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Protective Effect of Ascorbic Acid against Endosulphan Induced Testicular Toxicity in Goat *in vitro*

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Abstract: During the present investigation effect of vitamin C in rescue of degenerating changes induced by endosulphan have been observed. Small pieces of testicular tissue (approximately 1 mm^3) were divided into three groups (One control group + two experimental groups). Experimental group (A) was treated with 100 nmol mL^{-1} endosulphan concentration and experimental group (B) was supplemented with 100 nmol mL^{-1} endosulphan and 1000 μmol L^{-1} concentration of vitamin C (Ascorbic acid) and harvesting was carried out after 1, 4 and 8 h of exposure duration. Control was run simultaneously along with all the experimental groups. In the experimental group (A) treated with endosulphan there was alteration in histoarchitecture of seminiferous tubule. Endosulphan exposure after 1 h induced elevation in atretic spermatocytes from 22% in control testicular tissue to 54%, from 28 to 68% after 4 h and from 32 to 74% after 8 h of exposure duration. Testicular tissue exposed with endosulphan and supplemented with Vitamin C at dose level 1000 μmol L^{-1} in experimental group (B) restore the normal structure of the seminiferous tubule because of its scavenging property to scavenge the free radicals produced due to the oxidative stress. Due to the supplementation of vitamin C along with the exposure of endosulphan to the testicular tissue, atretic spermatocytes were declined from 54% in endosulphan exposed tissue [experimental group (A)], to 32% in vitamin C supplemented group [experimental group (B)] after 1 h, from 68 to 42% after 4 h and from 74 to 54% after exposure duration of 8 h.

Key words: Pesticide, endosulphan, ascorbic acid, testis, germ cells, goat, *Capra hircus*

INTRODUCTION

In the last 10 years, attention has focused on the global presence of endocrine-disrupting contaminants in the environment (Sonnenschein and Soto, 1998; Cooper *et al*., 1999). Endocrine disruptors such as vinclozolin, procymidone, linuron, p,p'DDE (1,1,1-dichloro-2,2-bis(p-chlorophenyl)ethane) may induce toxicity either by inhibiting (an antagonist) the action of hormones, enhance (an agonist), or others (phthalate esters) by reducing androgen synthesis, but it is likely that other modes of action are also involved in the toxicity induced by these xenobiotics (Kelce *et al*., 1997; Gray *et al*., 2001). Till date, approximately 60 chemicals have been identified as endocrine disruptors. Chemicals with hormonal activity fall into three broad classes: (1) synthetic chemicals used in industry, agriculture and consumer products, (2) synthetic chemicals used as pharmaceutical drugs and (3) natural chemicals found in human and animal food (phytoestrogens) (Pocar *et al*., 2003). Pesticides have unique status of all food residues because of their use in agricultural fields to meet worldwide food demands. Among all pesticides, organochlorine compounds are mostly persistent in the environment (Biswas *et al*., 2010). Presence of pesticides is more or less ubiquitous in the environment surrounding us and exposure to the same is more or less inevitable. On one hand they are necessary for mankind; on the other hand their exposure has produced a number of adverse effects including immunotoxicity (Gleichmann *et al*., 1989; Banerjee *et al*., 1996; Banerjee, 1999; Galloway and Handy, 2003; Pant *et al*., 2004; Wang *et al*., 2006). Pesticides may induce oxidative stress, leading to the generation of free radicals and alteration in antioxidants, oxygen free radicals, the scavenging enzyme system and lipid peroxidation (Banerjee *et al*., 1999; Kalender *et al*., 2004). However, in many cases the reactive non-radical species will end up as radicals, damaging biomolecules by oxidation. This type of reaction may be self-sustained leading to extensive cellular damage (Halliwell and Gutteridge, 1995). A few toxicological studies have addressed the possible relationship between reproductive toxicity and exposure to the chemicals that generate Reactive Oxygen Species (ROS)

