endosulfan 10mg/kgb.wt./day (30 days)	Endosulfan 10mg/kgb/wt/day (60 days)
Fibroblast like cell	48.50 (\pm 3.10)	50.10 (\pm 7.10)	55.20 (\pm 8.00)
Immature leydig cells	45.00 (\pm 2.80)	18.10 (\pm 3.50)	15.20 (\pm 4.20)
Mature leydig cells	44.00 (\pm 2.90)	20.20 (\pm 3.60)	18.20 (\pm 4.10)
Degenerating cells	62.24 (\pm 7.50)	112.00 (\pm 18.20)	120.00 (\pm 19.60)
Sertoli cells	36.7 (\pm 21)	40.2 (\pm 3.6)	30.2 (\pm 3.2)
Non Bsg	7.85 (\pm 0.55)	7.85 (\pm 0.44)	7.20 (\pm 0.8)
Bsg	3.75 (\pm 0.23)	5.58 (\pm 0.38)	6.24 (\pm 0.59)
Esc	42.6 (\pm 4.0)	52.7 (\pm 2.4)	50 (\pm 2.0)
Sc	82.4 (\pm 2.2)	76.2 (\pm 2.1)	70.2 (\pm 6.3)
Ssc	3.0 (\pm 0.9)	4.0 (\pm 1.2)	2.0 (\pm 1.0)
Est	220 (\pm 12)	240 (\pm 20)	140 (\pm 21)
Mst	78 (\pm 6)	50 (\pm 8)	20 (\pm 6)
Lst	160 (\pm 12)	164 (\pm 20)	90 (\pm 22)

Bsg: B Type Spermatogonia, Esc: Early primary spermatocytes, Est: Early round spermatids, Lst: Late elongated spermatids, Mst: Middle stage elongating spermatids, Non Bsg: Non type B spermatogonia, Sc: middle and late stage primary spermatocytes, Ssc: Secondary spermatocytes

Figures 1-4: Sperm dynamical and hormonal changes after Endosulfan treatment



1. Basal lamina
2. Spermatogonia
3. Spermatocyte 1st order
4. Spermatocyte 2nd
5. Spermatid
6. Mature Spermatid
7. Sertoli Cell
8. Tight Junction (Blood Testis Barrier)

Figure 5: Germinal epithelium of the testicle

At 60 days detachment of spermatocytes and spermatids become evident between the germ cells. It could also be concluded, if spermatocytes and spermatids were detached from the seminiferous epithelium in proportions, that key spermatogenic lesion also included impairment in meiosis (development of spermatids from spermatocytes) and spermiogenesis (transformation of elongated spermatids from round spermatids) as indicated by significantly smaller numerical ratios between round spermatid and spermatocytes and between elongated spermatids and round spermatids²⁸.

Degenerations (Pycnotic nuclei) or apoptosis of spermatocytes and spermatids as observed in the study indicated impairment in meiosis and spermiogenesis²⁹. These changes should be testosterone deficiency

specific²⁷. Despite impairment in spermiogenesis and spermatogenesis following endosulfan treatment, the no of type-B spermatogonia was increased. This might be the effects of increased levels since LH is known to act on Leydig cells and has no direct effect on spermatogenesis.³⁰⁻³²

Thus this study revealed that administration of endosulfan results in testicular injury. The underlying mechanism that causes endosulfan-induced damage may be due to disruption of the BTB in which the seminiferous epithelium consists of Sertoli cells and germ cells at different stages. Their development is overlying the tunica propria, which is composed of the non-cellular zone (Basement membrane type-1 collagen layer) and the cellular zone (peritubular myoid cell layer and the lymphatic vessel). The BTB is created by adjacent Sertoli cells near the basement

membrane divides the seminiferous epithelium into the basal and the adluminal compartment. The BTB is constituted by coexisting tight junction desmosome like junction and basal ectoplasmic specialization (basal ES which is the testes specific actin based a typical adherens junction types) which is one of the primary target of endosulfan toxicity in the testes³³.

CONCLUSION

Endosulfan induces damages to the testis which is the result of interaction of complex network. These findings have explained the disruption of BTB via stress activated P38 MAPK pathway which is the possible mechanism by which testicular injury occurred in the form of changes in sex hormones concentration, sperm density and motility. Inhibition of testicular spermatogenesis decreased seminiferous tubular diameter, volume and accessory sex organ weight which leads to infertility in male rat.

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