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Protective Role of Curcumin on Lindane Induced Reproductive Toxicity in Male Wistar Rats

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Abstract The aim of this study was to explore the adverse effects of lindane pesticide on testes and epididymus weight, sperm head counts, sperm motility, abnormal changes in sperm morphology, biochemical changes in endogenous antioxidants and oxidative enzymes in male wistar rats as well as to assess ameliorating role of 'curcumin'. Rats were exposed daily to lindane (30 mg/kgbw) for 14 and 28 days and administered with curcumin (100 mg/kgbw) in pretreatment, post treatment and combination groups. We observed decrease ($p < 0.001$) in testes and cauda epididymus weight, decrease ($p < 0.001$) in testicular sperm head count, increase ($p < 0.001$) in abnormal tail morphology (headless tail, multiple tail, broken tail, coiled tail and bent tail), abnormal head morphology (excessive hook, amorphous head, pin head, short head, blunt hook and detached hook) and decrease ($p < 0.001$) in sperm motility after lindane exposure, as compared to control. There was ($p < 0.01$) decline in superoxide dismutase, catalase and glutathione-s-transferase activity on lindane exposure, however, no change ($p > 0.05$) was observed in glutathione level. Lipid peroxidation was ($p < 0.01$) enhanced on lindane exposure as compared to control. Curcumin administration was able to ameliorate lindane induced reproductive toxicity in pretreatment, post treatment and combination groups. The study indicated that even after 14 days of metabolism, toxic effects of lindane were evident.

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Humans are constantly exposed to foreign chemicals or xenobiotics such as drugs, industrial chemicals, pesticides and other environmental pollutants. Most of these chemicals remain untested for their safety in humans and other animals. These chemicals are known for causing health hazards like cancer, organ toxicity, mutations, cardiac disorders, changes in pulmonary functions, adverse impact on the immune system, infertility and many other disorders. Pesticides are the most common xenobiotics present in the environment and causing toxicity.

γ-Isomer of 1,2,3,4,5,6 hexachlorocyclohexane, commercially known 'lindane' is common organochlorine pesticide widely used in agriculture, veterinary and public health programmes (Solomon et al. 1977). Lindane is known to accumulate in food chains, causing toxicity in wild/domestic animals and human beings (WHO Report 1991). Apart from contaminated food, human beings are exposed to lindane by inhalation, polluted water and dermal contact. The toxic effects of lindane on liver and brain of experimental animals are well documented (Arisi et al. 1994; Junqueira et al. 1994). Several studies showed that lindane have adverse effect on male reproductive system leading to infertility (Chitra et al. 2001; Sahoo et al. 2008). Owing to its lipophillic character, lindane is reported to accumulate in testes and cauda epididymus in rats, resulting in induction of oxidative stress, enhancement of ROS production and reproductive toxicity (Samanta and Chainy 1997).

As per recent studies it has been found that several phytochemicals possess very good antioxidant activity and protect cells from oxidative damage. Curcumin, the yellow coloring agent chief bioactive constituent of turmeric

rhizome (*Curcuma longa*) possesses a variety of pharmacological activities and the molecule is reported to be safe for human use (Strimpakos and Sharma 2008; Luthra et al. 2001). The antioxidant activity of curcumin is well established. It reduces oxidative stress in the cells by lowering lipid peroxidation and inducing cellular antioxidant enzymes (Ak and Gülcin 2008; Priyadarshini 1999). Since lindane induced toxicity in male reproductive system may be due to oxidative stress and ROS production, we planned to study the protective effect of curcumin on lindane induced reproductive toxicity on male wistar rats.

Materials and Methods

Pure lindane 99.6% and curcumin were purchased from Sigma–Aldrich (st. Louis, Mo. USA). All other chemicals were of AR grade and purchased locally. The inbreed wistar rats were obtained from animal research division, Central Drug Research Institute (CSIR), Lucknow (India). The spectrophotometric measurements were performed using Shimadzu UV-Spec 1700 and microscopic examinations on Olympus CH20i microscope.