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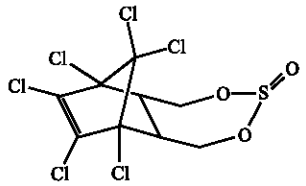
(Latchoumycandane *et al*., 2002). Roughly half of infertility cases are caused by male infertility (Sharlip *et al*., 2002). Endosulphan (6,7,8,9, 10-hexachloro-1,5,5a,6, 9,9a-hexahydro,6-9-methano 2,3,4, benzodioxathiepin-3-oxide) is a member of the cyclodiene group of organochlorine insecticide used worldwide in agriculture and is absorbed by both humans and animals through the intestinal tract, lungs and skin (Vale *et al*., 2003). Endosulphan induced alterations in male fertility. Endosulphan exposure resulted in degenerative changes in fine morphology of goat spermatogonia (Sharma *et al*., 2010). The antioxidant vitamins are the most important free radical scavengers in the extra-cellular fluids, trapping radicals in the aqueous phase and protect biomembranes from peroxidative damage (Chan, 1993; Yavuz *et al*., 2004). These vitamins also counteract the testicular oxidative stress induced by exposure to pro-oxidants such as arsenic, PCBs (Arochlor, 1254), cadmium, endosulphan and alcohol (Kumar *et al*., 2004; Maneesh *et al*., 2005; Rao *et al*., 2005; Chang *et al*., 2007; Gupta *et al*., 2004). In normal circumstances, there is an equilibrium between the generation of ROS and antioxidant strategies of the male reproductive tract, leaving only a critical amount of ROS required for normal sperm functions (Griveau and Le Lannou, 1997). Administration of Vitamin C with lead exposed animals exerts an obvious ameliorating as well as treatment effects (Raafat *et al*., 2009). Keeping in view the effect of pesticides on male reproduction, the present study was undertaken to find out the effect of antioxidant (Vitamin C) in preventing the hazardous effect induced by endosulphan in goat (*Capra hircus*) *in vitro*.

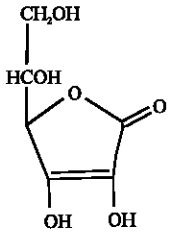
MATERIALS AND METHODS

Testis of mature goat (*Capra hircus*) were procured from slaughter houses around Kurukshetra (29°6′N, 76°50′E), Haryana, India. The material was brought to the Reproductive Physiology Laboratory, Department of Zoology, Kurukshetra University Kurukshetra at 4°C in normal saline during year 2009.

After decapsulation, the testis was cut into small pieces (approximately 1 mm^3) for culture.



ENDOSULPHAN (Structure-I)
Molecular formula: $C_9H_6Cl_6O_3S$
Molecular mass: 406.95 g moL^{-1}



ASCORBIC ACID (Structure-II)
Molecular formula: $C_6 H_8 O_6$
Molecular mass: 176.13 g moL^{-1}

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 1U mL^{-1} and streptomycin 100 g mL^{-1}). The tissue was divided into three groups (1 control group + 2 experimental groups). Experimental group (A) was supplemented with 100 nmol mL^{-1} concentration of endosulphan (Structure-I) and experimental group (B) was supplemented with 100 nmol mL^{-1} endosulphan and 1000 μmol L^{-1} concentration of vitamin C (Ascorbic acid) (Structure-II). The culture petri plates were kept at temperature 39°C, humidity 95 and 5% CO_2 concentration in an asceptic oven.

Control was run simultaneously along with all the experimental groups. Harvesting was carried out after 1, 4 and 8 h of exposure duration. Tissues 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).

RESULTS

During the present investigation ascorbic acid (1000 μmol L^{-1}) exerts an ameliorating effect on endosulphan (100 nmol mL^{-1}) induced testicular toxicity in *Capra hircus in vitro*. Histomorphological analysis of testicular sections revealed normal structure of seminiferous tubule with well organized arrangement of germ cells and somatic cells in control group (Fig. 1). In the experimental group (A) treated with endosulphan the seminiferous tubules were more or less normal in outline but atretogenic changes were observed after 1 h of

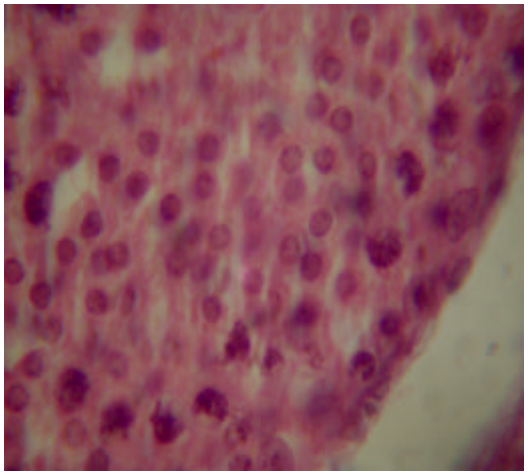


Fig. 1: Microphotograph of testicular sections showing normal structure of seminiferous tubule with well organized arrangement of germ cells and somatic cells in control (X 1000)

