

By SIP



भारतीय आयुर्विज्ञान अनुसंधान परिषद  
INDIAN COUNCIL OF MEDICAL RESEARCH

अन्सारी नगर, पोस्ट बॉक्स 4911, नई दिल्ली - 110 029  
ANSARI NAGAR, POST BOX 4911, NEW DELHI - 110 029

No:5/8/4-13(Env)/01-NCD-1

Dated: 31-5-11

To

Dr.C.Nithyananda Pai  
Convener  
Consumer Welfare Forum  
Bolwar,  
Puttur D.K. -574 201 (Karnataka)

Sub: RTI Application under reference Ad-hoc Research Project entitled "Effect of long term endosulfan exposure on male fertility".

Sir

Reference your RTI dated 11.5.2011 on the above mentioned subject, asking for complete details of the above mentioned project.

The reply to the your RTI application as provided by concerned Programme Officer is as under:

	Query	Reply
1	Complete details regarding effect of long term endosulfan exposure on male fertility - Project no.30/2/01 SIC/PI/N2001-02570/566-IRIS ID 2001-02570 (5/8/3-13(Env)/2001-NCD-1)	Report Enclosed
2	Did the ICMR fund this Project, if yes total amount of the Project	Yes, The total grant of Rs.8,68,022/- was released by the Council for this project under reference
3	The name of the Investigators of this project and date of completion of the project and date on which paper submitted to ICMR	Dr. Pratap Kumar, Prof. & Head, Deptt. of Obstetrics of Gynaecology, KMC, Manipal. Co-Investigators: Dr. Satish Kumar, Adiga, Dr. Satish Rao BS Dr. Ganesh Chandra Jagetia  Date of Sanctioning - 17.2.2003 Date of Completion - 16.2.2006
4	The results and conclusions of the investigation of the project.	Report enclosed
5	Whether any paper has been published on this project in any journal or publication.	No (as per information available from the PU) (as enclosed)

This is for your information & necessary action.

Yours faithfully,

(Tripti Khanna)  
Scientist 'E' & CPIO

for DG.

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**Kasturba Hospital, Manipal**  
(ISO 9001:2000 certified)  
(An associate hospital of the Manipal University, Manipal)

May 26, 2011

From  
Dr Pratap Kumar  
Professor  
Dept. of Obstetrics & Gynaecology  
Kasturba Medical College  
Manipal 576 104

To  
Dr Tanvir Kaur  
Scientist 'D'  
Indian Council of Medical Research  
Ansari Nagar  
Post Box 4911  
New Delhi 110 029

Dear Dr Kaur,

With reference to your fax dated 24-05-2011.

I am herewith sending the documents as requested by you.

I would like to state that this data has not been published in any journal.

Thanking you,

Yours sincerely,

*Pratap Kumar*  
Dr Pratap Kumar

*Pl. recd of  
Man  
30/5/2011*

*SO E*

## FINAL REPORT

**1. Title of the research scheme**

Effect of Long Term Endosulfan Exposure on Male Fertility

**2. Name, degree and designation of Principal Investigator**

Dr. Pratap Kumar, MD  
Professor and Head,  
Department of Obstetrics and Gynecology  
Kasturba Medical College,  
Manipal

**Co-Investigators**

- Dr. Satish Kumar Adiga, Ph.D,  
Associate Professor,  
Department of Obstetrics and Gynecology,  
Kasturba Medical College,  
Manipal
- Dr. Satisu Rao BS, PhD,  
Associate Professor,  
Department of Radiobiology,  
Kasturba Medical College  
Manipal
- Dr. Ganesh Chandra Jagetia Ph.D,  
Professor & Head,  
Department of Radiobiology,  
Kasturba Medical College,  
Manipal

