Effect of Endosulfan on Male Reproductive Development

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There is experimental evidence of adverse effects of endosulfan on the male reproductive system, but there are no human data. Therefore, we undertook a study to examine the relationship between environmental endosulfan exposure and reproductive development in male children and adolescents. The study population was composed of 117 male schoolchildren (10–19 years of age) of a village situated at the foothills of cashew plantations, where endosulfan had been aerially sprayed for more than 20 years, and 90 comparable controls with no such exposure history. The study parameters included recording of clinical history, physical examination, sexual maturity rating (SMR) according to Tanner stages, and estimation of serum levels of testosterone, luteinizing hormone (LH), follicle-stimulating hormone, and endosulfan residues (70 study and 47 control subjects). Mean ± SE serum endosulfan levels in the study group (7.47 ± 1.19 ppb) were significantly higher ($p < 0.001$) than in controls (1.37 ± 0.40 ppb). Multiple regression analysis showed that SMR scoring for development of pubic hair, testes, penis, and serum testosterone level was positively related to age and negatively related to aerial exposure to endosulfan (AEE; $p < 0.01$). Serum LH levels were significantly positively related to AEE after controlling for age ($p < 0.01$). The prevalence of congenital abnormalities related to testicular descent (congenital hydrocele, undescended testis, and congenital inguinal hernia) among study and controls subjects was 5.1% and 1.1%, respectively, but the differences were statistically nonsignificant. Our study results suggest that endosulfan exposure in male children may delay sexual maturity and interfere with sex hormone synthesis. Our study is limited by small sample size and nonparticipation. *Key words:* **endocrine disruptor, endosulfan, luteinizing hormone, male reproductive development, sexual maturity rating, testosterone.** *Environ Health Perspect* **111:1958–1962 (2003). doi:10.1289/ehp.6271 available via *http://dx.doi.org/* [Online 22 September 2003]**

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is a broad-spectrum insecticide and acaricide first registered for use in the United States in 1954 to control agricultural insect and mite pests on a variety of field, fruit, and vegetable crops. Technical-grade endosulfan is composed of two stereochemical isomers, α-endosulfan and β-endosulfan, in concentrations of approximately 70% and 30%, respectively. Use data from 1987 to 1997 indicate an average domestic use of approximately 1.38 million pounds of active ingredient per year [U.S. Environmental Protection Agency (U.S. EPA) 2002]. It has been found in at least 162 of the 1,569 current National Priorities List sites by the U.S. EPA (HazDat 2000). In India, it is widely used against a variety of agricultural pests. During 1999–2000, about 81,000 metric tons of endosulfan was manufactured in India, and in terms of tonnage its production was next only to mancozeb (103,000 metric tons) and monocrotophos (95,000 metric tons) (Anonymous 2001).

Oral LD50 (lethal dose sufficient to kill 50% of population) endosulfan in rats is 80 mg/kg, and it has been classified as a moderately hazardous (class II) pesticide [World Health Organization (WHO) 2002]. Neurotoxicity is the major end point of concern in acute endosulfan exposure in human beings and experimental animals. No data are available for subacute or chronic exposure to endosulfan in human subjects; however, the subacute and chronic toxicity studies of endosulfan in animals suggest that the liver, kidneys, immune system, and testes are the main target organs [Agency for Toxic Substances and Disease Registry (ATSDR) 2000].

