

**BEFORE THE NATIONAL GREEN TRIBUNAL,
SOUTHERN BENCH, CHENNAI,
ORIGINAL APPLICATION NO. 186 OF 2023 (SZ)**

IN THE MATTER OF

RAVINDRANATH SHANBHOUGUE

.....APPLICANT

VERSUS

UNION OF INDIA AND OTHERS

.....RESPONDENTS

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SUBMISSIONS ON BEHALF OF ADVOCATE OF PETITIONER

1. It is respectfully submitted that this Honble Court vide its Order dated 20/12/2023 directed the authorities to file their appropriate report after making a spot inspection.
2. That in compliance of the Order dated 20/12/2023, Senior Scientist of Central Pollution Control Board performed spot inspection on 28/12/2023 to assess the area under scrutiny.
3. That on or about 01/01/2024, the Central Pollution Control Board filed its affidavit which includes the Spot Inspection Report of the area under scrutiny.
4. That as per the affidavit of CPCB, applicant has learnt that Scientist of CPCB visited an abandoned well within the cashew plantation area of M/s PCK Ltd, where barrels believed to be

endosulfan barrels were disposed of unscientifically. The CPCB Scientist collected 2 soil samples from dumped and closed open wells, a concrete chemical mixing area, and 2 groundwater samples from open wells located close to the Adhur Estate Division Office and Nettanige- Mundnur, and one ground water sample from open well near Primary Health Centre, Ishwarmangal, Karnataka.

5. Applicant has also learnt that among the 32 wells located near the Kasargod cashew plantation where Endosulphan was used for pest control, 27 are open and five wells are reported as abandoned.
6. It is also respectfully submitted that followings are the main observations made by CPCB during their Spot Inspection:
 - Aerial spraying of endosulphan was carried out from 1983-2001. Physically no odours of this pesticide was felt by the team.
 - Only 20 barrels stored in locked room of division store of Adhur Estate was shown and no accountability of 258 used endosulphan barrels or proof of documentary evidence furnished.

- Based on information provided around 57.56 KL of endosulphan was consumed totally during the period of 1983-2001.
- According to M/s PCK Lts. Officials the abandoned wells are closed due to administrative reasons while local public claim these wells were used for unscientific dumping of used endosulphan barrels. Since ***no documentary evidence was furnished regarding safe disposal of used barrels*** this claim of the local public cannot be ignored.
- M/s PCK Ltd is not monitoring the water quality the 27 open wells to keep in check any possible contamination.
- ***No details regarding ultimate disposal of used barrels*** and unused stockpiles of endosulphan was provided by Udupi and Uttara Kannada as cashew plantations in these areas was also sprayed by the chemical.
- The team has collected soil sample from only 1 feet depth from closed well and the results are yet to be received to ascertain the actual damage to the environment.
- As per the public during the spraying of the pesticide mike announcement and sirens used to be made for alerting them, however, the team ***didn't observe any air pollution control***

barrier around the peripherals of plantation area, which could have avoided interaction of endosulphan aerosol entering the local population during the period of spray.

7. It is respectfully submitted that recently a Research Study titled as ***'A Study on the Distribution of Health Complications among the Endosulfan Pesticide Affected Victims of Karnataka'*** has been issued in a Journal namely ***'ANTROCOM JOURNAL OF ANTHROPOLOGY'***. For the sake of convenience of this Honble Court, relevant excerpts of the study is reproduced herein below:

'All over the world, several studies have established a connection between aerial spraying of Endosulfan pesticides and the growing health complications and disorders in human populations from toxic exposure (Savvy Soumya et al. 2017). The chemical pesticide Endosulfan was aerially sprayed widely on the government-owned cashew farms in the states of Kerala and Karnataka over two decades to control the tea mosquitoes that harmed the cashew fruits in the plantations. In the state of Kerala, the regular spraying of Endosulfan was started around 1981 by the Plantation

Corporation of Kerala (PCK) in the division of Kasaragod and was sprayed two to three times a year in the 4696 hectares of cashew plantations of the Kasaragod district. The number of people affected is increasing drastically from the Endosulfan toxic exposure after the aerial spraying continuously for 20 years in the cashew plantations and about surrounding 15 villages half a million people were continuously exposed to exposure (Asha, et al. 2012). In the year 2001, the Centre for Science and Environment conducted the Endosulfan tests to identify the prevalence of endosulfan toxic residues in the Endosulfan pesticide exposed village Padre in Kasaragod district in Kerala. After the examinations results showed the occurrence of poisonous consequences from Endosulfan pesticide. After the spraying of Endosulfan in the Kasaragod district of Kerala in 2011 about 9,000 individuals were affected by the deadly exposure, about 4,800 individuals were bedridden, and more than a thousand individuals have lost their lives and are resulted in the death of at least 4,000 people

along with induced chronic health ailments in more than 10,000 inhabitants.