**3. Name of the institution**

Kasturba Medical College,  
Manipal

**4. Year in which the scheme started**

2003

**5. Date from which scheme was started**

July 15, 2003

**6. ICMR Grant sanctioned and actual expenditure incurred from the year of inception till the date of termination**

Year	Grant sanctioned	Actual expenditure incurred
2003-2004	Rs. 3,61,500.00	Rs. 2,14,266.06
2004-2005	Rs. 3,74,220.00	Rs. 2,72,417.25
2005-2006	Rs. 2,10,476.00	Rs. 3,16,902.00

**7. Aims and objectives of the project**

- To assess the association between endosulfan exposure and alteration in the serum reproductive hormones like FSH, LH and Testosterone.
- To estimate the incidence of DNA damage (chromosomal damage) in peripheral blood lymphocytes
- To find relation between serum reproductive hormones and cytogenetic changes in the peripheral blood lymphocytes of exposed population.

**8. Details of the report of work done**

**a) Material and methods used**

A survey of the families exposed to endosulfan in Kasargod district was undertaken and 3 villages were short listed for endosulfan-exposed subjects and two near by unexposed villages were short listed for control subjects for the collection of blood samples. The health status of the whole families including children was recorded before the collection of blood samples.

**Selection of the subjects**

Subjects were selected randomly from the villages living in the area where endosulfan was sprayed for the past 15-20 years and they were given a set of questionnaire to find out the duration of exposure, their occupation, life style, habits, previous diseases, and problems related to reproduction. The subject fulfilling the criteria mentioned below was included in the study to investigate the effect of endosulfan.

*Inclusion criteria:*

1. Men who were living in the area of endosulfan exposure for more than 5 years.
2. Age <50 yrs.
3. N/ h/o endocrine illness

*Exclusion criteria:*

1. Subjects taking antidepressants, tranquilizers or antihypertensives were excluded.

**Collection of blood samples from subjects in the Endosulfan exposed area**

The blood was collected by venipuncture using vacutainers (heparinised and plain). Regular visits were

made to the endosulfan exposed and control villages for this purpose. The samples were brought immediately to the laboratory in ice packs and used for cytogenetic studies and reproductive hormone profile investigations.

#### **Cytogenetic studies: The Lymphocyte Culture**

The whole blood was collected from the donor in the heparinised vacutainers (Becton Dickinson, US). The erythrocytes were allowed to sediment & the buffy coat containing nucleated cells was used for lymphocyte culture at 37°C. Usually,  $10^6$  nucleated cells were inoculated into each culture tube containing 1 ml of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamine, PHA as mitogen and the following analysis were performed.

- a. Micronucleus assay
- b. Chromosome assay
- c. Sister chromatid exchange assay

##### **a. Micronucleus (MN) assay**

For micronucleus assay, to the culture tubes, 5 µg/ml of cytochalasin B was added to block cytokinesis at 44<sup>th</sup> hour and incubated again at 37°C till 72 hours. The tubes were centrifuged and supernatant was removed and the pellet was treated with a hypotonic solution of 0.56% KCl for one minute and the cells were then fixed with Carnoy's fixative (Methanol: acetic acid, 3:1). The cells were washed with the fixative for three times and finally the pellet was resuspended in 0.5 ml of fresh fixative and dropped on to acid washed slides. The cells were then stained using 4% Giemsa stain. Then 2000 binucleate cells were scored for the presence of one, two, three or more than three micronuclei under light microscope (Fenech & Morely, 1985). The results were expressed as Mean ± SEM.

##### **b. Chromosome Aberration (CA) assay**

For chromosome aberration assay, 10 µg/ml of clochicine was added at 46<sup>th</sup> hour of the culture to block cell cycle at metaphase and incubated again at 37°C till 52 hours. Then tubes were centrifuged and supernatant was removed and the pellet was treated with a hypotonic solution of 0.56 % KCl for 18 minutes and the cells were then fixed with Carnoy's fixative (Methanol: acetic acid, 3:1). The cells were washed with the fixative for three times and finally the pellet was resuspended in 0.5 ml of fresh fixative and dropped on to acid washed and chilled slides. Then chromosomes were stained with 4 % Giemsa stain and 200 metaphases were scored for structural as well as numerical aberrations under light microscope (Purrot and Lloyd, 1977). The results were expressed as Mean ± SEM.