The animals were kept in central animal house of Bundelkhand University, Jhansi under a well regulated light and dark (12–12 h) schedule of 24 h and were allowed free access to laboratory diet and tap water. Adult male rats (3–4 months old weighing about 200–300 g) were used for this study. Lindane and curcumin were dissolved in Dimethylsulfoxide (DMSO) and administered in rats by gavaging. Dosage of lindane (Ahmad et al. 2006) and curcumin (Lubbad et al. 2009; Perkins et al. 2002) were selected as per available literature and our preliminary results (unpublished data). All animal experiments were carried out as per the guidelines of Institutional Ethical Committee. Animals were divided in six groups of six rats each and treated as per given schedule (Table 1).

At the end of the experiment, rats were sacrificed as per institutional ethical committee guidelines, testes, cauda epididymus were removed, weighted and used for sperm head counts, sperm motility, sperm morphology study, biochemical and antioxidant enzyme estimations.

Sperm head counts were determined with hemocytometer using a method described by Choi et al. (2008) with necessary modifications. Testes was taken out in petridish, decapsulated, put in a tube and homogenized for 2 min with 5 mL phosphate buffer saline (PBS; pH 7.4) at high speed. The homogenized sample was transferred to 45 mL PBS (pH 7.4). Ten to 15 µL of homogenate was loaded into hemocytometer chamber (improved Neubauer, deep 1/10 mm), and after 5 min sperm heads were counted in RBC chamber at 40× magnification. Number of sperm count per gram of testes was equal to mean number of sperm head counted in five squares × hemocytometer factor × square factor × dilution factor/weight of testes.

Cauda epididymus was used for sperm head, tail abnormalities and motility studies. For sperm head and tail abnormalities cauda epididymus was removed and minced in 1 mL of 0.9% saline with the help of razor or scalpel. One milliliter of phosphate buffer (pH 7.4) with 10% formalin was added to it. Suspension was diluted with distilled water to volume suitable for performing assay. Twenty milliliter of above suspension was taken and 1.67% eosin, 10% nigrosin and 0.1 M sodium citrate were added and incubated at room temperature for 45–60 min. One drop of the suspension was taken on slide and smear was prepared for evaluation of head and tail abnormalities. The slides were then viewed under a light microscope at 40× magnification. A total of 200 sperms were examined on each slide. The head and tail abnormalities were expressed as a percentage (Turk et al. 2007). For sperm motility studies 100 mg of cauda epididymus was taken out in 10 mL of pre-warmed 37°C PBS in a test tube. Tube was kept in water bath and cauda was gently minced with scalpel or scissor. Incubated at 37°C for 15 min and pipetted gently 20 times up and down. The sample was diluted with pre-warmed 37°C PBS (1:200). Ten to 15 µL of homogenate was taken and placed on hemocytometer. Non-motile sperms were counted in WBC chamber and calculated by mean count in four chamber × 10^4 × dilution factor. Hemocytometer was kept at 40–50°C for 1 min (to kill the sperms) and then total number of dead sperms were counted (Williams 1993).

Table 1 Experimental schedule

Group-Ia	Vehicular control	Normal diet and DMSO for 14 days
Group-Ib	Vehicular control	Normal diet and DMSO for 28 days
Group-IIa	Sub-acute exposure	Lindane 30 mg/Kgbw once daily for 14 days
Group-IIb	Sub-acute exposure	Lindane 30 mg/Kgbw once daily for 28 days
Group-III	Pretreatment	100 mg/kgbw curcumin for 14 days followed by lindane 30 mg/Kgbw once daily up 14 days
Group-IV	Post treatment	Lindane 30 mg/Kgbw once daily for 14 days followed by 100 mg/kgbw curcumin for 14 days
Group-V	Combination	Lindane 30 mg/Kgbw co-administered with curcumin 100 mg/kgbw daily for 28 days
Group-VI	Metabolized	Lindane 30 mg/Kg bw for 14 days and then allowed to metabolize for 14 days

