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Effects of lindane on reproductive parameters in male rats

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ABSTRACT

The effects of lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane) on male reproductive parameters were studied in sexually mature rats. Epididymal sperm numbers and motility, as well as α-glucosidase activity in the epididymis, were determined after i.p. treatment of lindane at concentrations of 9 and 18 mg kg^{-1} body wt, twice a week for 60 days. The values of epididymal sperm numbers were significantly lower ($P<0.001$) in the animals treated with both doses compared to the control values. Sperm numbers decreased by 42% after treatment with the lower lindane dose, while the drop in sperm count in the animals exposed to the higher dose extended to about 50%. In addition, dose-related changes in epididymal sperm motility were detected in lindane-treated animals. Motility decreased by about 45% to 68% at 9 and 18 mg kg^{-1} body mass, respectively. Relative organ masses (epididymis, prostate, anterior pituitary and testis) were measured at the end of the treatment. A significant decrease ($P<0.01$) in prostate weight was detected in both groups treated, while the weight of the pituitary decreased ($P<0.05$) only in the animals treated with the higher dose. α-Glucosidase activity in the epididymides did not show any statistically significant change after exposure to lindane. A histopathological analysis of testicular tissue from treated rats showed cell disorganization. Cells were irregularly shaped, with marked intercellular space between the spermatogenic cells. Our results imply that organochlorine insecticides like lindane can cause reproductive disorders, and therefore more attention should be directed towards understanding the affects of persistent pesticide residues on reproductive outcomes.

Key words: lindane, reproductive toxicity, sperm parameters, rat

Introduction

Many chemicals, such as pesticides, industrial chemicals, plastics, plasticizers, pharmaceuticals and others present in the environment, have been shown to cause disruptive endocrine effects, yet currently, for many of them, there is no known structure/function relationship (DIAMANTI-KANDARAKIS et al., 2009). Like other persistent organic pollutants, lindane can enter the food chain and lipophylicity facilitates its accumulation

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in the various tissues of living organisms where, after absorption and distribution, it can easily reach the essential tissues of the reproductive system (WHO, 1991; DALSENTER et al., 1996; MENDEZ, 2003).

Lindane has been reported to cause impairment to many biological functions, including reproduction in humans and animals. It has adverse effects on various hormone-dependent reactions in the male reproductive system. The testes have been found to be highly sensitive target organs for lindane, which has been shown to disrupt testicular morphology (DIKSHITH et al., 1978; CHOWDHURY et al., 1987; DALSENTER et al., 1996; SRINIVASAN et al., 1988) and induce epididymal cellular degeneration (CHOWDHURY, 1994). It causes alterations in Leydig and Sertoli cells and impairs their functions (DALSENTER et al., 1996; SUWALSKY et al., 2000; SARADHA et al., 2008). Investigations have revealed (CHOWDHURY, 1994) that exogenous lindane treatment diminishes serum testosterone level, and it has been confirmed that lindane acts as an inhibitor on testicular steroidogenesis (WALSH and STOCCO, 2000; RONCO et al., 2001; SARADHA et al., 2008).

It is generally accepted that testosterone is converted into 5α-reduced metabolites, which interact with their specific receptors to become fully active (KNIEWALD et al., 1971; KNIEWALD and MILKOVIĆ, 1973). Both *in vitro* and *in vivo* lindane exposure interfere with androgen metabolism and with the formation of a 5α-dihydrotestosterone receptor complex in the prostate of rats (ŠIMIĆ et al., 1991; ŠIMIĆ et al., 1992). Reduced sperm count and an increased incidence of sperm abnormalities have been evidenced as the consequences of exposure to lindane (PRASAD et al., 1995; SAMANTA et al., 1999; SARADHA and MATHUR, 2006). Furthermore, as an endocrine disrupting chemical, it may interfere with male reproductive performance and fertility.

This study was conducted to examine the effects of lindane on male reproductive parameters related to spermatogenesis. Adult male rats were treated *i.p.* with two lindane doses (9 and 18 mg kg^{-1} body mass) twice a week for 60 days. The primary goal of the study was to measure the effects of lindane on sperm count, motility and morphological changes in the testis. In addition, the activity of α-glucosidase in the epididymis and relative weight of some androgen-dependent organs were monitored.