##### **c. Sister Chromatid Exchange (SCE) assay**

For SCE assay, at 24<sup>th</sup> hour of the lymphocyte culture, 5 µg/ml of 5-bromodeoxyuridine was added and kept at 37°C until 70 hours. The test tubes were covered with silver foils to avoid the exposure to bright light.

70<sup>th</sup> hour, 10 µg/ml of colchicine was added to block cell cycle at metaphase and incubated again at 37°C for 2 more hours. Then tubes were centrifuged and supernatant was removed and the pellet was treated with a hypotonic solution of 0.56 % KCl for 18 minutes and the cells were then fixed with Carnoy's fixative (Methanol: acetic acid, 3:1). The cells were washed with the fixative for three times and finally the pellet was resuspended in 0.5 ml of fresh fixative and dropped on to acid washed and chilled slides. Then chromosomes were stained with Fluorescent plus Giemsa (FPG) stain and 30 metaphases were scored for the presence of exchanges (Perry and Evans, 1975). The results were expressed as Mean ± SEM.

#### **Reproductive hormone profile investigations in serum – ELISA method**

Serum reproductive hormones like follicle stimulating hormone (FSH), leutinizing hormone (LH), testosterone and prolactin were estimated using Enzyme Linked Immuno Sorbent Assay (ELISA). Serum and calibrators were added to different micro well plates followed by the addition of respective enzyme reagents. The plates were incubated for one hour at room temperature followed by washing the plates with washing buffer. Then enzyme substrate was added to each well and kept for 15 minutes. The reaction was stopped by the addition of 0.1N HCl. The absorbance was taken at 450 nm using micro well plate reader. The standard graph was plotted with the absorbance values of calibrator. The hormone levels of the patient's serum samples were estimated by extrapolating their absorbance values on the standard graph. The results were expressed as Mean ± SEM.

#### **b) Observations**

During 36 months of the project work, we collected 424 blood samples out of which 313 were from endosulfan-exposed population and 111 are from control population. The age of individuals varied from 15 years to 50 years. All the results were expressed as Mean ± SEM. Pearson's bivariate correlation was studied to find the effect of confounding factors on different parameters.

The report includes results of,

- a. Reproductive hormone profiles of 311 exposed and 107 control subjects,
- b. Micronucleus data of 312 exposed and 109 control subjects
- c. Chromosomal aberration data of 307 exposed and 107 controls
- d. Sister chromatid exchange data of 307 exposed and 107 control subjects.

Table 1: Profiles of subjects associated with various confounding factors

Subjects	Control subjects	Exposed subjects
Smokers	19	64
Non-smokers	92	248
Tobacco	30	69
Non-tobacco	81	243
Alcoholic	34	46
Non-alcoholic	77	266
Vegetarians	14	38
Non-vegetarians	97	274
Mean age	29.32 ± 0.79	30.05 ± 0.55

Observations of Reproductive hormone profile

Fig 1: Serum FSH levels of control and endosulfan exposed populations of different age groups

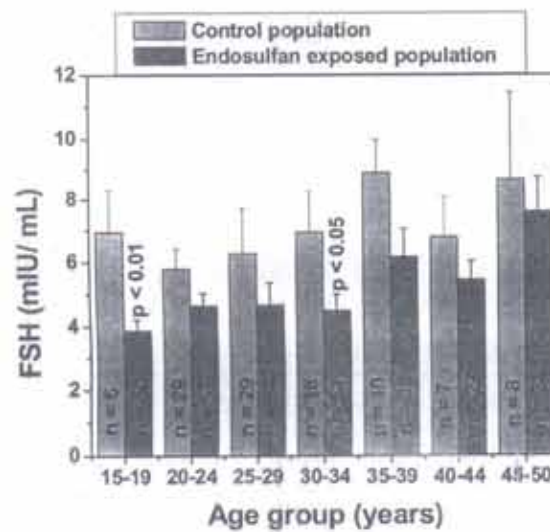


Fig 2: Serum LH levels of control and endosulfan exposed populations of different age groups