According to Kerala, government authorities listed that there are 6,212 (assessment by the People 10,000) victims who are prone to deadly exposure (Arjun Raghunath, 2019). The Karnataka Cashew Development Corporation Ltd. (KCDC), an organization undertaken by the government of Karnataka has aerielly sprayed 32,604 liters of highly toxic Endosulfan pesticide as an insecticide on the cashew plantations in the divisions of Puttur, Kumta, Kundapur, Moodbidri from the year 1980 to 2000 and in addition of 11,225 liters of Endosulfan was sprayed manually from the ground. After the trial spraying, more than 242 villages were exposed to the deadly toxic properties in the districts of Dakshina Kannada (3,762 persons), Udupi (1,557 persons), Karwar (1,793 persons) of Karnataka, and around 7,112 persons were severely got affected by the deadly exposure (Arjun Raghunath, 2019). In the Dakshina Kannada district, around 90 villages in five talukas cover more than a thousand

hectares of cashew farms. For about two decades, KCDC has sprayed the Endosulfan pesticide on the 3,500 hectares of cashew plantation areas and about 69 villages have reported the use of Endosulfan insecticide (Dayakar, et al. 2015; Badiger, et al. 2019). Keeping the above background in mind, the present paper aims to assess the nature and prevalence of disabilities and health complications among Endosulfan pesticide-affected victims in the selected village of Karnataka state, India.

Results and Discussion

Out of 160 samples, 38 were collected from Kokkada village with 23.8% of the total sample size. From Koyyur, 29 samples with 18.1%, 20 samples from Laila with 12.5%, 18 samples from Nidle with 11.3%, 18 samples from Patrame with 11.3%, 16 samples from Belaalu with 10%, 16 samples from Ujire with 10%, and 5 samples from Dharmasthala with 3.5% were collected for the study. Among all Kokkada village is the most affected village and Dharmasthala is the least affected by the Endosulfan pesticide.

.....The pesticide-affected victims are suffering numerous health problems. The majority of them are having Psychological, Gynecological, and Neurological illnesses compared to diseases. Many patients are continuously taking treatment for different complications. Table-2 shows the Age-wise distribution of patients against treatment by the specialized doctors. 51 persons from the age group of 0-25, 19 persons from the age group of 26-50, and 4 persons from the age group of 51-80 are consulting the doctors for regular treatment for Psychological illnesses. 2 persons from the age group of 0-25, 13 persons from the age group of 26-50, and 7 persons from the age group of 51-80 are availing treatment for Gynecological related disorders. For Neurological problems, 10 persons from the age group of 0-25, 3 persons from the age group of 26-50, and 2 persons from the age group of 51-80 are suffering from disorders. The chi-square value shows that there is a significant age difference in consultation of doctors for treatment among Endosulfan victims

...Many of the Endosulfan pesticide affected victims are suffering from the severe health complications such as Epilepsy, Moderate Mental Retardation, Mild Mental Retardation, Severe Mental Retardation, Seizure Disorder, Cerebral Palsy, Epileptic Seizures (Kutluhan et al., 2003), Bilateral Hearing Loss, Schizophrenia, Buccal Mucosa Carcinoma with SNHL, etc. The results found that out of 95 female victims most of them were suffering from the health complications related to Infertility (7 with 7.37%) and Severe Mental Retardation (6 with 6.32%), Cerebral Palsy (5 with 5.26%), Moderate Mental Retardation (22 with 23.16%), Mild Mental Retardation (13 with 13.68%), Carcinoma Ovary (3 with 3.16%), Buccal Mucosa Carcinoma with SNHL (3 with 3.16%). The statistical value shows that there are significant gender differences in the diagnosed disorders among the affected victims ($\chi^2 = 55.59$, $df = 39$, and $p < 0.05$).

Conclusion

Based on the findings, Pesticides are hazardous destructive toxic chemical substances which are resulted

in severe complications and damage to humans, animals, and the environment. The affected victims are suffering numerous health problems. The majority of the Endosulfan pesticide-affected victims are suffering from Psychological, Gynecological, and Neurological illnesses, etc. The majority of female victims are suffering from problems related to Infertility and Mental illnesses. It was found that more percentage of affected victims fell under the age group of 0-25. The chi-square analysis revealed that age-wise, gender-wise, speciality-wise, disability-wise and disorder-wise distribution of Endosulfan affected victims were statistically changed.