In recent years, there has been growing concern about toxicity of a number of chemicals, including pesticides, on the male reproductive system (Murray et al. 2001; Sharpe 2001). Reported effects of endosulfan on the male reproductive system in experimental animals have been variable, depending on species, age at exposure, dose, duration of exposure, and study end points. Routine gross and histopathologic examination of the reproductive organs of male mice that consumed doses of 7.3 mg/kg/day for 13 weeks (Hoechst. Unpublished data) or 2.5–5.0 mg/kg/day for 2 years [Hack et al. 1995; Hoechst. Unpublished data; National Cancer Institute (NCI) 1978] revealed no toxic effects. Later on, more detailed studies in adult rats exposed to 2.5, 5, and 10 mg/kg/day endosulfan for 5 days per week for 10 weeks showed reduced intratesticular spermatid counts, sperm abnormalities, and changes in the marker enzymes of testicular activities, such as lactate dehydrogenase, sorbitol dehydrogenase, γ-glutamyl transpeptidase, and glucose-6-phosphate dehydrogenase, providing further evidence of effects on spermatogenesis (Khan and Sinha 1996; Sinha et al. 1995). Exposure of younger animals (3 weeks old) showed marked depletion of spermatid count as well as decreased daily sperm production at a dose of 2.5 mg/kg/day (Sinha et al. 1997), which was earlier seen only at 5 mg/kg/day in adult rats by the same investigators (Sinha et al. 1995). More recent studies have shown that exposure of pregnant rats to endosulfan at 1 mg/kg/day from day 12 through parturition leads to decreased spermatogenesis in offspring (Sinha et al. 2001). Dalsenter et al. (1999) reported similar observations at 3 mg/kg/day but not at 1.5 mg/kg/day, and they attributed this to strain variation (Dalsenter et al. 2003). Thus, experimental studies suggest that endosulfan can affect the male reproductive system and also that these effects are likely to be greater if exposure occurs during the developmental phase.

Environmental exposure to a single chemical over a long period of time is very rare. We came across a situation where endosulfan was the only pesticide that had been aerially sprayed two to three times a year for more than 20 years on cashew nut plantations situated on hilltops in some villages of northern Kerala, India (Figure 1). The population living in the valley had a significant chance of exposure to this pesticide during aerial spray and subsequently through other contaminated environmental media. This population, therefore, provided a unique opportunity to study the long-term health effects of endosulfan. In this article, we report the effects of endosulfan on male reproductive development.

Materials and Methods

Selection of study and control areas. The exposed population was defined as schoolchildren who were permanent residents of the

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village situated below the cashew plantations where endosulfan had been sprayed aerially. This village had 12 first-order streams originating from the cashew plantations. Most of the habitations were along the valleys and close to the stream banks. Most of the inhabitants depended on runoff water for irrigation purposes. The control population was selected from schoolchildren of another village situated approximately 20 km away. The population of this village was comparable with the exposed population in socioeconomic status, ethnicity, and occupational characteristics but without any history of aerial endosulfan spray. The control village did not have streams.

Selection of study and control subjects. The main study was carried out to explore the effects of endosulfan exposure on growth and development in 619 schoolchildren of both sexes (5–19 years) and 416 comparable controls. All male children (272 exposed and 135 controls) older than 10 years were asked to participate in a sexual maturity rating (SMR) study; 117 (43%) exposed and 90 (67%) controls participated in SMR examination. For the hormone study, every other student who participated in the SMR study was requested to donate a blood sample.

Study parameters. The study parameters included recording of clinical history in a specially designed pro forma physical examination, assessment of SMR by Tanner's classification (Marshall and Tanner 1969), and estimation of serum levels of testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and endosulfan residues.

Figure 1. Cashew nut plantations on the hills and houses in the valley. Water streams formed in the hills pass through residential zones in the valley.

Ethical aspects. This study was approved by the ethics committee of the National Institute of Occupational Health. Parents, who were requested to accompany their children at the time of examination, were told the objectives of the study, and a consent form in local language was read aloud to them. The children were examined only after one of the parents gave written consent. In addition, special consent of the child was taken for the SMR study, and only in willing cases were blood samples collected. The SMR examination was carried out by pediatricians observing necessary privacy required for this delicate examination.

