

Clinical and Cytogenetic Effects in Habitants under Large Duration Exposure of Endosulfan

¹R. Saraswathy, ¹G. Alex, ¹B.M. Basil, ¹A.R.S. Badarinath, ¹R. Girish, ¹G. Ribu, ¹V.G. Abilash,
²G.D.J. Cruz and ³K.M. Marimuthu

¹Division of Biomolecules and Genetics, School of Bio Sciences and Technology,
VIT University, Vellore-632014, TamilNadu, India

²District Medical Officer, Kasaragod, Kerala, India

³Emeritus Professor, Madras University, Chennai, India

Abstract: No data is available on the genotoxic effects of long term exposure of endosulfan on human population. A study was undertaken to examine the environmental long term endosulfan exposure ranging from 6 years to 40 years on the clinical and cytogenetic nature of human. The study population was composed of eight families consisting of 35 individuals. They are permanent residents of the village situated below the cashew plantation where endosulfan had been sprayed aerially. Thus the residents had the exposure of endosulfan through aerial spray and subsequently through other contaminated environmental media for several years. Clinical nature of all the 35 subjects were recorded with the help of the physicians and 11 of them (31.4%) showed severe clinical deformity ranging from mental retardation to bone marrow cancer showing neurotoxicity and immunotoxic effects of endosulfan exposure. The cytogenetic studies were carried out using peripheral blood leucocyte cultures and giemsa banding technology and scoring chromosome aberrations as endpoint. It was observed that chromosome aberration per cell was 0.121 ± 0.09 which was significantly higher, when compared with the control samples showing 0.04 ± 0.0001 . This study is the first of its kind in literature. This study shows a clear relationship between the human health hazards and environmental pollutant.

Key words: Chromosome aberrations, endosulfan genotoxicity, environmental pollutant, neurotoxicity

INTRODUCTION

Since the early 1940's there has been a tremendous proliferation of man made chemicals into the environment. Of increasing concern is the fact that a majority of these chemicals has not been evaluated for potential danger to the present or to future generations either as mutagens, carcinogens or teratogens. One of the man made genotoxic chemicals, endosulfan, is sprayed onto crops and the spray travels long distances before it lands on crops, soil and water. It is an insecticide with estrogenic activity that is toxic to many fish and mammals. Some reports suggested that it could accumulate in aquatic animals and cause human fatalities. Exposure to endosulfan happens mostly from eating contaminated food, skin contact, breathing and drinking contaminated water.

Since endosulfan is relatively labile and is considerably less toxic to mammals compared with other cyclodiene insecticides (Goebel *et al.*, 1982; Sutherland *et al.*, 2002), it has been widely used as a broad-spectrum insecticide for the control of numerous

insects in a wide variety of food and non-food crops. It may also be used as a wood preservative.

Neurotoxicity and endocrine-disrupting potential of endosulfan were demonstrated in animal experiments (Hiremath and Kaliwal, 2002), *in vitro* assays (Kang *et al.*, 2001; Bisson and Hontela, 2002) and yeast-based reporter gene assays (Graumann *et al.*, 1999). 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].

Hence this study is undertaken in human subjects to fulfill the lacunae. To our knowledge the results presented here appear to be the first to demonstrate the genotoxic effect of long duration exposure of endosulfan on human population using cytogenetic and clinical parameters.

Endosulfan was the only pesticide that had been aerially sprayed two to three times a year for more than 20

Corresponding Author: R. Saraswathy, Division of Biomolecules and Genetics, School of Biosciences and Technology, VIT University, Vellore-632014, TamilNadu, India. Tel: 9443328424/91 416 2202390/2202373; Fax: 91-416-2243092 / 224041

years on cashew nut plantations situated on hilltops in some villages of northern Kerala, India. 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. Hence for this study the subjects were selected from Kasaragod district, a hilltop area in Kerala, India.

MATERIALS AND METHODS

Area of study: Endosulfan 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. As 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, therefore, it would provide a unique opportunity to study the long-term health effects of endosulfan, on human habitants of this area.