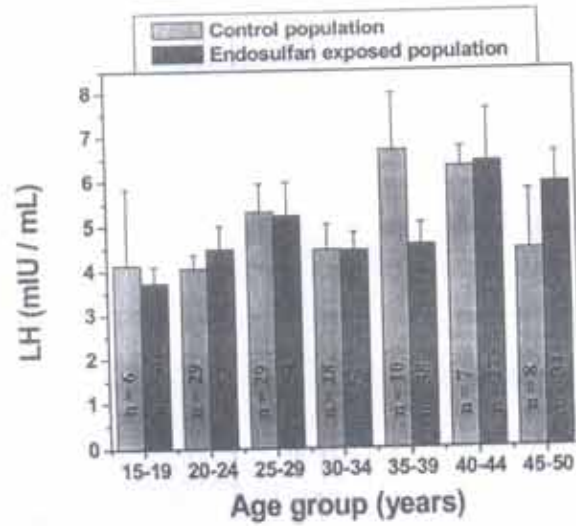


Fig 3: Serum prolactin levels of control and endosulfan exposed populations of different age groups

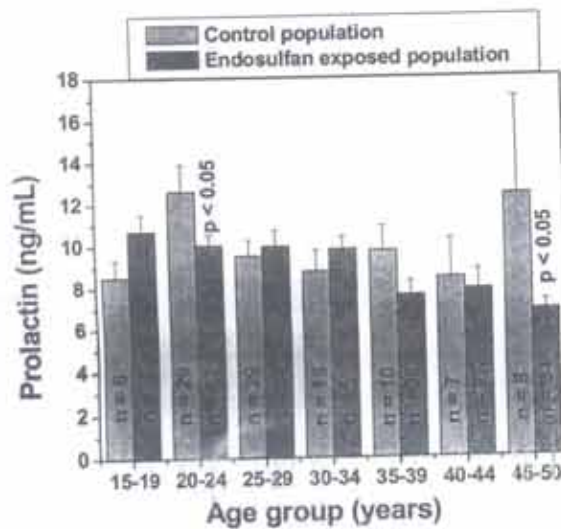




Fig 4: Serum testosterone levels of control and endosulfan exposed populations of different age groups

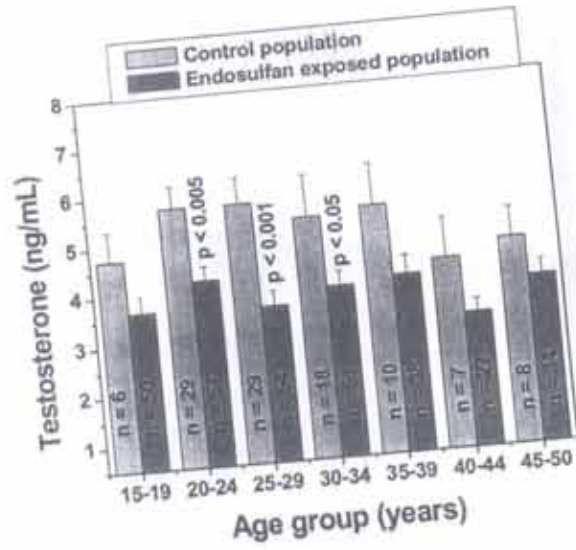
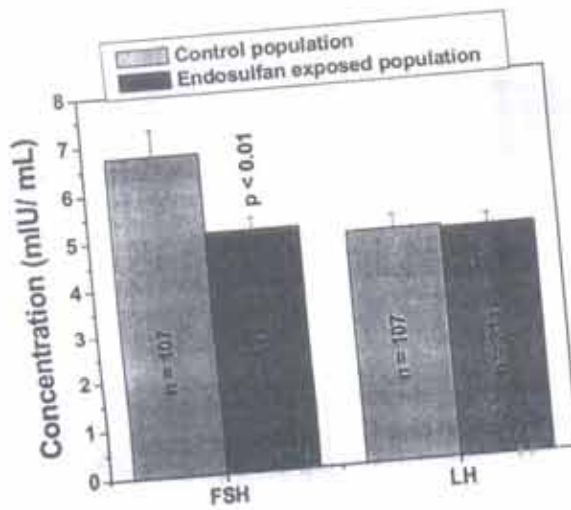
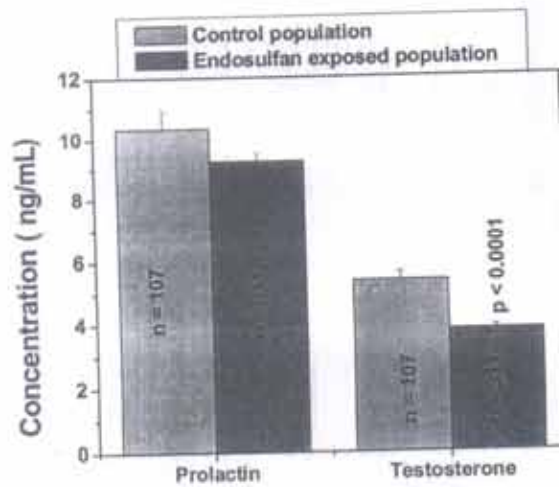