Collection, storage, and transport of blood samples. Five milliliters of venous blood were collected from each willing individual between 1000 and 1200 hr on the day of examination and was centrifuged at 5,000 rpm for 5 min in the field laboratory. Serum was separated and stored at –20°C in a nearby hospital. The serum samples were air-shipped under dry ice to the laboratories at National Institute of Occupational Health, Ahmedabad, India. To avoid observer bias, the samples were coded before being handed over for analysis.

Chemicals and standard control materials for analysis of endosulfan in serum samples. All the chemicals and reagents used in the extraction and cleanup of endosulfan residues were highly pure HPLC (high-performance solvents filtered through 0.2-µ filters and packed under nitrogen)-grade obtained from Qualigens Fine Chemicals (Glaxo India Ltd., Mumbai, India) and were checked for any pesticide contamination. Glassware used was free from residue contamination. Standard reference materials of α-endosulfan (99.0%), β-endosulfan (99.0%), and endosulfan sulfate (99.0%) were a gift from M/s Excel Ltd., (Mumbai, India), which is the largest manufacturer of endosulfan in India.

Extraction of residues from serum. Extraction was modified from techniques described in Dale et al. (1966) and U.S. EPA (1980). Briefly, serum (0.5 mL) was pipetted into a graduated stoppered centrifuge tube, 6 mL of hexane was added, and it was rotated in a slow-speed Roto-rac (National Institute of Occupational Health, Ahmedabad, India) for 2 hr. The organic layer was transferred into another graduated tube and was evaporated to dryness under a stream of nitrogen. The final volume was made up with hexane corresponding to the expected concentration of the residue. A suitable aliquot was injected into a gas chromatograph with an electron capture detector. We calculated the recoveries of endosulfan residues, which ranged from 88 to 102%. In addition, fortified samples were studied as a part of quality assurance and quality control.

Instrumentation and quantification. We used a gas chromatograph (model 6890) equipped with a Micro Electron Capture Detector, a capillary column (HP 5, 60 m, 0.25 mm inner diameter, film thickness 0.25 µm; all these items from Hewlett Packard Agilent Plus; Agilent Technologies, Little Fall, DE, USA), and N_2 (ultra high purity, 99.999% grade) as carrier gas for the quantification of endosulfan residues. The initial oven temperature was 80°C with ramp rate of 20°C per min to 200°C. The injector port temperature was 220°C (splitless mode), and detector temperature was 275°C. We quantified the the samples by comparing the peak area of each with those of their respective standards. The retention times of α-endosulfan, β-endosulfan, and endosulfan sulfate were at 38.9, 52.5, and 67.2 min, respectively (Figure 2A,B).

Hormone analyses. We estimated testosterone, LH, and FSH in 50 µL and 100 µL serum samples of study and control subjects using radioimmunoassay kits procured from Immunotech (Marseille, France). We used a Wallac 1470 Wizard autogamma counter (Perkin Elmer, Turku, Finland) to count radioactivity with detection efficiency of 78% for I^{125} and negligible cross talk with other isotopes. Equipment and glassware were segregated to prevent cross-contamination. The hormones were estimated in serial dilutions of serum along with parallel curves to standard. All the samples for hormone estimations were processed in one assay to rule out interassay variations. We performed a linearity study to assess the sensitivity of the hormone assays by serial dilution of a

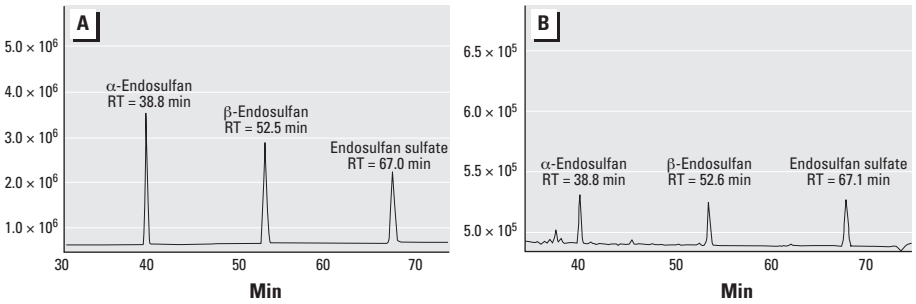

Figure 2. Chromatograms of α-endosulfan, β-endosulfan, and endosulfan sulfate. RT, retention time. (*A*) Standard chromatogram of α-endosulfan, β-endosulfan, and endosulfan sulfate. (*B*) Chromatogram of α-endosulfan, β-endosulfan, and endosulfan sulfate in blood sample of a study subject.