Materials and methods

Chemicals. Lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane, 99% purity) was obtained from Sigma-Aldrich Chemical, St. Louis, MO. Redistilled glycerol, purchased from Claro-Prom, Zagreb, Croatia, was used. Glasgow Minimum Essential Medium (MEM) was purchased from GIBCO BRL, Life Technologies, Paisley, Scotland. *p*-Nitrophenyl *α*-D-glucopyranoside (PNPG), and *p*-nitrophenol, spectrophotometric grade, were obtained from Sigma-Aldrich Chemical, St. Louis, MO.

Animals, treatment and tissues. Male Fischer strain rats aged 90 days were obtained from the Animal Unit, Faculty of Medicine, University of Zagreb and maintained in a temperature- and humidity-controlled room with an alternating 12 hour circadian cycle. Animals were housed in plastic cages, given pelleted food for laboratory animals (Institute for Animal Nutrition, Domžale, Slovenia) and water *ad libitum*. The exposure of the animals, randomly divided into three groups, consisted of intraperitoneal injections of lindane suspended in 0.4 mL of glycerol, twice a week over a period of 60 days. The lindane doses were 9 mg kg^{-1} body mass or 18 mg kg^{-1} body mass. The control group received the same volume of glycerol only. Body weights were recorded before the animals were destroyed. The rats were destroyed by decapitation under light ether anaesthesia, 24 hours after the last dose, and the pituitaries, prostates, testes and epididymides were removed and weighed immediately.

Epididymal sperm number and motility. The epididymal sperm number and motility were determined after the animals had been destroyed. Freshly removed epididymides were minced and flushed with 10 mL of MEM preincubated overnight at 37 °C in an atmosphere of 5% CO_2 in air. The number of sperm and motility were measured 2 hours later.

α-Glucosidase activity. The epididymis was minced with scissors, homogenized in five volumes of sodium phosphate buffer (pH 6.8) using a glass-Teflon tissue homogenizer (Kontes Glass Co., NJ) and centrifuged at 15000 g for 15 min (KALLA et al., 1997). A supernatant was used for the estimation of epididymal α-glucosidase activity. The assay was based on the determination of hydrolytic relapse of p-nitrophenol from synthetic substrate PNPG by α-glucosidase (CHAPDELAINE et al., 1978). The supernatant (50 µL) was incubated for a 4-hour period at 37 °C with a constant amount of PNPG (0.5 mg). The addition of 0.1 M Na_2CO_3 stopped the reaction and the quantity of *p*-nitrophenol was measured spectrophotometrically at 405 nm (ANONYM., 1992).

Light microscopy. The testes were fixed in Bouin's solution for 2 hours, dehydrated in graded series of alcohol and embedded in paraplast. The paraplast blocks were cut into 6 µm sections and mounted on slides. Sections were stained with haemalaun and eosin (FARMILO and STEAD, 1989).

Statistical analysis. A two-tailed Student's *t*-test was applied to evaluate the significance of the differences between the means and $P<0.05$ was considered to be significant.

Results

Lindane doses of 9 and 18 mg kg^{-1} body mass were administered to adult male rats by i.p. treatment twice a week for 60 days. On the 61st day after the beginning of lindane exposure, the rats were destroyed and the tissues of the anterior pituitary, ventral prostate,

testes and epididymides were immediately removed. The tissue weights expressed as relative organ weights are shown in Table 1. Changes in the relative weights of the testes and epididymides were not recorded. However, significantly lower relative weights of the anterior pituitaries *vs*. controls were found in the group treated with the higher dose of lindane ($P<0.05$). In both groups tested, a significant ($P<0.01$) decrease in the relative mass of the ventral prostate was also found.