Selection of study and control areas: The exposed populations were permanent residents of the village situated below the cashew plantations where endosulfan had been sprayed aerially. These villages had 12 first-order streams originating from the cashew plantations. Most of the habitations were along the valleys and close to the stream banks, and they depended on runoff water for irrigation purposes. A total of 6 panchayats in the district which had the incidence of severe cases were selected for this study. Eight families consisting of 35 individuals were selected for clinical studies and 16 subjects were used for cytogenetic analysis.

Study parameters: The study parameters included recording of clinical history with the help of the physicians in a specially designed proforma and pedigree analysis of the families and cytogenetic studies using chromosomal aberrations as endpoint.

Ethical aspects: The participants were told the objectives of the study, and a consent form in local language was read aloud to them and only in willing cases from whom blood samples were collected.

Cytogenetic studies:

Collection, storage and transport of blood samples: Five milliliters of venous blood were collected from each willing individual and were transported to Biomedical Genetics Research Lab at VIT University within 24 h for culturing. To avoid observer bias, the samples were coded before being handed over for analysis.

Table 1: The frequency and the nature of clinical deformities observed in endosulfan exposed individuals from Kasaragod area

S.No.	Family code	Diagnosis /No. of affecteds	No. of individuals studied in the family
1	ENS - 1	Mental retardation, seizures and cerebral palsy / 9 yr old male affected	5
2	ENS - 2	Mental retardation, deformities of hands and legs / 24 yr old male affected	4
3	ENS - 3	Osteogenesis imperfecta, limb deformities /9 yr and 12 yr females (sisters) affected	6
4	ENS - 4	Mental retardation, hepatosplenomegaly and hydrocephaly/ 6 yr old male affected	4
5	EN - 1	Mental retardation, paralysis, locomotor problem, speech problem, strabismus / 6 yr old male affected	5
6	EN - 3	Uterine, bone marrow cancer/ 45 yr old female affected	3
7	EN - 4	Optical atrophy, nystagmus/ 8 yr and 10 yr old males (brothers) affected	4
8	EN - 5	Muscular dystrophy/8 yr and 11 yr old males (brothers) affected 11 affecteds	4 35 individuals / 8 families

Chromosomal aberration analysis: Chromosome preparations were obtained from PHA (phytohemagglutinin) stimulated peripheral blood lymphocytes by using modified method of Hungerford (1965). At least fifty well spread metaphase plates were scored by direct microscopic analysis. Well spread metaphases were photographed under oil immersion objective lens (100X) Leica DM2000 microscope with metasystem camera & photomicrographs of banded spreads were karyotyped using automatic IKaros software (metasystems). The karyotype was described according to International System for human Cytogenetic Nomenclature (ISCN, 2005).

RESULTS

The clinical nature of the study subjects' and the frequency of chromosomal aberrations analysed in endosulfan exposed subjects are presented in Table 1 and 2, respectively.

Clinical examinations of all the 35 subjects belonging to 8 families were recorded with the help of the physicians. Eleven of them (31.4%) showed severe clinical deformity ranging from mental retardation to bone marrow cancer showing neurotoxicity and immunotoxic effects of endosulfan exposure. They are given below.

Family 1: Members studied were 5:

ENS-1-1: This family was reported to be living in Panathady village since 14 years.

Proband: The 9 year old boy showed delayed milestone since 3 months of age. He had large head (circumference- 44.5 cm), flaccid extremities, seizures and cerebral palsy. His chromosome complement showed 46, XY karyotype and 13.3% chromosomal aberrations were observed.

Parents of the proband: His parents were reported to be clinically normal and were not available for sample collection.

Grandparents of the proband: His grandparents (maternal) were reported to be clinically normal. The grandmother showed 10% chromosomal aberrations and there were no chromosomal aberrations observed in grandfather.

Relative of the proband: The maternal uncle aged 27 years was clinically normal; however 20% chromosomal aberrations were observed in him.

Family 2: Members studied were 4:

ENS-2-3: This family was reported to be living in Cheravathoor village, Kasaragod since 27 years.

Proband: The 24 year old male showed mental retardation, deformities of hands and legs.

