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Endosulphan Induced Changes in Fine Morphology of Goat Spermatogonia *in vitro*

R.K. Sharma, P.K. Chauhan and A. Fulia
Reproductive Physiology Laboratory, Department of Zoology,
Kurukshetra University, Kurukshetra-136119, India

Abstract: The aim of the present study is to analyze the effect of different doses of endosulphan on spermatogonia of *Capra hircus in vitro*. Small pieces (approximately 1 mm^3) of testicular tissue were divided into two groups (one experimental group and one control group). Experimental group was treated with different concentrations of endosulphan (1×10^{-3}, 1.0 and 100 nmol mL^{-1}) and was further divided into three subgroups (1), (2) and (3) exposed for 1, 4 and 8 h, respectively. The endosulphan exposure revealed increasing morphological alterations with increasing dose of endosulphan in spermatogonia. At dose level 1×10^{-3} nmol mL^{-1} of endosulphan, light microscopic changes in spermatogonia were characterized by condensed nuclei which were darkly stained with haematoxylene eosin. Hyalinization and chromolysis, vacuolization in spermatogonia were clearly observed. Vacuolization in spermatogonia were also enhanced as the exposure duration extended. Endosulphan at concentration level 1×10^{-3} nmol mL^{-1} induced decline in spermatogonial diameter from 7.245±0.201 in control to 6.75±0.231 μm after 1 h, 6.5± 0.2115 μm after 4 h and 6.45±0.2233 μm after exposure duration of 8 h. At the same dose level of endosulphan atretic spermatogonia were 34, 44 and 52% after exposure duration of 1, 4 and 8 h, respectively. Due to increase in the endosulphan concentration upto 100 nmol mL^{-1}, atretogenic changes induced in spermatogonial cells were characterized by increased fragmentation and pycnosis of nucleus. Ultrastructurally, chromolysis, vacuolization, hyalinization, condensation of nucleus, apoptotic vacuoles and shrinkage of cytoplasm was generally observed. These changes were prominent at higher dose level and extended exposure duration.

Key words: Histopathology, pesticide, testis, germ cells, *Capra hircus*

INTRODUCTION

The wide spread use of pesticides in public health and agriculture has caused severe environmental pollution and health hazards including cases of severe, sub chronic and chronic human poisoning (Ellenhorn *et al.,* 1997; Abdollahi *et al.,* 1997; Pajoumand *et al.*, 2002). These pesticides have been recorded to affect non-target organisms including human beings (Cantelli-Forti *et al.,* 1993; Chaudhuri *et al.*, 1999). The ability of endosulphan to affect the functioning of various endocrine systems is responsible for a wide variety of the

Corresponding Author: R.K. Sharma, Reproductive Physiology Laboratory, Department of Zoology, Kurukshetra University, Kurukshetra-136119, India

health hazards (Chapin *et al.*, 1996; Mohd, 2001). In recent years, there have been growing concerns about the toxicity of a number of pesticides on the reproductive system. There are pronounced structural defects in histological examinations of ovarian tissues in rats which were administered endosulfan and malathion (Koc *et al.*, 2009). A series of research papers and reports indicated that the male reproductive system in humans and certain wildlife species have been seriously affected in recent decades. Reported effects included reduced sperm production, improper development of the penis, cryptorchidism, testicular tumors, etc. are described in a report commissioned by the Danish Environmental Protection Agency (Toppari *et al.*, 1995). Endosulphan introduced in the 1950's, it emerged as a leading chemical which is used against a broad spectrum of insects and mites in agriculture and allied sectors (Cooper and Kavlock, 1997). Studies on pesticides levels in blood samples of school children showed that there were detectable levels of lindane, diazinone, heptachlor and chlorpyrifos. About 7% of the samples analysed were found to be contaminated with pesticides in trace levels (Mohd, 2001). Kurutas *et al.* (2006) observed the toxic effects of short and long term exposure to endosulphan pesticide on mice. Even though endosulphan exposure had no significant effect on total body weight of mice ($p>0.05$), it increased significantly the weight of liver and hepato/somatic index (HSI: liver weight/body weight) (Kurutas *et al.*, 2006).