Biochemical and antioxidant enzyme estimations were performed from testes. Testes was removed, cleaned of the adhering tissue and weighted. Ten percent testicular homogenate (w/v) was prepared in normal saline using homogenizer and the homogenate was centrifuged at 800×*g* for 30 min at 4°C (Sujatha et al. 2001). The supernatant was used for biochemical estimations. Protein was estimated by the method of Lowry et al. (1951). Lipid peroxidation was estimated as per method of Wright et al. (1981) with necessary modifications. Testes homogenate (1.0 mL) was diluted with 1.0 mL of distilled water and mixture was stirred at 37°C for 30 min, 1.0 mL trichloroacetic acid (TCA) was added and the mixture was centrifuged at 1,500×*g* for 5 min. The supernatant was mixed with 1.0 mL of 0.67% thiobarbituric acid (TBA) on a boiling water bath for 10 min and then the A_{532} was determined. The results were expressed as nmol malondialdehyde (MDA) formed/min/mg protein at 37°C. GSH was assayed by the method of Jollow et al. (1974). Briefly, 1.0 mL of post-mitochondrial supernatant PMS (10%) was precipitated with 1.0 mL of sulphosalicylic acid (4%). The sample were kept at 4°C for at least 1 h and then centrifuged at 1,200×*g* for 15 min at 4°C. The assay mixture contained 0.1 mL filtered aliquot and 2.7 mL phosphate buffer (0.1 M, pH 7.4) in a total volume of 3.0 mL. The yellow color developed was read immediately at 412 nm using spectrophotometer. The results were expressed as mmolGSH/gm tissue. GST activity was measured by the method of Habig et al. (1974). Briefly, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) was added to buffer containing 1 mM GSH and an aliquot of testes homogenate. Upon addition of CDNB, the change in absorbance at 340 nm was measured as a function of time. The results were expressed as nmol CDNB conjugate formed/min/mg protein. CAT activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture contained 2.40 mL of phosphate buffer (50 mM, pH 7.0), 10 µL of 19 mM hydrogen peroxide and 50 µL testes homogenate. The decrease in absorbance was measured immediately at 240 nm against blank for 3 min at 10 s intervals. The results were expressed as nmol H_2O_2 consumed/min/mg Protein. SOD activity was determined by measuring the inhibition of autoxidation of epinephrine at pH 10.2 at 30°C according to Misra (1989). The results were expressed as Unit/mg Protein.

Results were expressed as Mean ± SEM. Data was subjected to one way analysis of variance (ANOVA). The treatment groups were compared with control group using Dunnett's test. All the statistics were carried out in GraphPad InStat Software Inc., v. 3.06, San Digeo, USA.

Results and Discussion

DMSO for 14 days (group-Ia) and 28 days (group-Ib) showed similar results, hence group-Ib is taken as vehicular control for all comparison and it is indicated as group-I in tables. All comparisons were made with Group-I unless

Table 2 Effect of lindane and curcumin on testis and cauda epididymus weight

Parameters	Group-I	Group-IIa	Group-IIb	Group-III	Group-IV	Group-V	Group-VI
Cauda epididymus (g)	0.22 ± 0.00	0.16 ± 0.00**	0.15 ± 0.00***	0.19 ± 0.00•	0.19 ± 0.00•	0.18 ± 0.00•	0.17 ± 0.00**
Testis (g)	1.61 ± 0.00	0.51 ± 0.00**	0.49 ± 0.00***	1.36 ± 0.00•	1.36 ± 0.00•	1.52 ± 0.00•	0.51 ± 0.00**

Results shown as mean ± SEM, *** ($p < 0.001$), ** ($p < 0.01$), • ($p > 0.05$)