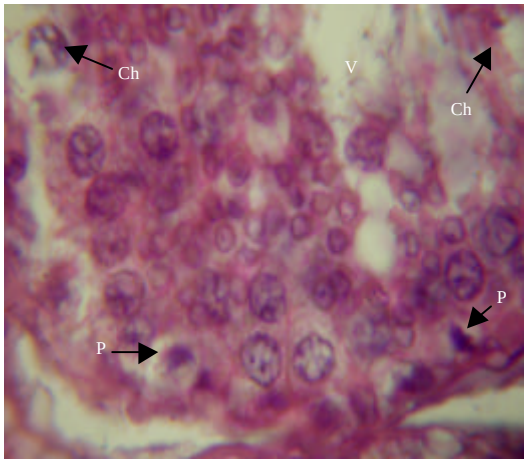


Fig. 2: Testicular section treated with endosulphan (100 nmol mL^{-1}) after 1 h of exposure duration showing pycnotic nuclei (P) and chromolysis (Ch). Vacuoles (V) of various shapes and sizes were observed in cytoplasm of germ cells and somatic cells (X 1000)

exposure duration. The pycnotic nuclei, chromolysis, vacuoles of various shapes and sizes were observed in cytoplasm of germ cells and somatic cells (Fig. 2). As the exposure duration of endosulphan was increased from 1 to 4 h the number of pycnotic nuclei was increased. Hyalinization was observed in spermatogonia and Sertoli cells. Fragmented nuclei and chromolysis were observed in spermatogonium, spermatocytes and Sertoli cells. As the exposure duration of endosulphan further enhanced

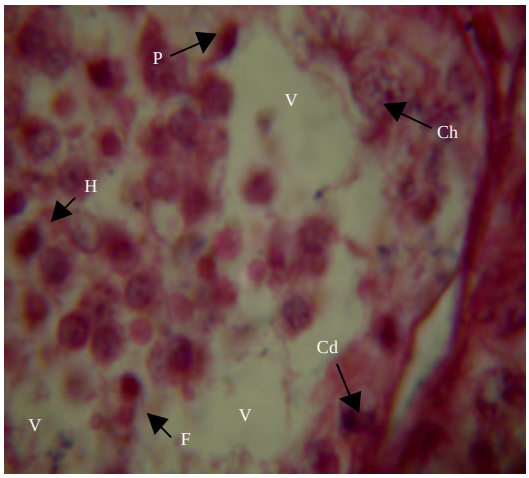


Fig. 3: Light micrograph of testicular tissue exposed with endosulphan (100 nmol mL^{-1}) showing fragmented nuclei (F), chromolysis (Ch), condensed nuclei (Cd) and vacuoles (V) of larger size in spermatogonium, spermatocytes and Sertoli cells. Hyalinization (H) was observed in spermatids, spermatogonia and Sertoli cells after 8 h of exposure duration (X 1000)

from 4 to 8 h these degenerating changes further increased. Endosulphan induced detachment of seminiferous cells from the basement membrane. Seminiferous tubules became atrophied and dislodging of different types of cells of seminiferous tubules was observed after 8 h of exposure durations. Chromolysis, pycnosis, condensation, hyalinization and fragmentation were frequently observed. Intra cytoplasmic and intra nuclear vacuoles were evident. Vacuoles became enlarged with the enhancement of exposure duration (Fig. 3). Atretic percentage of germ cells and somatic cells increased in the time dependent manner. Endosulphan induced elevation in atretic spermatocytes from 22% in control testicular tissue to 54% after 1 h of exposure, from 28 to 68% after 4 h and from 32 to 74% after 8 h of exposure duration. Chi-square values between control and endosulphan treated group [experimental group (A)] were analyzed and all the variations recorded were statistically significant (χ^2 0.05) (Table 1). Endosulphan induced increase in atretic Sertoli cells from 18% in control to 62% after 1 h, 24 to 70% after 4 h and 32 to 78% after 8 h of exposure duration. Chi-square values between control and endosulphan treated group [experimental group (A)] were analyzed and all the variations recorded were statistically significant (χ^2 0.05) (Table 2).