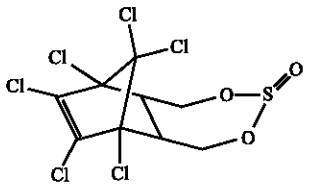
Keeping in view the effect of endocrine disruptors on reproduction, the present study on the effect of endosulphan (endocrine disruptors) on spermatogonia of goat (*Capra hircus*) *in vitro* has been analyzed to assess the damage of reproductive potential in domestic animals.

MATERIALS AND METHODS

The mature goat (*Capra hircus*) testis were procured from the slaughter houses around Kurukshetra (29°6' N, 76°5' E) and Delhi (28°38'N, 77°12'E). The material was brought to the Reproductive Physiology Laboratory, Department of Zoology, Kurukshetra University Kurukshetra at 4°C in normal saline during the year 2009.

Testicular Tissue Culture

After washing with the normal saline the testis was decapsulated and cut into small pieces (approximately 1 mm^3) for culture. After washing three times with TCM-199, small pieces of testicular tissue were immediately placed on nucleopore filter and floated on medium. The medium was prepared by mixing TCM-199 and antibiotics (200 unit penicillin 100 1 U mL^{-1} and streptomycin 100 g mL^{-1}). The tissues were divided into two groups (one experimental group and one control group). Experimental group was treated with different concentrations of endosulphan (1×10^{-3}, 1.0 and 100 nmol mL^{-1}) (Structure 1) and was further divided into three subgroups (1), (2) and (3) exposed for 1, 4 and 8 h, respectively at temperature 39°C, humidity 95 and 5% CO_2 concentration and the control was run simultaneously.



Structure 1:

Endosulphan

- **IUPAC Name:** 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide
- **Chemical formula:** $C_9H_6Cl_6O_3S$
- **Molecular mass:** 406.95

Harvesting of testicular tissues was carried out after the specified durations.

Histomorphology

Tissue from all the groups were processed for the histomorphological studies. Paraffin embedded tissue from all experimental and control was cut at 5 μm thickness and after dewaxing in xylene, the sections were passed through decreasing grades of alcohol and stained with haematoxylene. After that the sections were gradually dehydrated up to the 70% alcohol and stained with eosin, after further dehydration up to absolute alcohol the sections were cleared with clearing agent (xylene) and finally mounted with DPX (Pearse, 1968).

Transmission Electron Microscopy

Simultaneously harvested testicular tissue was also fixed for transmission electron microscopic studies in 2.5% glutaraldehyde in 0.2 M phosphate buffer saline (pH 7.2 to 7.4) at 4°C for 24 h. The trimming of samples to appropriate size was done in fixative. The samples were then post-fixed in 1.3% osmium tetraoxide for 2 h at 4°C. The fixed material was dehydrated through a graded series of acetone, the sample were cleared with epoxy propane and embedded in epoxy resin. Before proceeding to ultrathin sectioning, the blocks were thick sectioned (1 μm) serially and successive groups of five serial thick sections were examined under phase contrast microscope to select the required portion. The blocks were finally trimmed and the serial 60-90 nm sections were cut with glass razors. The thin sections were stained with uranyl aetate followed by lead citrate (Hertig and Adams, 1967). The sections were examined and photographed under electron microscope model installed CM-10 Philips at All India Institute of Medical Sciences, New Delhi.

RESULTS

Histomorphology

Histologically, the testis was comprised of seminiferous tubules and interstitium. In the control group, a large number of seminiferous tubules were packed in the loose connective tissue. Seminiferous tubule of sexually mature goat was characterized by the presence of a series of well defined cellular associations and stages. These orderly sequences of different type of cells give rise to the cycle of the seminiferous epithelium and were divided into the stages on the basis of relative positions of germ cells within the seminiferous tubule in control (Fig. 1a). Spermatogonia are the small sized cells present in the basal part of the seminiferous epithelium and were characterized by large nucleus lying in direct contact with basal lamina. Nucleus was spherical to elongate in shape. Spermatogonia were also in direct contact with Sertoli cells through the cytoplasmic extensions (Fig. 1a).