Table 1. Relative organ masses (mg /100 g body mass) of rats after *in vivo* treatment[a] with lindane

Tissue	Control (N = 6)	Lindane treatment	
		9 mg kg^{-1} body mass (N = 6)	18 mg kg^{-1} body mass (N = 5)
Pituitary	3.99 ± 0.304[b]	3.36 ± 0.377	2.92 ± 0.342 **
Ventral prostate	76.97 ± 8.000	49.29 ± 3.343 *	44.97 ± 3.299 *
Testis			
Left	483.28 ± 17.604	450.61 ± 52.062	429.70 ± 32.837
Right	497.02 ± 17.339	520.45 ± 20.153	455.03 ± 12.971
Epididymis			
Left	144.39 ± 5.227	144.45 ± 8.869	138.00 ± 10.447
Right	150.86 ± 5.320	161.68 ± 4.659	147.01 ± 7.851

[a] Intraperitoneal treatment twice a week for 60 days. [b] Values are means ± SEM. Statistically significant differences (Student's *t*-test) *vs* control: *$P<0.01$; **$P<0.05$.

Sperm numbers and motility were measured separately for the left and right epididymides immediately after the animals were destroyed, 24 hours after the last lindane treatment (on day 61). The results in Table 2 show that treatment with either the higher or lower lindane dose induced a significant decrease ($P<0.001$) in epididymal sperm numbers and sperm motility. The sperm number in the control group was about 16.5×10^6, whereas in the groups of rats exposed to lindane it was 9.5×10^6 after treatment with the lower dose, and 8.3×10^6 with the higher dose. Epididymal sperm motility in the control group of animals was about 49.8%, whereas in lindane-exposed rats, the motility of sperms significantly declined. The changes were dose-related (Table 2). The lower dose (9 mg kg^{-1} body mass) diminished sperm motility by about 44.7%, and the higher dose (18 mg kg^{-1} body mass) provoked a decline of about 68.2% compared to control values.

Table 2. Sperm count and motility in rat epididymis after the last day of lindane treatment [a]

Group	Sperm No. $\times 10^6$	Motility (%)
Control (N = 6)		
Left	17.17 ± 0.856	50.33 ± 2.390
Right	15.83 ± 1.238	49.17 ± 3.745
Lindane 9 [b] (N = 6)		
Left	9.50 ± 0.645**	26.00 ± 3.559**
Right	9.50 ± 0.847**	29.00 ± 2.191*
Lindane 18 [c] (N = 5)		
Left	9.00 ± 0.812**	15.00 ± 3.101**
Right	7.60 ± 1.304**	16.60 ± 6.177*

[a]Intraperitoneal treatment twice a week for 60 days. [b]Lindane dose = 9 mg kg^{-1} body mass. [c]Lindane dose = 18 mg kg^{-1} body mass. Values are means ± SEM. Statistically significant difference (Student's *t*-test) *vs* control: *P<0.01; **P<0.001.

The activity of α-glucosidase determined in the epididymides 24 hours after the last lindane treatment (on day 61) is presented in Table 3. α-Glucosidase activity is expressed as units per gram of wet tissue and units per milligram of protein content in the epididymis, where one unit corresponds to one nanomole of *p*-nitrophenol liberated per hour. Similar protein concentrations were found in the epididymis in both treated groups and control animals (~10 mg mL^{-1}). The calculated values for α-glucosidase activity (U mg^{-1} of protein) in the epididymis did not reveal any differences between the controls and both lindane-treated groups. Also, changes in α-glucosidase activity expressed as units per gram of wet epididymis were not detected after the treatment with either the lower or the higher lindane dose (Table 3).

Table 3. α-Glucosidase activity in rat epididymis after *in vivo* treatment[a] with lindane

Group	Epididymal protein conc. (mg mL^{-1})	α-Glucosidase activity [d]	
		U mg^{-1} protein	U g^{-1} tissue
Control (N = 6)			
Left	10.13 ± 0.227	1.57 ± 0.097	38.14 ± 1.124
Right	9.85 ± 0.403	1.45 ± 0.095	38.74 ± 0.839
Lindane 9 [b] (N = 6)			
Left	10.18 ± 0.546	1.44 ± 0.095	34.54 ± 1.224
Right	9.75 ± 0.229	1.37 ± 0.034	38.38 ± 2.002
Lindane 18 [c] (N = 5)			
Left	10.42 ± 0.174	1.34 ± 0.097	32.71 ± 3.170
Right	10.50 ± 0.469	1.44 ± 0.122	37.94 ± 2.370

[a]Intraperitoneal treatment twice a week for 60 days. [b]Lindane dose = 9 mg kg^{-1} body mass. [c]Lindane dose = 18 mg kg^{-1} body mass. [d]U = One unit corresponds to one nmol of *p*-nitrophenol liberated per hour. Values are means ± SEM.