Fig 5: Overall mean and standard error of reproductive hormones in control and endosulfan exposed groups





The level of significance was determined using ANOVA. The hormones FSH ( $p < 0.01$ ), and Testosterone ( $p < 0.0001$ ) levels were significantly lower in endosulfan exposed populations compared to control populations. But we did not find any significant difference in LH and prolactin levels between endosulfan exposed and control populations.

Pearson's r-value in control group: It was  $-0.207$  ( $p < 0.05$ ) for FSH and alcohol. For LH and smoking r-value was  $0.246$  ( $p < 0.05$ ).

Pearson's r-value in endosulfan exposed group: It was  $0.218$  ( $p < 0.01$ ) for FSH and age and  $0.124$  ( $p < 0.05$ ) for FSH and smoking. For LH and age, the r-value was  $0.144$  ( $p < 0.05$ ) and for LH and smoking r-value was  $0.158$  ( $p < 0.01$ ). For prolactin and age, r value was  $-0.249$  ( $p < 0.01$ )

### Observations of micronucleus (MN) assay

Fig 6: Profiles of micro nucleated binucleate cells (MNBNC) in control and endosulfan exposed subjects with different age groups

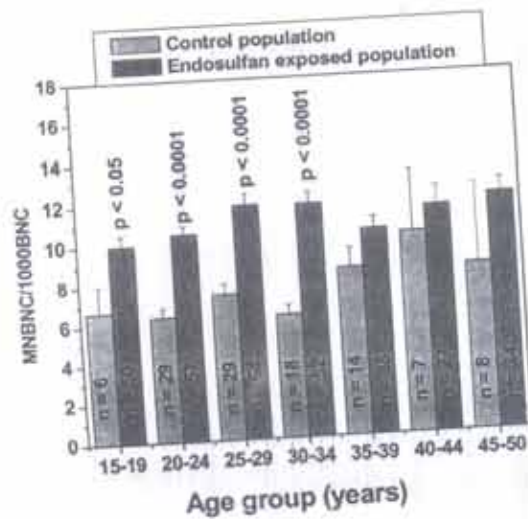
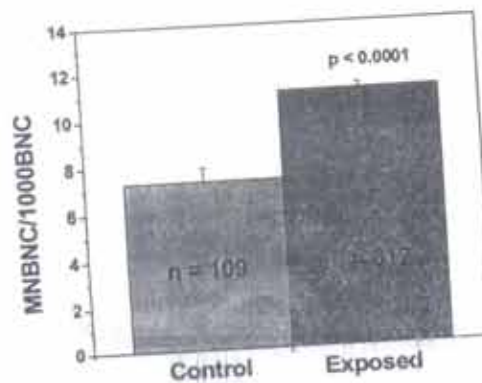