high level with the zero standards. The sensitivity to testosterone, LH, and FSH was 2.5 ng/dL, 0.45 IU/L and 0.02 IU/L, respectively.

Statistical analysis. The statistical software SPSS, release 6.1.4 (SPSS Inc., Chicago, IL, USA), was used for statistical analysis of data and for making graphs. For the multiple regression analysis, age and aerial exposure to endosulfan (AEE; study = 1, control = 0) were taken as independent variables, and parameters of SMR and serum hormone levels were taken as dependent variables. Exact age in years on the date of examination was used as the age variable.

We used the following multiple regression equation: SMR score = $b_0 + b_1$ age + $b_2 \times$ AEE, where b_0 is the regression constant and b_1 and b_2 are the regression coefficients of age and exposure, respectively, fitted for SMR scores of pubic hair, testes, and penis. Similarly, multiple regression equations were fitted for testosterone, LH, and FSH levels.

Results

Table 1 shows the comparison of the mean ± SD values of the age and some of the growth-related basic parameters in study and control subjects participating in the SMR study. There are no statistically significant differences in the mean values of these parameters in two groups. The mean values of height, weight, body mass index, and skin-fold thickness of the nonparticipating subjects in study and control groups with respect to age were also comparable, indicating that the nonparticipation of the subjects may not have had significant effects on the outcome of the study.

Clinical examination revealed six (5.1%) cases of congenital malformations in the study group, consisting of undescended testis (two), congenital hydrocele (three), and congenital inguinal hernia (one), and only one case (1.1%) of congenital inguinal hernia in the control group. The differences in prevalence of congenital malformation were statistically nonsignificant.

SMR score. A summary of the multiple regression analysis of parameters of SMR score on age and AEE is presented in Table 2. The values of R^2 corresponding to SMR of pubic hair, testes, and penis are 0.48, 0.43, and 0.43, respectively, are statistically significant ($p < 0.001$), and indicate that considerable proportion of variance of SMR scores can be attributed to age and AEE. The statistically significant negative regression coefficient (b_2) of AEE in all three equations signifies delayed sexual maturity in the study group compared with the control group. As expected, age had a significant positive regression coefficient.

These effects of AEE are further elaborated graphically in Figures 3–5. In Figure 3, the findings for SMR for pubic hair is plotted against age for study and control individuals. The regression lines drawn for the study and control groups indicate that the SMR score for the study group is lower for the same age. Similar observations were made for the SMR score for testicular (Figure 4) and penis development (Figure 5).

Serum hormone levels. Table 3 shows the summary of the multiple regression analysis of serum testosterone levels against age, AEE, and serum LH levels. The value of R^2 is 0.61, which means that 61% of the variations observed in serum testosterone levels can be explained on the basis of age, AEE, and serum LH levels. Very small p-values (< 0.001 in all cases) of the regression coefficient indicate that statistically these parameters are significant in determining the serum testosterone levels. The positive sign of the regression coefficient for age and LH indicates that with increase in age and serum LH levels, there is an increase in the serum testosterone levels. This is a well-known physiologic fact. On the other hand, the regression coefficient for AEE has a negative sign, which means the serum testosterone levels of individuals belonging to the study group are statistically lower than expected from age and serum LH levels. The levels of FSH were slightly higher for the age in study group; however, the differences were statistically nonsignificant.

