Indian J Physiol Pharmacol 2005; 49 (3) : 331–336

# L-ASCORBIC ACID AMELIORATES POSTNATAL ENDOSULFAN INDUCED TESTICULAR DAMAGE IN RATS

MANJULA RAO[a]*, K. NARAYANA[b], SUSAN BENJAMIN[a] AND K. L. BAIRY[c]

[a]*Department of Physiology, Kasturba Medical College, Manipal – 576 104*

[b]*Department of Anatomy, Center for Basic Sciences, Kasturba Medical College, Bejai, Mangalore – 575 004*

[c]*Department of Pharmacology, Kasturba Medical College, Manipal – 576 104*

**( Received on November 20, 2004 )**

**Abstract :** The aim of the present study is to investigate the effect of L-ascorbic acid on postnatal exposure of endosulfan induced testis damage in the rat. Four groups of seven day old male Wistar rats were treated with 3, 6, 9 and 12 mg/kg endosulfan orally (10 pups/group), from postnatal day 7 to 60 at intervals of 24 h. For 2 more groups (n = 10/group), endosulfan (9 mg/kg and 12 mg/kg) was administered along with L-ascorbic acid (20 mg/kg). The sperm morphology, sperm count and sperm motility was analyzed in all the groups on postnatal day 70. Endosulfan significantly affected the testicular function enhancing the incidence of abnormal spermatozoa, decreasing the sperm count and sperm motility in a dose dependent manner. Abnormalities were of both head and tail and increase in their frequency was more than two-fold of the control value. Sperm count abruptly decreased in 12 mg/kg group and sperm motility decreased up to 50% of the control value. L-ascorbic acid has nullified the toxic effects of the pesticide significantly, but not to the control level. Endosulfan induces the testicular damage following postnatal exposure and L-ascorbic acid prevents the adverse effects considerably in the rat.

**Key words :** L-ascorbic acid, pesticides, gonadotoxicity, sperm morphology, sperm count

## INTRODUCTION

Endosulfan is an organochlorine pesticide and acaricide of the cyclodiene subgroup, which acts as a poison to a wide variety of insects on contact. It is used basically on a wide variety of crops including tea, coffee, fruits, vegetables and

*Corresponding Author : E-mail : klbairy@yahoo.com

food grains. Endosulfan is highly toxic and regardless of routes of its exposure, it results in poor coordination, imbalance, difficulty in breathing, gagging, vomiting, diarrhea, agitation, convulsions, loss of consciousness, and central nervous system disorders (1, 2). Chronic exposure results in liver enlargement, seizures, reduced growth and survival, changes in kidney structure and blood parameters (2). Endosulfan is known to get excreted from the mammary gland via the human breast milk in a comparatively higher rate than other pesticides (3).

Exposure at the dose levels of 1.5 to 3.0 mg/kg in pregnant rats resulted in decrease of sperm count and alterations in the cytoarchitecture of the testis in offspring, although, the authors believe that only high doses of endosulfan have some effects on male reproduction (4). In the pubertal rats, endosulfan (1 mg/kg), disrupted the testicular function, by altering the enzyme like acid phosphatase, and nucleic acid levels (5). Endosulfan was found to be mutagenic *in vitro* inducing micronuclei and sister-chromatid exchanges in HepG2 cells (6). Its genotoxic effects were assessed in mouse by dominant lethal and sperm morphology assays and both tested positive although the results are equivocal, but the sperm motility was not affected (7, 8). Even though the human exposure levels are unknown, endosulfan rapidly breaks down into its metabolites, and residues are found in the vegetables and fruits (2). Reports on fertility in endosulfan treated male rats invited some controversy by providing contradictory conclusions.

Pandey et al (7) reported that there was some dominant mutations, whereas Dzwonkowska and Hubner concluded that there were no such effects in mice (8). In adult mice, ascorbic acid successfully ameliorated the genotoxicity at 20 mg/kg (9), and reduced the frequencies of meiotic chromosomal damage induced by the pesticides including endosulfan (10). All these studies were conducted in animals to evaluate the effects of ascorbic acid. Besides, the studies on interactions of ascorbic acid (if any) with pesticide treated mammalian systems are very few. Therefore, the present study was designed to evaluate the effects of prolonged postnatal exposure of endosulfan on male reproductive parameters and its interactions with ascorbic acid in rats.

## MATERIALS AND METHODS

**Animals:** Inbred male Wistar rats (7 days old) were maintained in the animal house of Kasturba Medical College, Manipal, under all ethical guidelines. They were housed in polypropylene cages with the paddy husk bedding at 28 ± 1°C and 50 ± 5% humidity. They were fed on laboratory chow and tap water ad libitum. The rats were divided into 9 groups of ten rats each as shown in Table I. The institutional animal ethical committee approval was obtained before starting the experiment.