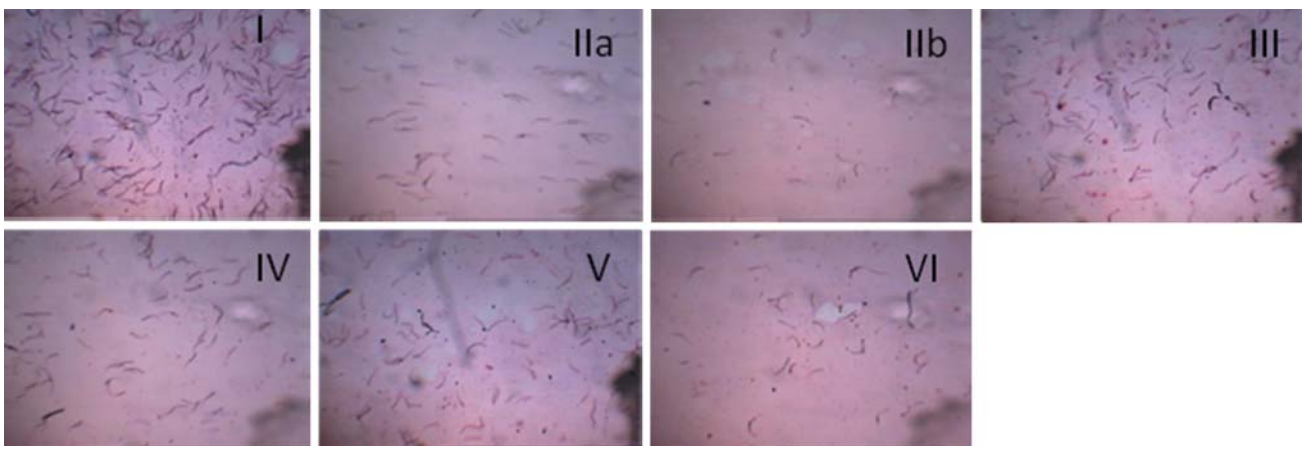

Fig. 1 Sperm count in different groups on lindane exposure and curcumin treatment

Table 3 Effect of lindane and curcumin on sperm head counts, percentage of sperm motility, live sperm and sperm abnormalities

Parameters	Group-I	Group-II a	Group-II b	Group-III	Group-IV	Group-V	Group-VI
Sperm head count $\times 10^4$	9,129.82 ± 1.98	1,381 ± 5.36**	1,033.33 ± 2.64***	9,034.05 ± 2.73•	8,830.17 ± 2.01•	9,040.49 ± 2.70•	1,452 ± 3.91**
Sperm motility (%)	90.06 ± 0.04	12.81 ± 0.05***	9.29 ± 0.02***	86.74 ± 0.04*	87.97 ± 0.04•	89.47 ± 0.06•	13.32 ± 0.05***
Live sperm (%)	91.12 ± 0.08	13.77 ± 0.04**	10.34 ± 0.03***	88.64 ± 0.09•	88.63 ± 0.02•	90.76 ± 0.37•	14.84 ± 0.03**
Sperm abnormalities (%)	12.15 ± 0.11	61.43 ± 0.10**	68.58 ± 0.37***	32.73 ± 0.03•	31.636 ± 0.47•	31.82 ± .03•	59.85 ± 0.00**

Results shown as mean ± SEM, *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$), • ($p > 0.05$)

Table 4 Effect of lindane and curcumin on sperm tail and head abnormalities

	G-I	G-IIa	G-IIb	G-III	G-IV	G-V	G-VI
Tail morphology							
Headless tail (%)	2	22	24	8	7	10	20
Multiple tail (%)	0	1	1	0	0	0	1
Broken tail (%)	0	1	1.5	0.5	0.4	0	1.5
Coiled tail (%)	2	5.5	6.5	4	5	3	2
Bent tail (%)	5	9	10	7	6.5	8	9
Head morphology							
Excessive hook (%)	1	1	1.5	1	1	0.5	1.5
Amorphous hook (%)	0	1	2	0.5	1	0	1
Pin head (%)	0	3	4	1.5	1	0	1
Detached hook (%)	2.5	18	19	11.5	10.5	13	17

and otherwise specified. Significant decrease in testes and cauda epididymus weight was observed in group-IIa ($p < 0.01$) group-IIb ($p < 0.001$) whereas non significant change was observed in group-III, IV and V ($p > 0.05$). Lindane metabolism after 14 days of exposure in group VI showed significant ($p < 0.01$) decrease in weight (Table 2).

Significant decrease in sperm head counts was observed in lindane exposure in group-IIb ($p < 0.001$) and in group-IIa and group-VI ($p < 0.01$). Group-III, IV, and V showed non significant ($p > 0.05$) decrease in sperm head counts, exhibiting protective effects of curcumin. Slides prepared for studying structural abnormalities in sperm clearly shows marked reduction in sperm counts in group IIa, IIb and Group-VI as compared to group-I, while group-III, IV, and V showed ameliorating effect of curcumin in sperm counts as compared to lindane exposed groups (Fig. 1). Sperm motility was decreased significantly in group-IIa ($p < 0.001$), group-IIb ($p < 0.001$), and group-VI ($p < 0.001$), while the decrease in sperm motility in group-III, IV, and V was non significant ($p > 0.05$). Significant increase in sperm abnormalities was observed in group-IIa, VI ($p < 0.01$) and group-IIb ($p < 0.001$), while there was non-significant increase in sperm abnormalities in group-III, IV, and V ($p > 0.05$) where curcumin was co-administered with lindane (Table 3).