Parents of the proband: His parents were reported to be clinically normal. Proband's mother 43 years old showed 10% chromosomal aberrations and father 48 years old showed 6.66% chromosomal aberrations.

Family 3: Members studied were 6:

ENS-3-6: 77 years old female living in Kumbadaje village, Kasaragod for more than 40 years was reported to be clinically normal. Her chromosome karyotype was 46, XX and 5% chromosomal aberrations were observed. She worked in the field. Her two grand daughters were born with limb deformities and osteogenesis imperfecta.

Family 4: Members studied were 4:

ENS-4-1: This family was reported to be living in Kanjambady village, Kasaragod since 6 years.

Proband: The 6 year old boy showed mental retardation, hydrocephaly and hepatosplenomegaly. His chromosome complement showed 46, XY karyotype and 5% chromosomal aberrations were observed.

Parents of the proband: His parents were reported to be clinically normal. Proband's mother 34 years old showed

13.3% chromosomal aberrations and father 35 years old showed 44% chromosomal aberrations.

Family 5: Members studied were 5:

EN-1-1: This family was reported to be living in Kamhangod village for more than 30 years.

Proband: The 6 year old boy showed mental retardation, paralysis (right side), speech problem, strabismus and locomotory problem. His chromosome complement showed 46, XY karyotype and no chromosomal aberrations were observed.

Parents of the proband: His parents were reported to be clinically normal and his mother 29 years old showed 6.66% chromosomal aberrations. Father was not available.

Grandparents of the proband: His grandmother (paternal) was reported to be clinically normal. The grandmother 65 years old showed no chromosomal aberrations and grandfather died due to myocardial infarction.

Relative of the proband: His brother aged 8 years was clinically normal; no chromosomal aberrations were observed.

Family 6: Member studied was 3:

EN-3-2: 52 years old male living in Rajapuram panchayat for more than 40 years was reported to be clinically normal. His chromosome karyotype was 46, XY and 10% chromosomal aberrations were observed.

Family 7: Members studied were 4:

EN-4-3: 34 years old male living in Enmakaje panchayath for more than 30 years was reported to be clinically normal. His chromosome karyotype was 46, XY and 15% chromosomal aberrations were observed. He worked in the field. His children (2 boys) have optical atrophy and nystagmus.

Family 8: Members studied were 4:

EN-5-3: This family lived in Bellur panchayath for more than 14 years and the parents are reported to be clinically normal. Two sons aged 8 years and 11 years were affected with muscular dystrophy.

The cytogenetic studies were carried out using peripheral blood leucocyte cultures and giemsa banding technology and scoring chromosome aberrations as endpoint. It was observed that a chromosome aberration per cell was 0.121 ± 0.09 which was significantly higher, when compared with the control samples showing 0.014 ± 0.0001 aberrations per cell (Table 2).

Table 2: Frequency of chromosomal aberrations analysed in endosulfan exposed patients

S No.	Patients	sex/age	No. of metaphases analysed	No. of chromosomal aberrations	Aberration per cell
1.	ENS 1-1	M/9	50	7	0.14
2.	ENS 1-3	M/56	50	2	0.04
3.	ENS 1-4	M/27	50	10	0.2
4.	ENS 1-5	F/48	50	5	0.1
5.	ENS 2-3	M/48	50	4	0.08
6.	ENS 2-4	F/43	50	7	0.14
7.	ENS 3-6	F/77	50	4	0.08
8.	ENS 4-1	M/6	50	4	0.08
9.	ENS 4-2	F/34	50	7	0.14
10.	ENS 4-3	M/35	50	22	0.44
11.	EN 1-1	M/6	50	3	0.06
12.	EN 1-2	F/29	50	4	0.08
13.	EN 1-3	M/8	50	4	0.08
14.	EN 1-4	F/65	50	4	0.08
15.	EN 3-2	M/52	50	5	0.1
16.	EN 4-3	M/34	50	5	0.1
			800	97	0.121

Endosulfan exposed patients: Aberration per cell = 97/800 = 0.121; S.D. = ± 0.09 , Control: Aberration per cell = 7/500 = 0.014; Mean \pm SD = 0.014 ± 0.0001

DISCUSSION

Endosulfan is a pesticide belonging to the organochlorine group of pesticides, under the Cyclodiene subgroup. It emerged as a leading chemical used against a broad spectrum of insects and mites in agriculture and allied sectors. It acts as contact and stomach poison and has a slight fumigant action. It is used in vegetables, fruits, paddy, cotton, cashew, tea, coffee, tobacco and timber crops. It is also used as a wood preservative and to control tsetse flies and termites. It is not recommended for household use. Intentional misuse of endosulfan for killing fish and snails has also been reported. Endosulfan was also reported as used deliberately as a method of removing unwanted fish from lakes before restoring.