Endosulphan disrupts the testicular architecture and thus affects the functions in adult goat. Vitamin C

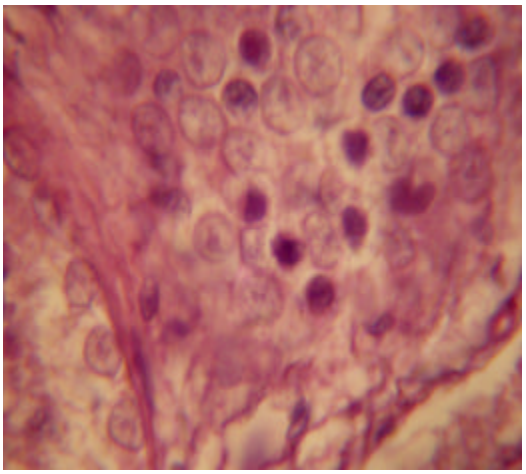


Fig. 4: Microphotograph of testicular tissue treated with endosulphan (100 nmol mL^{-1}) and supplemented with vitamin C (1000 μmol L^{-1}) showing rescue of atretogenic changes induced by endosulphan after 1 h of exposure duration (X 1000)

Table 1: The comparison of a number of atretic spermatocytes between control versus endosulphan (100 nmol mL^{-1}) exposed group (A) and endosulphan (100 nmol mL^{-1}) treated group (A) versus endosulphan supplemented with vitamin C group (B) showing Chi-square values after 1, 4 and 8 h of exposure durations

Parameters	Endosulphan	Endosulphan+Vitamin C
1 h	5.4329*	4.9367*
4 h	8.0127*	6.8282*
8 h	8.8513*	4.3402*

*Statistically significant difference (χ^2 0.05)

Table 2: Chi-square values between atretic Sertoli cells observed in control versus endosulphan (100 nmol mL^{-1}) treated group (A) and endosulphan (100 nmol mL^{-1}) treated group (A) versus endosulphan supplemented with vitamin C group (B) after 1, 4 and 8 h of exposure durations

Parameters	Endosulphan	Endosulphan+Vitamin C
1 h	20.1667*	10.3059*
4 h	21.2364*	11.6017*
8 h	21.3738*	7.42857*

*Statistically significant difference (χ^2 0.05)

treatment at dose level 1000 μmol L^{-1} in experimental group (B) restore the normal structure of the seminiferous tubule (Fig. 4). The frequency of pycnotic nuclei, chromolysis, fragmented nuclei, in spermatogonia, spermatocytes, spermatid and Sertoli cells were declined to the large extent. There was a significant decline in atretic germ cells and somatic cells in the seminiferous tubules (Fig. 5, 6). Due to the supplementation of vitamin C along with the exposure of endosulphan to the testicular tissue atretic spermatocytes were declined from 54% in endosulphan exposed tissue [experimental group (A)], to 32% in endosulphan supplemented with vitamin C group [experimental group (B)] after 1 h, from 68% to

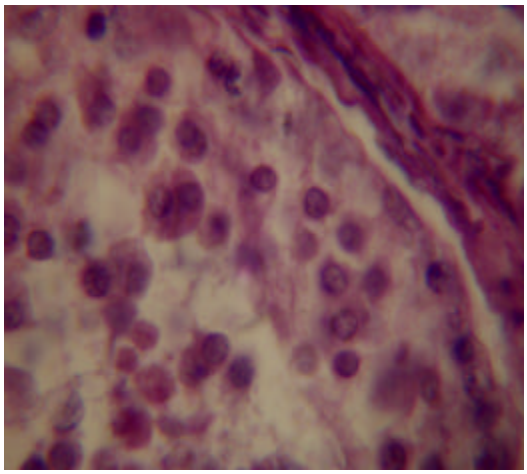


Fig. 5: Testicular tissue treated with endosulphan (100 nmol mL^{-1}) and supplemented with vitamin C (1000 μmol L^{-1}) showing decline in number of pycnotic and fragmented nuclei in spermatogenic cells after 4 h of exposure durations (X 1000)

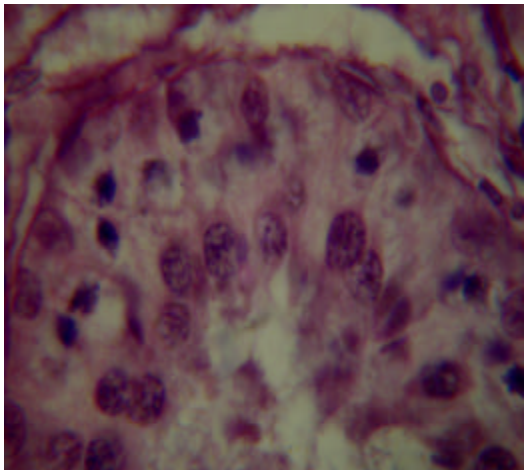


Fig. 6: A portion of testicular tissue treated with endosulphan (100 nmol mL^{-1}) and supplemented with vitamin C (1000 μmol L^{-1}) showing restoration of testicular structure after 8 h of exposure duration (X 1000)