**Chemicals and treatment:** Endosulfan (Dhanusulfan, 35 Ec, Meerut Agro Chemicals Industries Ltd., Meerut, India), was mixed in pea nut oil, given orally at dose levels of 3, 6, 9, and 12 mg/kg body weight equivalent to 1/6, 1/3, ½, and 1/1, 5 of oral $LD_{50}$ in rats (l), from post natal day 7 to 60 at interval of 24 h. L-ascorbic acid

(Loba Chemicals (P) Ltd., Mumbai, India) was dissolved in distilled water and given at a dose of 20 mg/kg, equivalent to twice that of therapeutic dose (9). One group (n = 10/group) was treated with ascorbic acid alone and other 2 groups were given 9 and 12 mg/kg endosulfan and L-ascorbic acid.

**Sperm morphology assay, sperm count and sperm motility :** Animals were sacrificed by overdose of anesthesia (Pentobarbitone sodium, 45 mg/kg) on postnatal day 70. Laparotomy was conducted and the reproductive tract was exposed. The weight of the animals was noted before sacrificing them. The testis and epididymis were removed, weighed and placed in phosphate buffered saline (PBS; pH 7.2), and then epididymis was separated and minced in 1 ml of PBS and filtered through 80µ nylon mesh. To the filtrate one drop of 1% (w/v) eosin Y was added and kept for 30 minutes. Sperm morphology assay was performed as per the standard method described for rats (11).

One drop of the stained filtrate was placed on a clean glass slide and a smear was prepared. The slides were coded and screened for sperm abnormalities. One thousand sperms per animal were observed and classified into normal and different abnormal types (11). The sperm count was conducted by diluting the sperm suspension in phosphate buffered saline in a leucocyte haemocytometer. An aliquot of stained sperm suspension was taken up to 0.5 mark in a haemocytometer and diluted (1:40) with PBS up to mark 11. The sample was then charged into Neubauer's chamber and sperms were counted in 8 squares except the central area and multiplied by $5X10^4$ to express the total count per epididymis (12). Number of motile spermatozoa was counted and recorded as percentage sperm motility.

**Statistical analysis :**

For each group mean ± S.E.M. was calculated and the data were analyzed by one way ANOVA followed by Bonferroni's post test using software package, GPIS, 1990, version 1.13. $P<0.05$ was considered as the level of significance.

## RESULTS AND DISCUSSION

Table I shows the data on effect of endosulfan, and L-ascorbic acid on body and weight of testis in rats. Endosulfan at different doses induced significant ($P<0.05$) decrease in body, and testis weight. In endosulfan treated rats effect was dose dependent and more reduction was observed as the dose was increased. The administration of L-ascorbic acid to the rats exposed to 9 and 12 mg/kg endosulfan increased ($P<0.05$) the testis weight compared to that of 9 and 12 mg/kg endosulfan alone treated rats.

There was significant ($P<0.05$) decrease in sperm count, motility and significant ($P<0.05$) increase in sperm abnormality in rats exposed to endosulfan (3–12 mg/kg) in a dose dependent manner. Qualitative analysis of the different types of abnormal sperm revealed the presence of amorphous, bookless, coiled, banana shaped and double headed /doubled tailed forms. A quantitative analysis of the different sperm abnormality was assessed. Concurrent administration of L-ascorbic acid to endosulfan treated rats significantly reduced the effect of

TABLE I: Effect of endosulfan and L-ascorbic acid on rat testis.

| *Drug/dose mg/kg* | *Body weight (g)* | *Testis weight (g/100 g body weight)* | *Sperm count* | *Sperm motility (%)* |
|---|---|---|---|---|
| Control (saline) | 240±2.58 | 0.70±0.01 | 42.19±0.77 | 34.00±1.30 |
| Vehicle (Peanut oil) | 238±2.90 | 0.69±0.01 | 41.74±0.92 | 33.33±1.34 |
| Asc | 236±2.60 | 0.69±.01 | 42.01±0.61 | 34.70±1.27 |
| End (3 mg/kg) | 180±3.33* | 0.54±0.02* | 37.10±0.38* | 24.50±1.34* |
| End (6 mg/kg) | 159±3.14* | 0.51±0.01* | 35.21±0.49* | 23.21±0.81* |
| End (9 mg/kg) | 148±3.88* | 0.46±0.02* | 30.53±0.97* | 19.70±0.86* |
| End (12 mg/kg) | 127±3.01* | 0.44±0.02* | 13.55±1.02* | 15.91±0.79* |
| End (9 mg/kg) + Asc | 181±3.66*# | 0.57±0.02*# | 36.33±1.13*# | 26.60±1.10*# |
| End (12 mg/kg) + Asc | 166±3.39*@ | 0.54±0.02*@ | 32.11±085*@ | 24.00±1.31*@ |

Mean ± S.E. from 10 animals/point. Asc = Ascorbic acid, End = Endosulfan.