Endosulfan was introduced at a time when environmental awareness and knowledge about the environmental fate and toxicology of such chemicals were low and not mandatory as per national laws. But now it is being detected as an important cause of pesticide poisoning in many countries.

Endosulfan has been in world-wide use since its introduction in the 1950's. It was considered a safer alternative to other organochlorine pesticides in many countries in all regions since the 1970's. But in the last two decades many countries have recognized the hazards of wide application of endosulfan.

For instance, cases of endosulfan poisoning have been reported from many parts of the world. Accidental and intentional exposure leading to human fatalities and environmental tragedies has occurred. The following are some of the major cases of poisoning.

Human poisonings: The worst of all the cases so far reported are from three nations; Cuba, Benin and India. In Cuba- Endosulfan was responsible for the death of 15 people in the western province of Matanzas, Cuba in February 1999. A total of 63 people became ill after consuming food contaminated with endosulfan according to Cuban authorities (Anonymous, 1999). In Borgou province in Benin, endosulfan poisoning caused many deaths during 1999-2000 cotton season. Official records state that at least 37 deaths occurred and 36 were taken seriously ill. In the same region in 1999 a boy died after eating corn contaminated with endosulfan (Ton *et al.*, 2000). In South India- People in 15 villages in Kasaragod in the South Indian State of Kerala were subjected to continuous exposure to endosulfan which was aerially sprayed three times every year for 24 years. Congenital Birth defects, reproductive health problems, Cancers, loss of immunity, neurological and mental diseases were reported among the villagers. Following a public outcry a number of health based scientific studies confirmed that the health problems were directly linked to the exposure to endosulfan (Thanal Conservation Action and Information Network, Thiruvananthapuram, Kerala, India, 2001; Anonymous, 2002; Quijano, 2002).

In this study also the hazardous nature of endosulfan exposure on the human population is confirmed. Out of 35 subjects studied 11 of them (31.4%) had severe clinical deformity (Table 1).

For the clinical and pedigree analysis we examined the thoroughly every one of 35 subjects with the help of qualified physicians. Out of the 11 physically deformed subjects, 4 of them had mental retardation showing a neurotoxic effect of endosulfan. Neurotoxicity and endocrine-disruption potential of endosulfan was also demonstrated in animal experiments (Hiremath and Kaliwal, 2002) *in vitro* assays (Kang *et al.*, 2001; Bisson and Hontela, 2002). Endosulfan activity was observed in human Jurkat T-cell (Kannan *et al.*, 2000; Kannan and Jain, 2003). And hence this pesticide has been banned or restricted its use. A ban on endosulfan exists in the South Indian state of Kerala (imposed through a Court Order), which came as a result of a public pressure following the poisoning of many villages due to aerial spraying of the chemical.

Endosulfan has also been detected from human tissues. It has been detected from cord blood samples obtained at the time of delivery and in human sera. Alarmingly high levels of endosulfan residues were observed in human blood and breast milk in a study in Kasaragod in Kerala, India.