In Figure 6, serum testosterone levels are plotted against age for study and control individuals. Regression lines drawn for the study and control individuals indicate that the average serum testosterone levels for the study group are lower for the same age. Similarly, in Figure 7, serum LH levels are plotted against

Table 1. Comparison of the mean (± SD) age and some of the growth-related basic parameters in study and control subjects participating in the SMR examination.

Parameters	Control (n = 90)	Study (n = 117)
Age (years)	13.10 ± 2.12	12.80 ± 2.07
Height (cm)	141 ± 10.60	139 ± 13.30
Weight (kg)	30.70 ± 7.44	29.50 ± 8.93
Body mass index	15.30 ± 1.98	15.00 ± 2.11
Skin-fold thickness (mm)	7.31 ± 2.15	7.40 ± 2.28

Table 2. Summary of the multiple regression analysis of parameters of SMR against age and history of exposure to endosulfan through aerial spray.

Dependent variable	R^2	Intercept (b_0)	Age b_1	Age SE	AEE b_2	AEE SE
SMR score						
Pubic hair	0.48**	–2.54**	0.36**	0.03	–0.43**	0.11
Testes	0.43**	–2.07**	0.32**	0.03	–0.32*	0.11
Penis	0.43**	–2.00**	0.32**	0.03	–0.37*	0.12

No. of observations: study = 117; control = 90.
*$p < 0.01$. **$p < 0.001$.

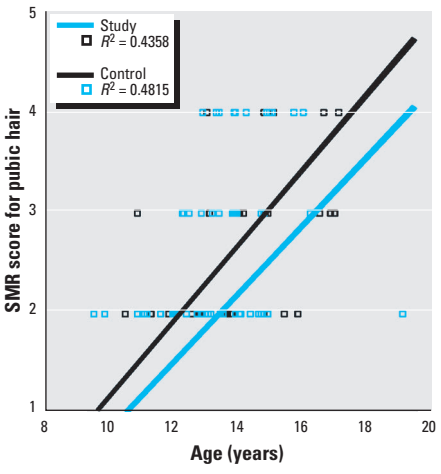

Figure 3. SMR score for the development of pubic hair according to age and AEE.

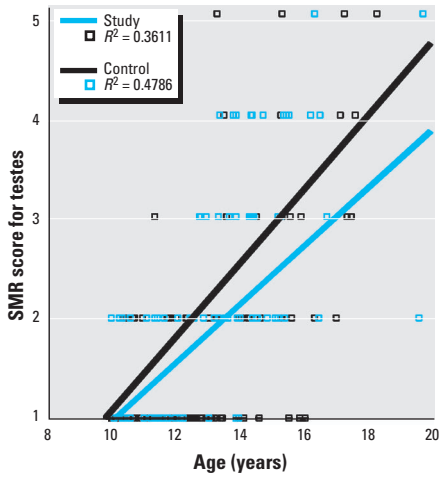

Figure 4. SMR score for the development of testes according to age and AEE.

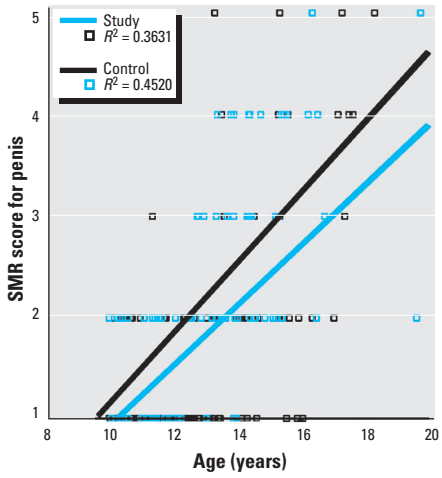

Figure 5. SMR score for the development of the penis according to age and AEE.

age in study and control individuals. The regression lines indicate that average serum levels for the same age are higher in the study group.