The endosulphan exposure (1×10^{-3}, 1.0 and 100 nmol mL^{-1}) revealed increasing morphological alterations in spermatogonia. At dose level 1×10^{-3} nmol mL^{-1} endosulphan induced atretic changes in spermatogonia, characterized by condensed nuclei which were darkly stained with haematoxylene eosin. Hyalinization and chromolysis in spermatogonia were clearly observed. Fragmented nucleus was observed in spermatogonia when exposure duration increased from 1 to 8 h (Fig. 1b). Vacuolization in spermatogonia were also enhanced as the exposure duration increased.

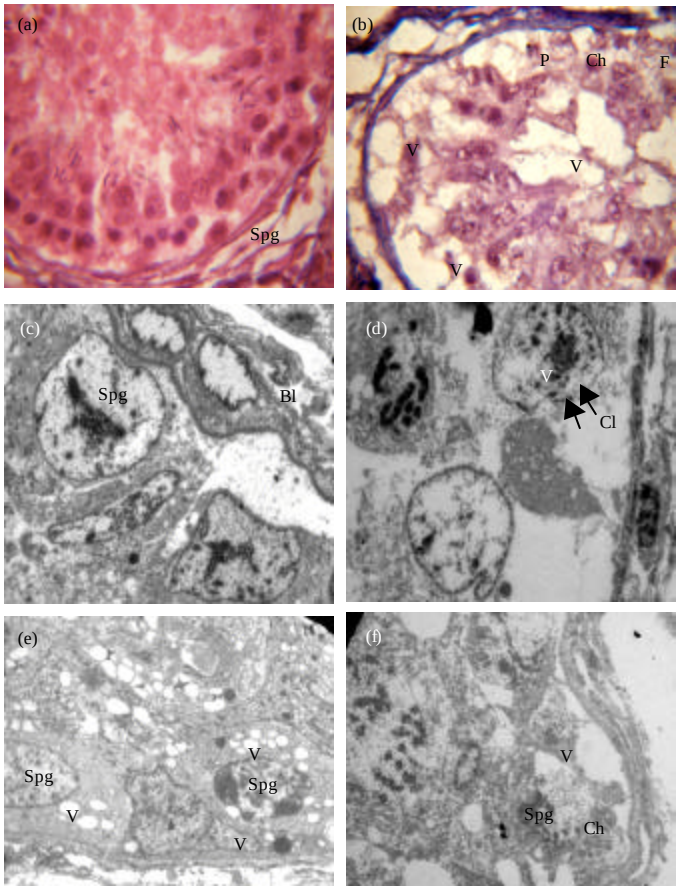


Fig. 1: (a) Light micrograph of control testicular tissue exhibiting normal arrangement of different types of germ cells (spermatogonia, spermatocytes, spermatid, sperms) and somatic cells (Sertoli cells) in sexually mature goat characterized by the presence of well defined cellular associations and stages (X1000), (b) microphotograph of testicular tissue treated with endosulphan (1×10^{3} nmol mL^{-1}) for 8 h showing large number of pycnotic nuclei (P), chromolysis (Ch), fragmented nuclei (F) in spermatogonia, large sized vacuoles (V) (intra nuclear and intra cytoplasmic) (X 1000), (c) fine micrograph of control testicular tissue showing spermatogonium (Spg) covered by the limiting membrane/basal lamina (Bl) which further consisted of different layers. Inside the nucleus, homogeneous chromatin material was evenly distributed within the nucleoplasm (X3600), (d) transmission electron micrograph of testicular tissue treated with endosulphan (1.0 nmol mL^{-1}) for 4 h showing large sized vacuoles (V) in the cytoplasm of spermatogonia (Spg). Degree of cytoplasmic degeneration triggered. Clumps of chromatin material (Cl) were also observed (X3500), (e) ultrastructure of testicular tissue treated with endosulphan (1.0 nmol mL^{-1}) for 4 h vacuoles (V) in the cytoplasm of spermatogonia (Spg). Degree of cytoplasmic degeneration enhanced (X 3500) and (f) fine micrograph of testicular tissue culture treated with endsulphan (1.0 nmol mL^{-1}) for 8 h exhibiting necrotic spermatogonium with ruptured nuclear membrane (arrow). Chromolysis (Ch) in nuclear material was clearly visible. Swollen and disrupted mitochondria were observed. Vacuoles (V) of various shapes and sizes were noticed. Pycnotic nuclear body was seen after 8 h culture (X3500)