42% after 4 h and from 74 to 54% after exposure duration of 8 h. Chi-square values between endosulphan exposed group and endosulphan supplemented with vitamin C [experimental group (B)] were analyzed and 4.936, 6.828 and 4.340 values were recorded after 1, 4 and 8 h of exposure durations respectively and all the variations recorded were statistically significant (χ^2 0.05) (Table 1). There was also decline in atretic Sertoli cell percentage after supplementation of Vitamin C. After supplementation

of vitamin C along with the exposure of endosulphan to the testicular tissue atretic Sertoli cell were declined from 62% in endosulphan exposed tissue [experimental group (A)], to 30% in vitamin C supplemented group [experimental group (B)] after 1 h, from 70 to 36% after 4 h and from 78 to 52% after exposure duration of 8 h. Chi-square values between endosulphan exposed group and endosulphan supplemented with vitamin C [experimental group (B)] were analyzed and 10.306, 11.6017 and 7.4285 values were recorded after 1, 4 and 8 h of exposure durations, respectively and all the values recorded were statistically significant (χ^2 0.05) (Table 2).

DISCUSSION

During the present investigation administration of 1000 μmol L^{-1} (Ascorbic acid) exerts an ameliorating effect on endosulphan (100 nmol mL^{-1}) induced testicular toxicity in *Capra hircus in vitro*. The present investigation is in consistent with the findings of Uzunhisarcikli *et al*. (2007) in which methyl parathion exposure resulted in necrosis and edema in the seminiferous tubules and interstitial tissues where as vitamins C and E supplementation reduces testicular toxicity induced by methyl parathion, but it does not protect completely. The results of the present findings that supplementation of vitamin C (1000 μmol L^{-1}) induced decline in atretogenic changes in germ cells present in the seminiferous tubule strongly supports the earlier finding of Narayana *et al*. (2005), who observed that decreased ascorbic acid concentration in the testes was well correlated with decreased sperm count and increased sperm abnormalities, indicating a close relation between them. Similar results were observed by Saalu *et al*. (2006) where melatonin a highly effective endogenous antioxidant free radical scavenger was found to alleviate the deleterious effects of cryptochidism on metabolic activity of the testes. Anderson *et al*. (1994) reported that there were small protective effects of Vitamin C at low doses and exacerbating effects at high doses. Our results demonstrated that there was decline in chromolysis, pycnosis, condensation and fragmentation which showed that there was ameliorating effect due to the supplementation of vitamin C on testicular toxicity induced by endosulphan. The results of present investigation showed that there was decline in atretic percentage of spermatocytes after exposure of endosulphan. Atretic percentage after 1 h of exposure of endosulphan induced elevation in atretic spermatocytes from 22% in control testicular tissue to 54%, from 28 to 68% after 4 h and from 32 to 74% after 8 h of exposure duration. These observations strongly supports the findings of Acharya *et al*. (2002) who observed the generation of significantly higher quantities of Reactive Oxygen Species (ROS) in Cd-treated Swiss mice testes which have detrimentally affected the germ cells consequently posing significant decline in sperm count compared to the untreated controls. However, supplementation of vitamin C and E to Cd-treated mice, drastically reduced the generation of ROS, thereby significantly escalating sperm count and declining the frequency of abnormal sperm population (Acharya *et al*., 2002). Our results are in agreement with the findings of Ata *et al*. (2007) in which ameliorating effect of oral ascorbic acid was evaluated against changes in sperm parameters in New Zealand White (NZW) rabbits treated with endosulphan. Ascorbic acid treatment showed significant amelioration when coupled with endosulphan (Ata *et al*., 2007). Ameliorations were up to control levels in all cases except for sperm motility. The data suggested that ascorbic acid has beneficial influences in neutralizing the toxic effects of endosulphan in the spermatologic parameters of NZW males (Ata *et al*., 2007). Our results that endosulphan induced testicular damage can be improved by the supplementation of vitamin C strongly supports the findings of Maneesh *et al*. (2005) who observed that Ascorbic acid treatment significantly decrease in oxidative stress induced by ethanol in the testis. Decrease in steroidogenesis due to ethanol exposure can also be reversed by treatment with ascorbic acid (Maneesh *et al*., 2005).

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