However, several independent studies have shown that endosulfan is genotoxic, using aquatic animals for evaluation (Lajmanovich *et al.*, 2005). Data from *in vitro* and *in vivo* mutagenicity studies generally provide

evidence that endosulfan is mutagenic, clastogenic, induces effects on cell cycle kinetics and DNA strand breaks (Yadav *et al.*, 1982; Lu *et al.*, 2000; Jamil *et al.*, 2004). Endosulfan was also found to cause chromosomal aberrations in hamster and mouse, sex-linked recessive mutations in Drosophila, and dominant lethal mutations in mice (Velázquez *et al.*, 1984; Naqvi and Vaisnavi 1993; Jamil *et al.*, 2004). It is also known to cause mutations in mammals (Pandey *et al.*, 1990). It may induce mutations in human, if exposure is great. It is also a potential tumor promoter (Fransson-Steen *et al.*, 1992). The genotoxic effect of endosulfan on erythrocytes of amphibian tadpoles was reported (Lajmanovich *et al.*, 2005). It is important to note that the occurrence of nuclear morphological aberrations in *H. puchella*, as well as the general degenerative changes in erythrocytes during the tadpole stages studied, corresponded to a period of intense hematopoiesis with active cell division in the circulating blood (Lajmanovich *et al.*, 2005).

The immunotoxic and genotoxic effect of endosulfan, an organochlorine insecticide, on sheep peripheral blood leukocytes was examined in vitro conditions. Our studies also provide evidence both for genotoxic and immunotoxic effects of endosulfan. The immunotoxic effect was evaluated by assays of the metabolic activity of phagocytes and assays for lymphocyte activation-the Leucocyte Migration-Inhibition Assay (LMIA) and lympho-proliferation. The significant inhibitory effect of endosulfan on metabolic activity of peripheral blood phagocytes was registered at the actual concentrations of 10(-3), 10(-4)M. At 10(-3)M the migration of leukocytes was inhibited, both in activated and non-activated with phytohemagglutinin (PHA) leukocyte suspensions ($p<0.01$) in LMIA.

This indicated the direct cytotoxic effect of endosulfan on the polymorphonuclears and monocytes of which the intensity of migration is an indicator of lymphocyte activation with mitogen. At the concentration of 10(-4) M an immunotoxic effect, ie. Significant decrease of lymphocyte activation with mitogen was recorded in LMIA. Lymphoproliferation test showed the significant inhibition of proliferation for PHA-stimulated lymphocytes at 10(-3) M and 10(-4) M. Micronucleus assay evaluated the genotoxic potential of endosulfan. Higher concentrations of insecticide 10(-5)M, 10(-6)M resulted in a significant dose dependent increase in the number of micronuclei (Pistl *et al.*, 2003).

In this study the genotoxic effects of endosulfan was evaluated by using chromosomal aberrations as the end point in the leucocyte cultures of the subjects exposed to endosulfan for several years. It was observed that the chromosomal aberrations per cell in an endosulfan exposed subject is 0.121 ± 0.09 which was significantly much higher than that of the control (0.014 ± 0.0001).

CONCLUSION

There exists contradicting results of the environmental samples of endosulfan residues present in the soil and water samples obtained after endosulfan spray that may cause genotoxic effects in human health. Centre for Science and Environment (CSE) New Delhi analysed biological and environmental samples for Endosulfan residues on 2001, about one and a half month after the last aerial spray of Endosulfan carried out. The results published in the magazine "Down to Earth" showed that the concentration of Endosulfan in three water samples were 7 to 51 times higher than the Maximum Residue Limit (MRL). Very high levels of Endosulfan were reported in samples of human blood, human breast milk, vegetables, spices, cow's milk, animal tissues, cashew, cashew leaves and soil. In one of the soil sample the levels of Endosulfan were 391 times higher than MRL.

Fredrick Institute of Plant Protection and Toxicology (FIPPAT) at the request of PCK carried out evaluation of Endosulfan residues in 106 samples of human blood, one cow milk sample, one fish sample, 30 water samples, 29 soil samples and 28 cashew leaf samples collected from 18.3.2001 to 02.05.2001 from Padre village. Their results show that there are no residues of Endosulfan in any of the blood samples, cow milk and water samples. However, some residue of Endosulfan was detected in soil and leaf samples. This study provide evidence for a significant increase in the chromosomal aberration in the human exposed to endosulfan, yet, there is no direct evidence to attribute to these effects to endosulfan pollution, but there is no evidence to completely deny it also. Hence it requires *in vivo* studies to confirm it. Hence it is planned to do *in vivo* studies using endosulfan on human leucocyte cultures.

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