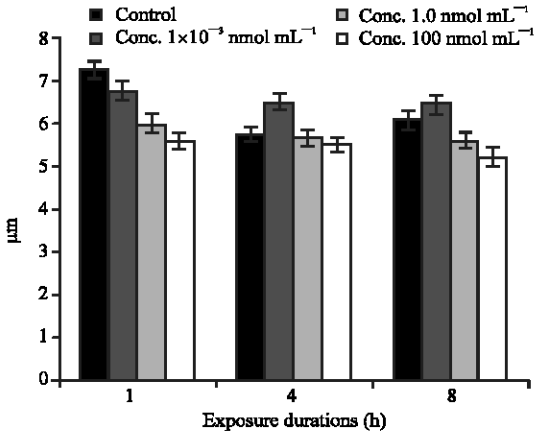


Fig. 2: Effect of different concentrations of endosulphan on diameter of spermatogonia after varying exposure durations

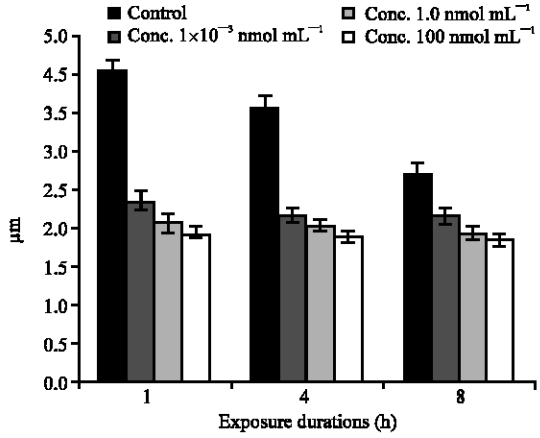


Fig. 3: Effect of endosulphan on nuclear diameter of spermatogonia after different exposure durations

Endosulphan concentration 1×10^{-3} nmol mL^{-1} induced decline in spermatogonia diameter from 7.245±0.201 in control to 6.75±0.231 µm after 1 h, 6.5± 0.2115 µm at 4 h and 6.45±0.2233 µm after exposure duration of 8 h. The variations recorded at 4 and 8 h were statistically significant ($p\leq0.05$) (Fig. 2). At same dose level, endosulphan also induced a decline in nuclear diameter from 4.5±0.147 in control to 2.60±0.152 µm after 1 h, 2.4±0.1141 µm after 4 h and 2.4±0.1124 µm after exposure of 8 h. All the variations recorded were statistically significant ($p\leq0.05$) (Fig. 3). At advanced stage of atresia (1a), a decline in nuclear diameter of spermatogonia from 3.5±0.1701 in control to 2.55±0.1141 µm after 1 h, 2.9±0.1235 µm after 4 h and 2.5±0.1526 µm after 8 h of exposure duration were noticed. The values recorded at 1 and 8 h were statistically significant ($p\leq0.05$). At the same dose level of endosulphan atretic cell after exposure duration of 1, 4 and 8 h were 34, 44 and 52% and Chi-square values were also recorded (Table 1).

Table 1: The comparison of a number of atretic spermatogonia between control (1, 4, 8 h) and different concentrations of endosulphan (1×10^{-3}, 1.0 and 100 nmol mL^{-1}) showing Chi-square values

Parameters	Concentration (1×10^{-3} nmol mL^{-1})	Concentration (1.0 nmol mL^{-1})	Concentration (100nmol mL^{-1})
1 h	1.2142	3.663*	19.4847*
4 h	2.1021	7.9545*	21.2364*
8 h	2.5974	10.256*	24.00*

*Statistically significant difference from control ($p\leq0.05$)