Endosulfan exposure. Endosulfan was detected in serum samples of 78% of the children in the study group and 29% of the children in the control group. Table 4 shows the serum endosulfan levels in the study and control groups. The levels of endosulfan in the study group children are significantly higher ($p < 0.001$).

Discussion

Our study results, after controlling for age, showed significantly lower SMR scores and serum testosterone levels and higher levels of serum LH in the study group compared with controls. To link these changes with endosulfan exposure, we should look at two issues: biologic plausibility of the cause–effect relationship and pathways of endosulfan exposure.

Biologic plausibility. There are reports of testicular toxicity of endosulfan manifested as decreased spermatogenesis and testicular hormone synthesis (steroidogenesis), as evidenced by a decrease in spermatid count in testes and in sperm count in the cauda epididymis and by changes in marker enzymes for testicular steroidogenesis in adult animals (Chitra et al. 1999; Singh and Pandey 1989, 1990; Sinha et al. 1995). These effects were seen at much lower dosages and shorter durations if exposures occurred during the prenatal or prepubertal periods (Dalsenter et al. 1999; Sinha et al. 1995, 1997, 2001). Singh and Pandey (1990) also reported profound decreases in the levels of plasma LH, FSH, and testosterone associated with decrease in testicular testosterone in pubertal rats exposed to endosulfan for 30 days. Thus, our observations of low testosterone levels in male children conform with the animal studies. Lower SMR scores appear to reflect lower serum testosterone levels for age. In our study, it is not possible to confirm disturbed spermatogenesis observed in animal studies.

The higher prevalence of congenital abnormalities related to testicular descent observed in the study group should not be overlooked simply because it failed to achieve statistical significance (which may be due to small sample size), because there is indirect evidence of endosulfan exposure associated with undescended testes in a human population from Spain. Garcia-Rodriguez et al. (1996) reported a higher incidence of hospital admissions to University of Granada Hospital for cryptorchidism from districts near the Mediterranean coast, where there is intensive use of pesticides. A subsequent study reported endosulfan isomers and/or metabolites in adipose tissue of 40% of children who were admitted to the same hospital for a variety of reasons (Olea et al. 1999), indicating that significant endosulfan exposures occurred in the region. In the present study, there is a definite history of endosulfan exposure that is likely to have occurred during the prenatal period.

Pathways of endosulfan exposure in the study population. In our study, we estimated endosulfan residues in biologic and environmental samples. The practice of aerial spraying of endosulfan was discontinued in December 2000. Serum endosulfan residue levels were significantly higher in the study population than in the control group even 10 months after the last aerial spray (October 2001). Moreover, endosulfan residues were detected in water (≥ 0.03 ppb) and pond sediments (≥ 0.3 ppb) only in the study area 1.5 years (June 2002) after the last aerial spray. This signifies that low-level endosulfan exposures continued to occur probably by translocation from the hilltops to the valley in the study area long after the aerial spray. This is supported by the report of Regional Remote Sensing Service Center (RRSSC), Bangalore, India (Nageswara Rao PP. Personal communication). On the basis of analysis of satellite pictures of the study area, the RRSSC reported, "The watershed characteristics are favorable for any aerially sprayed toxicant to reach the soil-water-plant continuum in a very short span of time and get accumulated" (Nageswara Rao PP. Unpublished report). Endosulfan has a half life of 60–800 days in soil (ATSDR 2000). Frank et al. (1982) have also reported that because of its persistence in soil, endosulfan residues were detected in water samples throughout the year (outside the spray season) with storm runoff. The results of several laboratory and greenhouse studies indicate that α- and β-endosulfan are strongly adsorbed to soil (Bowman et al. 1965; El Beit et al. 1981a, 1981b). The study area has an annual rainfall of 140 inches. Twelve first-order streams originate from the cashew plantations. It is likely that endosulfan sticking on the soil is carried by runoff water during most of the year.