*P<0.05 versus control, # P<0.05 versus 9 mg/kg Endosulfan alone, @P<0.05 versus 12 mg/kg Endosulfan alone.

TABLE II: Effect of endosulfan and L-ascorbic acid on sperm morphology in the rat.

| *Drug/dose* | *Normal sperm* | *Head abnormalities* | | | | *Tail abnormalities* | | *%sperm abnormality* |
|---|---|---|---|---|---|---|---|---|
| | | *Am* | *Hl* | *Bn* | *Dh* | *Cl* | *Dt* | |
| Control | 987.50±1.26 | 4.62±1.05 | 5.12±0.66 | 0.75±0.25 | – | 2.00±0.77 | – | 1.25±0.13 |
| Vehicle | 989.00±1.42 | 4.37±0.88 | 312±0.58 | 2.50±3.20 | – | 1.00±0.77 | – | 1.10±0.14 |
| Asc | 987.30±2.09 | 4.00±0.53 | 5.14±0.76 | 2.00±0.37 | – | 1.28±0.35 | 0.28±0.18 | 1.27±0.15 |
| End (3 mg/kg) | 959.30±8.51 | 13.60±6.00 | 8.14±0.50 | 5.80±1.90 | – | 12.80±6.50 | 0.40±0.40 | 4.07±1.41 |
| End (6 mg/kg) | 948.70±1.91 | 17.41±2.40 | 8.60±1.60 | 7.00±1.75 | 0.14±0.14 | 17.28±5.16 | 0.85±0.55 | 5.13±0.67* |
| End (9 mg/kg) | 934.60±8.62 | 22.85±0.92 | 10.70±1.30 | 10.61±0.65 | 0.20±0.12 | 19.80±3.12 | 1.22±0.32 | 6.54±0.69* |
| End (12 mg/kg) | 892.90±5.48 | 36.71±6.77 | 22.77±8.62 | 21.88±1.71 | 1.11±0.35 | 22.00±2.74 | 2.60±0.42 | 10.71±0.56* |
| End 9 + Asc | 953.00±3.42 | 17.00±1.58 | 5.00±1.51 | 12.61±0.50 | 0.20±0.20 | 10.60±0.40 | 1.60±1.02 | 4.20±0.33*# |
| End 12 + As | 938.60±4.11 | 22.41±2.08 | 7.00±0.50 | 15.00±0.31 | 0.20±0.20 | 15.00±0.40 | 1.80±0.24 | 6.14±0.22*@ |

All values are expressed as mean ± S.E. from 10 animals.

*P<0.05 versus control, # P<0.05 versus 9 mg/kg Endosulfan alone, @P<0.05 versus 12 mg/kg Endosulfan alone.

End = endosulfan, Asc = L-ascorbic acid, Am = Amorphous, Hl = Hook less, Bn = Banana shaped, Dh = Double headed, Cl = Coiled, Dt = Double tailed.

endosulfan on the sperm count, motility and abnormality (Tables I and II).

The results of this study substantiate the results in earlier reports, but for the sperm motility, which indicates that the pesticide affected the tail structure and consequently its function. The fertility-efficiency could be further affected by the altered sperm morphology. Several reasons have been accounted for sperm shape abnormalities, but point mutations, Y

chromosome defects and testicular dysfunctions are known to have some relation, though none of them has been individually proved absolutely responsible (11, 13, 14). The exposure protocol for long period was adopted here since the effects of long term exposure have to be evaluated on the reproductive system, largely because the pesticides remain continuously in contact with the human and animal life. Endosulfan treatment up to 60 days has resulted in decrease of testicular weight due to cytotoxicity of this chemical. This was evident in terms of decreased sperm count at all dose levels. Though L-ascorbic acid increased the organ and body weights, it could not neutralize the adverse effects of endosulfan completely. Endosulfan is rapidly degraded into water soluble compounds and eliminated in mammals with very little absorption in the gastrointestinal tract (1), but its (β-isomer is not eliminated that soon, compared to the α-isomer. This might account at least in part for the observed effects of endosulfan here. Hence, the negative results observed in a few studies might be due to its varied degree of metabolism.