Effect of lindane and curcumin on sperm head and tail morphology was assessed. The study showed 22% (group-IIa) and 24% (group-IIb) head less tails after lindane exposure indicating lindane induced abnormality in sperm structure. Ten percent and 20% headless tails were observed in group-V and Group-VI, respectively. The percentage of headless tails was very low in group-III, and IV, indicating protective effect of curcumin. Multiple and broken tail were seen after lindane exposure (group-IIa,

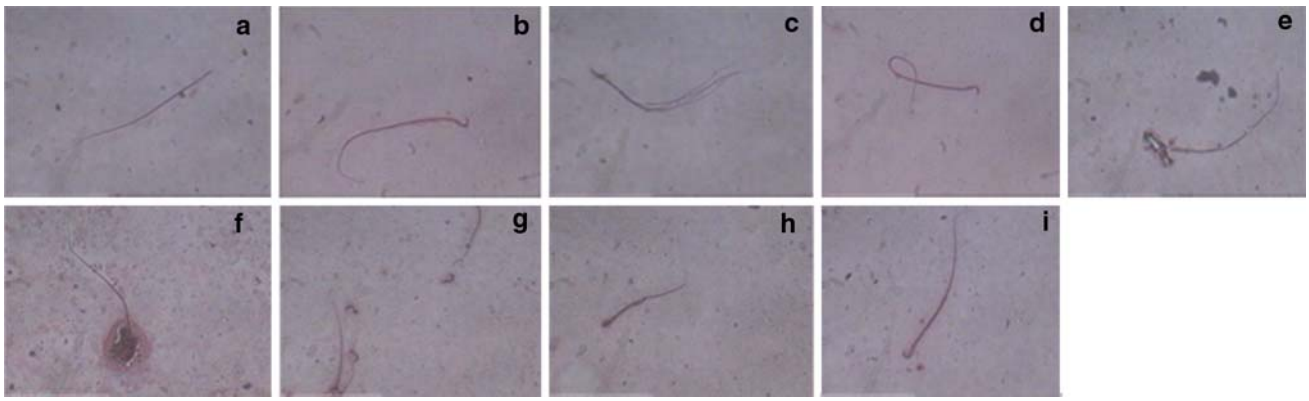

Fig. 2 Abnormal sperm tail and head morphology after lindane exposure. **a** Headless tail, **b** bent tail, **c** multiple tail, **d** coiled tail, **e** broken tail, **f** amorphous head, **g** detached head, **h** pin head, **i** excessive hook

Table 5 Effect of lindane and curcumin on biochemical parameters and antioxidant enzymes of testes

Parameters	Group-I	Group-IIb	Group-III	Group-IV	Group-V	Group-VI	Group-VII
LPO	13.68 ± .03	18.60 ± 0.16**	18.88 ± 0.12**	14.96 ± 0.23**	15.25 ± 0.09**	15.80 ± 0.06**	18.17 ± 0.05**
GSH	90.31 ± 0.01	89.79 ± 0.19*	89.65 ± 0.18*	89.19 ± 0.10**	89.39 ± 0.09**	86.77 ± 0.20**	90.02 ± 0.29*
SOD	66.82 ± 0.02	54.38 ± 0.16**	53.93 ± 0.03**	62.99 ± 0.21**	62.74 ± 0.15**	54.57 ± 0.31**	53.51 ± 0.17**
CAT	104.00 ± 0.02	79.56 ± 0.17**	79.38 ± 0.19**	102.12 ± 0.57**	102.63 ± 0.16**	81.35 ± 0.30**	79.91 ± 0.03**
GST	0.53 ± 0.00	0.44 ± 0.00**	0.43 ± 0.00**	0.52 ± 0.00*	0.53 ± 0.00*	0.49 ± 0.01**	0.41 ± 0.00**

Results shown as mean ± SEM, * ($p > 0.05$), ** ($p < 0.01$)

IIb, and VI) whereas no multiple and broken tail were spotted in control and groups treated with curcumin (group-III, IV, and V). The percentage of coiled and bent tail was high in group-IIa, IIb, and VI as compared to control, while curcumin treated groups were having low percentage of coiled and bent tail as compared to lindane exposed groups. No amorphous and pin head were reported in control, however, in group-IIa and group-IIb, 3% and 4% pin heads were reported, respectively. The percentage of amorphous and pin head was very low in all curcumin treated groups. Like wise the percentage of detached hooks was very high in lindane exposed groups as compared to curcumin treated groups (Table 4). Sperm head and tail morphology on lindane exposure are shown in Fig. 2.