Fig 7: Profiles of overall MNBNC in control and endosulfan exposed population



The level of significance was determined using one-way ANOVA. There was significant increase in micro nucleated binucleate cells of endosulfan-exposed population ( $p < 0.0001$ ) compared to controls. The Pearson's r-value was 0.246 ( $p < 0.01$ ) for micronuclei and age. It was  $-0.123$  ( $p < 0.05$ ) for micronuclei and non-vegetarians in endosulfan exposed populations. In endosulfan-exposed population, the

r-value was 0.174 ( $p < 0.01$ ) for micronuclei and alcohol intake

**Observations of chromosome aberration (CA) assay.**

Fig 8: Profiles of chromosome aberrations (CA) in control and endosulfan exposed subjects with different age groups

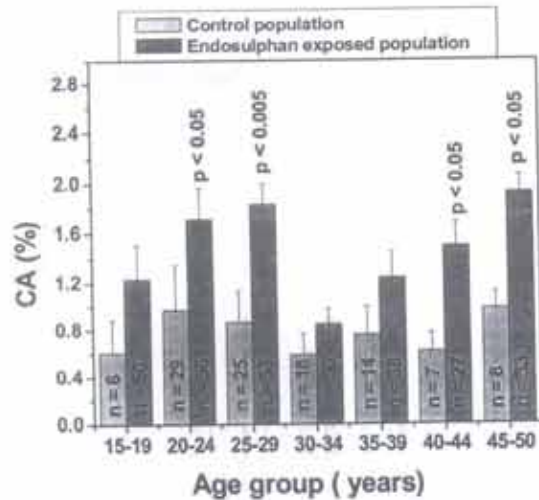
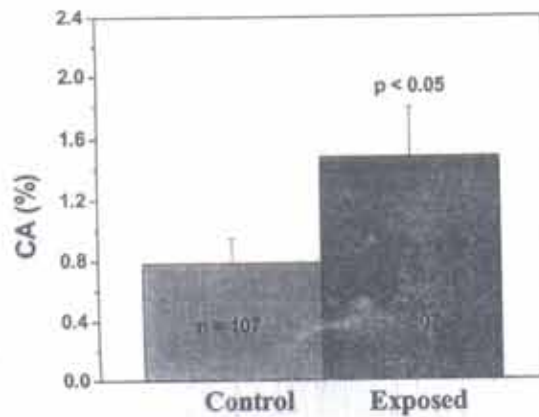


Fig 9: Profiles of overall CA in control and endosulfan exposed population



The level of significance was calculated by using two-tailed t test. The difference between chromosome aberrations of endosulfan-exposed population is statistically significant ( $*=p < 0.05$ ) compared to control

population. The Pearson's r-value is 0.265 ( $p < 0.05$ ) for chromosome aberrations and alcohol intake in control populations.

### Observations of sister chromatid exchanges (SCE) assay.

Fig 10: Profiles of sister chromatid exchanges (SCE) in control and endosulfan exposed subjects with different age groups