Concomitant administration of ascorbic acid resulted in decreased sperm abnormality and elevated sperm count and motility. L-ascorbic acid was able to decrease the chromosomal damages and micronucleus formation induced by endosulfan or other pesticides (9, 10, 15). Though the precise mechanism is not known, L-ascorbic acid being a reducing agent, might have reduced the active metabolites of endosulfan into inactive ones. This type of action of L-ascorbic acid has been reported in *in vivo* models exposed to cyclophosphamide (16) or mitomycin C (17). These chemicals or their metabolites exhibited their adverse effects by an electrophilic attack on the nucleophilic sites of DNA (18). Although L-ascorbic acid functioned as an antioxidant and showed nucleophilic character (19), it could compete with the mutagen for sites on DNA. Alkylation of ascorbic acid could compete with the alkylation of DNA or protein and if present in sufficient quantity, ascorbic acid might act as an antimutagen by this mechanism (18). Thus, we conclude that endosulfan acts as a potent gonadotoxic agent and since this chemical is extensively used as spray in agriculture, the users should be aware of the toxic effects of the chemical. L-ascorbic acid however considerably prevents the gonadotoxic effects of endosulfan in the rat.

## REFERENCES

1. Smith AG. Chlorinated Hydrocarbon Insecticides. In: Handbook of Pesticide Toxicology. Eds: Hayes WJ Jr, Laws ER Jr, Academic Press Inc., New York, NY, 1991; 3–6.

2. Dutta HM, Arends DA. Effects of endosulfan on brain acetylcholinesterase activity in juvenile bluegill sunfish. *Environ Res* 2003; 91: 157–162.

3. Sanghi R, Pillai MK, Jayalekshmi TR, Nair A. Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. *Hum Exp Toxicol* 2003; 22: 73–76.

4. Dalsenter PR, Dallegrave E, Mello JR, Langeloh A, Oliveira RT, Faqi AS. Reproductive effects of endosulfan on male offspring of rats exposed during pregnancy and lactation. *Hum Exp Toxicol* 1999; 18: 583–589.

5. Chitra KC, Latchoumycandane C, Mathur PP. Chronic effect of endosulfan on the testicular

functions of rat. *Asian J Androl* 1999; 1: 203–206.

6. Lu Y, Morimoto K, Takeshita T, Takeuchi T, Saito T. Genotoxic effects of alpha-endosulfan and beta-endosulfan on human HepG2 cells. *Environ Health Perspect* 2000; 108: 559–561.

7. Pandey N, Gundevia F, Prem AS, Ray PK. Studies on the genotoxicity of endosulfan, an organochlorine insecticide, in mammalian germ cells. *Mutation Res* 1990; 242: 1–7.

8. Dzwonkowska A, Hubner H. Studies on commercial insecticides with the dominant lethal mutations test. *Pol J Occup Med Environ Health* 1991; 4: 43–53.

9. Khan PK, Sinha SP. Impact of higher doses of L-ascorbic acid in modulating pesticide genotoxicity. *Teratog Carcinog Mutagen* 1994; 14: 175–181.

10. Khan PK, Sinha SP. L-ascorbic acid mediated amelioration of pesticide genotoxicity in murine spermatocytes. *Cytobios* 1994; 80: 199–204.

11. Narayana K, D'Souza UJA, Rao KPS. Ribavirin induced sperm shape abnormalities in Wistar rat. *Mutation Res* 2002; 513: 193–196.

12. Narayana K, D'Souza UJA, Rao KPS. Effect of ribavirin on epididymal sperm count in Wistar rat. *Indian J Physiol Pharmacol* 2002; 46: 97–101.

13. Wyrobek AJ, Gordon LA, Burkhart MW, Francs RW, Kapp Jr., Letz G, Mailing HV, Topham JC, Whorton MD. An evaluation of the mouse sperm morphology test and other sperm tests in non-human mammals. *Mutation Res* 1983; 115: 1–72.

14. Styrna J, Imai HT, Moriwaki K. An increased level of sperm abnormalities in mice with a partial deletion of the Y chromosome. *Genet Rev Comb* 1991; 57: 195–199.

15. Khan PK, Sinha SP. Antimutagenic efficacy of higher doses of L-ascorbic acid. *Mutation Res* 1993; 298: 157–161.

16. Ghaskadbi S, Rajamachikar S, Agate C, Kapadi AH, Vaidya VG. Modulation of cyclophosphamide mutagenicity by L-ascorbic acid in the *in vivo* rodent micronuclei assay. *Teratogen Carcinog Mutagen* 1992; 12: 11–27.

17. Krishna G, Nath J, Ong T. Inhibition of cyclophospamide and Mitomycin C induced sister chromatid exchanges in mice by L-ascorbic acid. *Cancer Res* 1986; 46: 2670–2674.

18. Vijayalaxmi KK, Venu R. *In vivo* anticlastogenic effects of L-ascorbic acid in mice. *Mutation Res* 1999; 438: 47–51.

19. Edgar JA. Ascorbic acid and biological alkylating agents. *Nature* 1974; 248: 136–137.