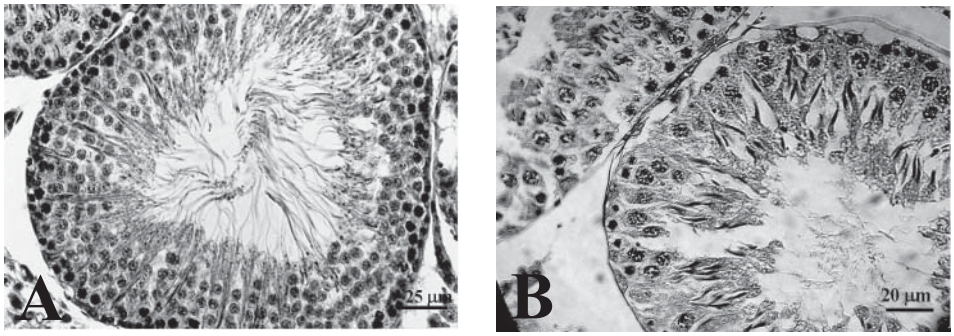

Fig. 1 Histological changes in testicular tissue after lindane i.p. treatment: (A) control; (B) 18 mg kg^{-1} body mass twice a week over 60 days. Magnification 400x.

The morphological changes in the testicular tissue between the control group and the group treated with 18 mg lindane kg^{-1} body mass, twice a week for 60 days, were analyzed by haemalaun-eosin staining (Fig. 1). Nearly all stages of spermatogenesis are shown (Fig. 1A) in a cross-sectioned seminiferous convoluted tubule of a control rat. Sperm align at a place close to the lumen or are free in the lumen, and their long, thin tails are oriented towards the lumen tubules. In Fig. 1A the spermatides are barely visible as small, longitudinal cells with dark nuclei located close to the lumen. However, there are visible holes within the cells in the tubules of lindane-treated animals (Fig. 1B). Spermatogenesis is still present, but the number of cells is smaller per unit of area. The spermatocytes are connected to the lumen and visible, indicating cell disorganization.

Discussion

In the present study, adult male rats were treated *i.p.* with lindane at either 9 or 18 mg kg^{-1} body mass. The duration of treatment was twice a week for 60 days in order to cover the time needed for spermatogenesis. The *i.p.* LD_{50} values for lindane in rats has been reported (CHOWDHURY et al., 1987) to be 36 mg kg^{-1} body mass, and in our experiments, the doses applied were 2-4 times lower. At the end of the treatment period, the rats were generally in good condition, except for a slight reduction in body weight in both lindane-treated groups. Nevertheless, changes in the relative masses of the testes and epididymides were not detected.

We found a statistically significant ($P<0.001$) fall in epididymal sperm count in both treated groups. After lindane doses of 9 mg kg^{-1} body mass, there was 42.5% inhibition in the left and right epididymides compared with control values. The higher lindane dose (18 mg kg^{-1} body mass) provoked a 45.5% drop in sperm numbers in the left epididymis and 54% in the right epididymis, compared with controls. Sperm motility was altered in both treated groups. At the lower lindane dose, it was inhibited by 47.8% in the left and 41.8% in the right epididymis, while the higher lindane dose provoked an even greater

fall in sperm motility, 69.9% in the left and 66.7% in the right epididymis, compared to control values. The lindane dose of 18 mg kg^{-1} body mass resulted in between 19% and 22% more inhibition of sperm motility compared to the lower dose of 9 mg kg^{-1} body mass.

α-Glucosidase, produced mainly by the epididymal ephitelium, is a normal constituent of semen, which significantly correlates to sperm count, and its activity is low in cases of epididymal obstructions (KRAUSE and BOHRING, 1999). A number of studies have shown positive correlations between the seminal levels of α-glucosidase activity and sperm motility (VILJOEN et al., 1990; FOURIE et al., 1991), but these results have been contradicted by other reports (GUERIN et al., 1990; KRAUSE and BOHRING, 1999; ELZANATY et al., 2002). In the present study, we did not detect changes in α-glucosidase activity, but at the same time, sperm motility was significantly lowered.