Further enhancement of endosulphan concentration to 1.0 nmol mL^{-1}, condensation, pycnosis and nucleus fragmentation were enhanced. Vacuolization and chromolysis were also increased as the concentration and exposure duration increased. Endosulphan dose level 1.0 nmol mL^{-1} induced characteristic changes in cell diameter. As the exposure duration was increased, a further decline in cell diameter of spermatogonia was noticed from 7.245±0.201 in control to 6.00±0.205 μm after 1 h, 5.65±0.1957 μm after 4 h and 5.6±0.1686 μm after 8 h of exposure of endosulphan (Fig. 2). The variations recorded at 8 h were statistically significant ($p\leq0.05$). Endosulphan exposure with dose level 1.0 nmol mL^{-1} also induced a decline in spermatogonial nuclear diameter from 4.5±0.147 in control to 2.30±0.111 μm after 1 h, 2.25±0.09934 μm after 4 h and 2.15±0.0895 μm after 8 h of exposure duration (Fig. 3). All the variations recorded were statistically significant ($p\leq0.05$).

At advanced stage of atresia (1a), a decline in nuclear diameter of spermatogonia from 3.5±0.1701 in control to 2.00±0.1357 μm after 1 h, 1.85±0.1410 μm after 4 h and 1.82±0.1163 μm were recorded after exposure duration of 8 hours and all these values were statistically significant ($p\leq0.05$). Endosulphan treatments at dose level 1.0 nmol mL^{-1}, atretic cells after exposure duration of 1, 4 and 8 h were 42, 58 and 68%, respectively. Chi-square values at 1, 4 and 8 h were 3.663, 7.9545 and 10.256, respectively and all the variations recorded were statistically significant (χ^2 0.05) (Table 1).

Due to increase in the endosulphan concentration upto 100 nmol mL^{-1}, atretogenic changes induced in spermatogonial cells were characterized by increased fragmentation and pycnosis of nucleus. Spermatogonia revealed lobed nuclei and vacuolated cytoplasm. Shrinkage of nucleus was also increased as the exposure duration was extended up to 8 h. The morphometric variations revealed a decline in cell dimensions while frequency of atretic cells increased with increasing endosulphan dose and exposure durations (Fig. 1b).

As dose level increased upto 100 nmol mL^{-1}, a further decline in cell diameter from 7.245±0.201 in control to 5.60±0.1974 μm after 1 h, 5.5±0.1581 μm after 4 h and 5.2±0.2096 μm after exposure duration of 8 h of endosulphan were recorded. All the variations recorded were statistically significant ($p\leq0.05$) (Fig. 2). Endosulphan at dose level 100 nmol mL^{-1} also induced further decline in diameter of spermatogonial nucleus from 4.5±0.147 in control to 2.15±0.0895 after 1 h, 2.10±0.08182 μm after 4 h and 2.05±0.09361 μm after exposure of 8 h. All the variations recorded were statistically significant ($p\leq0.05$) (Fig. 3). At advanced stage of atresia (1a), a decline in nuclear diameter was noticed from 3.5±0.1701 in control to 1.4±0.1064 after 1 h, 1.10±0.03699 μm after 4 h and 1.10±0.06399 μm after exposure duration of 8 h. After endosulphan exposure at dose level 100 nmol mL^{-1}, atretic cells at 1, 4 and 8 h were 68, 76 and 84%, respectively. Chi-square values revealed that all the variations were statistically significant (χ^2 0.05) (Table 1).

Transmission Electron Microscopic Study

Testicular germ cells were characterized by their large spherical nucleus manifesting finely granular nucleoplasm in case of type A spermatogonium. The spermatogonia were laid on the basal lamina of seminiferous tubule and were in direct contact with the Sertoli cells through cytoplasmic extension. In the nucleoplasm moderately electron dense granules were

forming small clumps near the nuclear membrane of spermatogonium in control group (Fig. 1c). Due to the endosulphan exposure at dose level 1.0 nmol mL^{-1}, chromolysis were clearly observed in the spermatogonia. Intranuclear vesicle was also noticed in spermatogonium. Nuclear membrane was disrupted at certain places. Ruptured organelles were evident near the nucleus of spermatogonia. Intracytoplasmic vacuoles were observed under electron microscope. These necrotic changes tended to gradually increase in time dependant manner. At certain places, complete chromolysis was seen. Detachment of spermatogonium from basal lamina was observed. Necrotic spermatogonium showed swollen, disrupted mitochondria. Pycnotic nuclear body was observed in the spermatogonium after 8 hours of exposure duration. Exposure of endosulphan resulted in uneven distribution of the cytoplasm within the spermatogonium. As the exposure duration increased, intercellular association was disrupted (Fig. 1d-f).