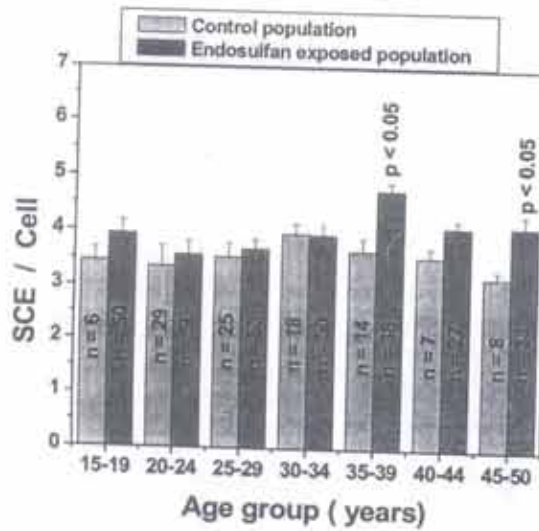
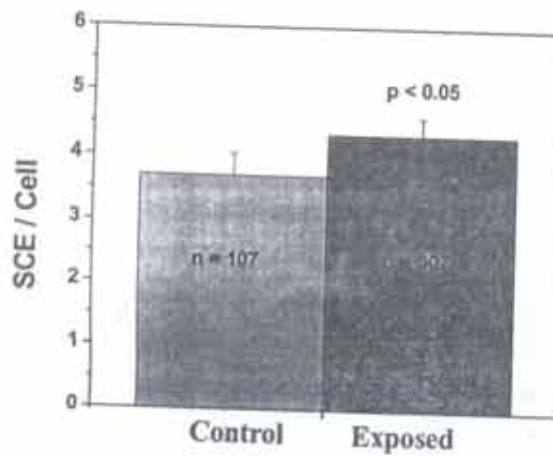


Fig 11: Profiles of overall SCE in control and endosulfan exposed population



The level significance of the data was analyzed by t-test. The mean number of exchanges in endosulfan exposed population is marginally significant ( $p < 0.05$ ) when compared to controls).

### c) Conclusions

1. Serum FSH and testosterone levels among endosulfan-exposed population were significantly lesser than the unexposed control population though no significant difference in LH and prolactin levels between control and endosulfan exposed populations.
2. The micronuclei frequency in the endosulfan-exposed population is significantly higher than the control populations. Similarly chromosome aberrations in the endosulfan exposed population is also significantly higher compared to unexposed control populations. In addition a significant increase was found in the sister chromatid exchanges in endosulfan exposed subjects compared to control populations. Saiyed et al (2003, vol-111, Environmental health perspectives 1958-1962) earlier measured the endosulfan level in these populations and were shown to be  $7.47 \pm 1.19$ ppb. Comparing with the study of near unexposed populations which is having similar life style, which shows lower levels of genetic damage it can be concluded that endosulfan exposed population carries higher amount of cytogenetic damage and also shows reduced levels of serum reproductive hormones.
3. Even though there were significant decline in the reproductive hormone levels and higher amount of cytogenetic damage, no comparable correlation could be drawn between serum reproductive hormones and cytogenetic changes in the peripheral blood lymphocytes of exposed population.

### 9. Abstract

The present study was carried out on the adult male population in the villages of Kasargod district where endosulfan has been extensively used for last twenty years. Earlier survey reports on the population showed increased cancer incidence, abortions, bone malformations in newborn children and neurological symptoms but there were no scientific studies reported on the cytogenetic damage carried by the healthy population in this endosulfan exposed area. In the present study, 313 endosulfan exposed subjects were studied for the cytogenetic damage and reproductive hormone profile and were compared with the 111 control subjects from the nearby villages where no endosulfan exposure was reported. The present study revealed reproductive hormones like follicle stimulating hormone ( $p < 0.005$ ) and testosterone ( $p < 0.001$ ) levels were significantly reduced in

endosulfan exposed populations compared to control populations. The cytogenetic study also revealed significantly higher amount of genetic damage (for micronucleus count  $p < 0.0001$ , for chromosome aberrations,  $p < 0.05$  and for sister chromatid exchange  $p < 0.05$ ) compared to unexposed control subjects. No correlations were found between reproductive hormone profile and cytogenetic damage. Comparing with the study of nearby unexposed populations with similar life style, which shows lower levels of genetic damage it could be concluded that endosulfan exposed population carries higher amount of cytogenetic damage and also shows reduced levels of serum reproductive hormones.

#### 10. List of publications

The study will be communicated for publication and will be informed in due time.

Signature: 

Date:

Designation: Professor and Head,  
Dept. of Obstetrics and Gynecology