Lindane exposure significantly increased testes MDA level in group IIa, IIb, and VI ($p < 0.01$) as compared to control. However, the difference in MDA level in IIa and IIb was non-significant ($p > 0.05$). Post, pre and co-administration of curcumin in group III, VI, and V significantly ($p < 0.01$) reduced lipid peroxidation as compared to group IIb and it was very similar to control group. The variation in level of GSH on lindane exposure was non-significant ($p > 0.05$) whereas significant ($p < 0.01$) decrease in SOD, CAT, and GST activity was observed as compared to control (Table 5).

The results of this study prove that lindane is toxic to the reproductive system of male wistar rats. Dalsenter et al. (1996) reported that testes are highly susceptible to lindane as it crosses blood testes barrier and depresses spermatogenesis. Lipophillic nature of lindane may leads to its accumulation in testes and resulting in enhancement in lipid peroxidation (Samanta and Chainy 2002). In our studies also we observed increase in lipid peroxidation on lindane exposure. The increase in lipid peroxidation may be due to oxidative stress and enhanced reactive oxygen species (ROS) production because of lindane accumulation in testicular tissue. The increase in lipid peroxidation and generation of ROS may reduce cell viability (Verma et al. 1992; Antunes-Madeira et al. 1993). In earlier reports endosulfan exposure to rats have shown reduction in weight of accessory sex organs (Chitra et al. 1999). The decrease in androgen level on lindane exposure has been reported by Mathur and Chatopadhyay (1982). The decrease in weight of testes and cauda epididymus in our study may be due to ROS induced cell damage resulting in impairment of steriodogenesis leading to low androgen production. We observed decrease in SOD and CAT activity on lindane exposure. Similar finding were observed by Samanta et al. (1999) in rat testes and Chitra et al. (2001) in rat epididymus. Decrease in SOD activity will favour increase in production of ROS which in turn will inactivate CAT (Kono and Fridovich 1982) causing accumulation of H_2O_2 in tissue. The higher level of H_2O_2 promotes formation of free radical (OH^-) in the presence

of Fe^{2+} (Halliwell and Gutteridge 1984) resulting in breakage of biological membranes and cytotoxicity. In our study we observed decrease in GST activity on lindane exposure. Lindane induced reduction in GST activity was reported by Anilakumar et al. (2006) in rat liver. GST helps in metabolism of lindane in mammalian system (Portig et al. 1979). The decrease in GST activity might be responsible for lindane accumulation and multiple toxicity in reproductive system. The study showed significant decrease in sperm head counts, sperm motility and significant increase in sperm abnormalities (abnormality in head and tail morphology) on lindane exposure. These findings may be again due to high level of lipid peroxidation and ROS production in testes resulting in impairment of spermatogenesis. Alteration in the spermatogenesis by chemicals leads to abnormalities in sperm (Hugenholtz and Bruce 1979; Krzanowska 1976). The observed anomalies in the sperm number and structure may be due to lindane induced damage to germ cells as lindane is proven mutagen, carcinogen and teratogen (Wolff et al. 1987). It has been reported earlier that lipid peroxidation and enhanced ROS production in testes and epididymus adversely affects the sperm motility (Ichikawa et al. 1999). Hence, decrease in sperm motility in our study may be due to lindane induced oxidative stress. In group VI lindane was administered for first 14 days and then allowed to metabolize for next 14 days. The study showed that even after 14 days of metabolism, toxic effects of lindane were evident.

Curcumin is a well known antioxidant possessing excellent ROS scavenging activity (Ak and Gülcin 2008; Reddy and Lokesh 1994; Sreejayan and Rao 1996) and a modulator of xenobiotic metabolizing enzymes. Pre, Post and co-administration of curcumin significantly ameliorated lindane induced variations in weight of testes and cauda epididymus, lipid peroxidation, sperm quality and quantity and activity of antioxidant enzymes in present study. In conclusion ROS are involved in the mechanism of lindane induced testicular toxicity. Antioxidants of herbal origin like curcumin possessing excellent ROS scavenging activity provides a wide scope for their use as chemopreventive agents in reducing toxic effects of pesticides and other xenobiotics.

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