DISCUSSION

During the present investigation, *in vitro* evaluation of different doses of endosulphan on mature goat (*Capra hircus*) testis was studied. Three dose levels, 1×10^{-3}, 1.0, 100 nmol mL^{-1} of endosulphan were used for 1, 4 and 8 h of exposure *in vitro* on the goat testicular tissue. The results of the experimental study revealed that endosulphan affected the testicular histomorphology and induced severe degeneration in spermatogonia. The present investigation revealed that the exposure of endosulphan to the testicular tissue at dose levels (1×10^{-3}, 1.0, 100 nmol mL^{-1}) caused the germ cells degeneration. Pycnotic nuclei, chromolysis, hyalinization, nuclear fragmentation and vacuolization were observed in most of the degenerating spermatogonia. The associations in spermatogonia and somatic cells were disrupted due to the exposure of endosulphan. Basal lamina became uneven, irregular and was distorted at few places. Extensive sloughing of spermatogonia was evident. All the degenerative changes increased in time and dose dependent manner. Similar set of pathological effects of pesticides on the reproductive system of experimental animals have already been recorded (Salem and Abd-Elghaffar, 1998; Okamura *et al*., 2005; Presibella *et al*., 2005; Assayed *et al*., 2008). The testicular tissue of rats treated with Cypermethrin insecticide induced degeneration and desquamation of spermatocyte series and spermatids giant cells into the lumen of seminiferous tubules and spermatogonial cells exhibited pycnotic nuclei and necrobiotic changes in their cytoplasm (Assayed *et al*., 2008). Any toxic insults to testes can result in a multiplicity of effects. Since the testis is compartmentalized into spermatogenic (seminiferous tubules) and steroidogenic (Leydig cells, interstitial) components, such effects can occur individually or in combination (Salem and Abd-Elghaffar, 1998).

The endosulphan induced changes in nuclear material resulting in chromosome condensation, chromolysis, nuclear fragmentation and pycnosis. These observations are in consistent with the earlier findings of Nehez *et al.* (1982) that pesticide induced alteration in nuclear material. In a preliminary study by Nehez *et al*. (1982), it has been recorded that two pesticides with two nitro radicals, the dinitro-*o*-cresol-containing pesticide (Krezonit E) and the trifluralin-containing (Olitref), produced chromosomal aberrations after a single intraperitoneal dose of 20 and 2000 mg kg^{-1}, respectively. It is also evident from present histological study that endosulphan induced vacuolization, chromolysis, nuclear fragmentation are in agreement with the findings of Dunnick *et al.* (1984), who have observed that DMMP (Dimethyl methylphosphonate) induced similar histopathological changes in the testes of the animals exposed with high-dose. These changes were characterized by blockade of spermatogenesis and enhanced degeneration, vacuolization and necrosis of cells in the

spermatogenic tubules. The present study showed that atretic cell percentage enhanced with the increase in exposure duration and also increased as the dose of endosulphan enhanced. At 1×10^{-3} nmol mL^{-1} dose level of endosulphan, the atretic cell percentage after exposure duration of 1, 4 and 8 h were 34, 44 and 52%. After endosulphan exposure at dose level 100 nmol mL^{-1}, atretic cell percentage at 1, 4 and 8 h increased to 68, 76 and 84%, respectively. These findings are in agreement with the findings of Bustos-Obregon and Hartley (2008) in which the effect of the agro pesticide, parathion upon apoptosis in mouse seminiferous tubules were analyzed in young mice and in adult animals (Bustos-Obregon and Hartley, 2008).

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