Exposure to technical grade hexachlorocyclohexane by dermal exposure, 5 days per week for 120 days, resulted in a significant accumulation of its isomers, including lindane, in the testes and sperm of treated rats (PRASAD et al., 1995). In rats dosed orally either with 6 mg kg^{-1} body mass for 5 days, or with a single dose of 30 mg kg^{-1} body mass, lindane was still detected in the brains and testes 2 weeks after treatment (DALSENTER et al., 1996). Reports suggest (PANT et al., 2007) that lindane readily penetrate the blood testis barrier, directly affecting spermatogenesis. The accumulation of lindane and its isomers in target sites may possibly be responsible for various biochemical alterations, resulting in reduced spermatogenesis, leading to a decrease in sperm count and motility, and an increase in morphological abnormalities (PRASAD et al., 1995). Lindane has been reported to intercalate into the sperm membrane and alter the molecular dynamics of the bilayer (SILVESTRONI and PALLESCHI, 1999). A significant association between hexachlorocyclohexane isomers and some DDT metabolites in the semen of infertile humans with semen quality problems has been established (PANT et al., 2007). The biochemical and histological effects in the testes of rats following treatment with lindane have been reported (DIKSHITH et al., 1978; SRINIVASAN et al., 1988). Seminiferous tubules and Leydig cells degenerated during treatment with doses of 8 mg kg^{-1} daily, over a 10-day period (CHOWDHURY et al., 1987). The atrophy of seminiferous tubules carrying necrosed spermatogenic cells was observed after lindane-treatment (SRINIVASAN et al., 1988).

Testicular tissue was analyzed morphologically by light microscopy (Fig. 1). In the lindane-treated rats, the cells were irregularly shaped and there was marked intercellular space between the spermatogenic cells. Spermatogenesis was still present, but cell disorganization was found.

According to the present results and previously published reports (PRASAD et al., 1995; DALSENTER et al., 1996; RONCO et al., 2001; SARADHA et al., 2008), much more attention should be paid to the possible effects of environmentally persistent pesticides,

even if they are banned in most developed countries, in view of the fact that they may induce changes at the cellular level to crucial stages in the reproductive processes.

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SAŽETAK

Učinci lindana (γ-1,2,3,4,5,6-heksaklorociklohеksan) na reprodukcijske pokazatelje istraživani su u spolno zrelih štakora. Broj i pokretljivost spermija, te aktivnost α-glukozidaze u epididimisu određivani su nakon i.p. primjene lindana u koncentracijama od 9 i 18 mg kg-1 tjelesne mase dvaput tjedno tijekom 60 dana. U usporedbi s kontrolnim vrijednostima broj spermija u epididimisu značajno je bio smanjen (P<0,001) u obje lindanom obrađene skupine životinja. Nakon davanja manje doze broj spermija je bio niži za 42%, dok je kod više doze smanjenje iznosilo oko 50%. Također su u pokretljivosti spermija utvrđene promjene ovisno o dozi lindana. Smanjenje pokretljivosti iznosilo je 45%, odnosno 68% uz primijenjene doze od 9 i 18 mg kg-1 tjelesne mase. Relativne mase organa (epididimis, prostata, hipofiza i testis) mjerene su po završetku obrade. Značajno smanjenje težine prostate (P<0,01) utvrđeno je u obje obrađene skupine, dok je težina hipofize bila smanjena (P<0,05) samo uz višu dozu lindana. Izloženost lindanu nije prouzročila statistički značajne promjene aktivnosti α-glukozidaze u epididimisu. Patohistološka analiza tkiva testisa pokazala je propadanje stanica. Stanice su poprimile nepravilne oblike uz dosta međustaničnog prostora između spermatogenih stanica. Ovi rezultati pokazuju da organoklorirani insekticidi poput lindana mogu izazvati reprodukcijske poremećaje, te da je potrebno više pažnje usmjeravati razjašnjenju učinaka ostataka postojanih pesticida na reprodukcijske ishode.

Ključne riječi: lindan, reprodukcijska toksičnost, pokazatelji kvalitete spermija, štakor