We have measured endosulfan levels only once in serum samples. These individual endosulfan measures may or may not accurately reflect the chronic levels and/or levels during critical developmental phases. However, the effect of this would be to decrease power of the study (via an increase in random misclassification of exposure) and thus bias toward the null. We believe that even single estimations of serum endosulfan levels validate that children exposed to endosulfan via aerial spraying do, on average, have higher exposures than children in the control group.

Finally, it is important to discuss and resolve the following weaknesses of the study. First is nonparticipation in SMR study: 57% of the exposed and 33% of the control participants did not agree to undergo SMR examination. However, growth-related end points (height, weight, and skin-fold thickness) were

Table 3. Summary of the multiple regression analysis for serum testosterone levels against age, history of AEE, and serum LH levels.

Dependent variable	Age		Exposure		LH	
	b	SE	*b*	SE	*b*	SE
Testosterone	0.37**	0.06	–0.62**	0.21	1.09**	0.20

No. of observations: study = 67; control = 46. Overall $R^2 = 0.61$. **$p < 0.001$.

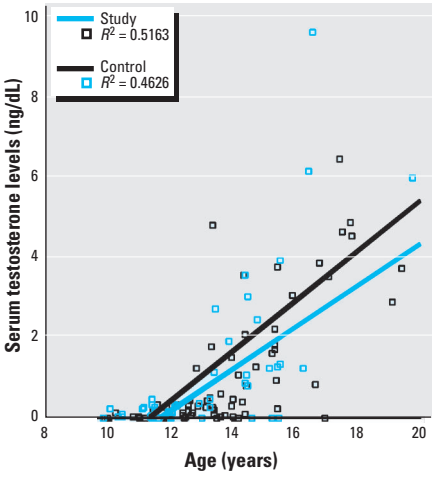

Figure 6. Serum testosterone levels according to age and AEE.

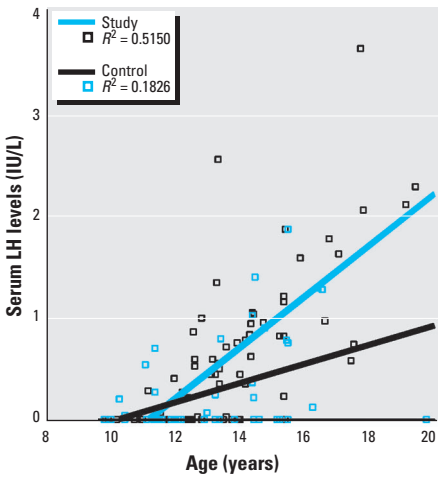

Figure 7. Serum LH levels according to age and AEE.

Table 4. Mean ± SE levels (ppb) of serum endosulfan in study and control subjects.

	Control ($n = 45$)	Study ($n = 70$)
α-Endosulfan	0.87 ± 0.23	4.24 ± 0.74**
β-Endosulfan	0.40 ± 0.17	1.77 ± 0.36**
Endosulfan sulfate	0.10 ± 0.08	1.47 ± 0.33**
Total endosulfan	1.37 ± 0.40	7.47 ± 1.19**

**$p < 0.001$.

comparable among the nonparticipating groups. It is still difficult to envisage the overall effect of nonparticipation. Second is one-time collection of blood samples for hormone analysis: Sex hormone secretion is pulsatile in nature, with a lot of diurnal variation. To minimize this effect, we collected blood samples in all cases between 1000 and 1200 hr. Because the variation in the blood hormone levels is random, the overall effect of natural variation is likely to be minimized. However, the increased random variability of hormone would have decreased the power of the study via an increase in random misclassification of testosterone levels, which in turn would bias toward the null.

Our study results suggest that endosulfan exposure may delay sexual maturity and interfere with hormone synthesis in male children. To validate these findings, a study with larger sample size is essential. We do not know the significance of these findings. Long-term follow-up of the children is essential to understand the implications.

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