TRUE COPY OF THE RESEARCH STUDY titled as ‘ ‘A Study on the Distribution of Health Complications among the Endosulfan Pesticide Affected Victims of Karnataka’ published in a Journal namely ‘ANTROCOM JOURNAL OF ANTHROPOLOGY’ is annexed and marked hereto as Annexure A1/6.

8. It is also respectfully submitted that recently another RESEARCH PAPER titled as 'Exposure to Endosulfan can cause long term effects on general biology, including the reproductive system of mice' was published in a Journal namely 'Frontiers'. It is respectfully submitted that the said Research Paper has found that Endosulfan exposure leads to persistent DNA Breaks in the reproductive organs of Mice as the same induces Reproductive Toxicity.

True copy of the RESEARCH PAPER titled as 'Exposure to Endosulfan can cause long term effects on general biology, including the reproductive system of mice' was published in a Journal namely 'Frontiers' is annexed and marked hereto as Annexure A1/7.

9. That observing the above two Research Studies in collaboration with the findings of Inspection Report of Central Pollution Control Board, it is submitted before this Honble Tribunal to constitute a Committee comprising of Scientists of Central Pollution Control Board, Scientists of Karnataka State Pollution Control Board, Scientists of Kerala State Pollution Control Board, Officer not

below the rank of Secretary – State of Kerela, State of Karnataka and Union Environment Ministry.

10. That to find the actual contamination of the Well water, there is an urgent need to do analysis of the Water of the 27 Open Wells and as such it is respectfully submitted that the samples of the Well water may be collected under the supervision of Central Pollution Control Board and the analysis of the same may be done by CSIR laboratory, situated in State of Kerala.

11. That in light of the claim of Local public that Unscientific Dumping of Used Endosulfan barrels was carried out in past by M/s. PCK limited in 5 wells which are closed presently due to administrative reasons, it is respectfully submitted that the said Committee should conduct the sampling upto 100 feet in all the said five abandoned well and the State of Kerala must be directed to support the committee with requisite logistic for ascertaining the claims of the Local Public.

12. That the Committee may be directed to monitor the disposal of the unused Barrels of the Endosulfan which is under the custody

of Kasargod District Administration and Palakkad District Administration.

13. It is respectfully submitted that the Honble Supreme Court in **Vellore Citizens Welfare Forum vs Union Of India & Ors on 28 August, 1996: AIR 1996 SUPREME COURT 2715 has held that** that "Precautionary Principle" and Polluter Pays principle are essential features of Sustainable Development. The "Precautionary Principle" - in the context of the municipal law - means. (i) Environment measures - by the State Government and the statutory Authorities must anticipate, prevent' and attack the causes of environmental degradation.(ii) Where there are threats of serious and irreversible damage lack of scientific certainty should not be used as the reason for postponing, measures to prevent environmental depredation. (iii)The "Onus of proof" is on the actor or the developer/industrial to show that his action is environmentally benign. Case of the applicant is that ***the precautionary principle and the polluter pays principle have been accepted as part of the law of the land.***

14. It is also respectfully submitted that as per **Article 47 of the World's Largest Democracy** it is the Duty of the State to raise the

level of nutrition and the standard of living and to improve public health. The State shall regard the raising of the level of nutrition and the standard of living of its people and the improvement of public health as among its primary duties and in particular, The State shall endeavour to bring about prohibition of the consumption except for medicinal purposes of intoxicating drinks and of drugs which are injurious to health.

15. That **Article 48A (g) of the Constitution of INDIA** talks about **the** Protection and improvement of environment and safeguarding of forests and wild life and as such states that the State shall endeavour to protect and improve the environment and to safeguard the forests and wildlife of the country.

16. That **Article 51A (g) of the Constitution of INDIA** stipulate **the need** to protect and improve the natural environment including forests, lakes, rivers and wild life, and to have compassion for living creatures.

17. That **The Water (Prevention and Control of Pollution Act 1974** *prohibits the use of streams and wells for disposal of polluting matters.*

18. That Section 03 of the the Environment (Protection) Act talks about the Power of Central Government to take measures to protect and improve environment. It is respectfully submitted that under the said section the Central Government shall have the power to take all such measures as it deems necessary or expedient for the purpose of protecting improving the quality of the environment and preventing controlling and abating environmental pollution including

(ix) carrying out and sponsoring investigations and research relating to problems of environmental pollution;

(x) Inspection of any premises, plant, equipment, machinery, manufacturing or other processes, material or substances and giving, by order, of such direction to such authorities, officers or persons as it may consider necessary to take steps for the prevention, control and abatement of environmental pollution;

(xiv) such other matters as the Central Government deems necessary or expedient for the purpose of securing the effective implementation of the provisions of this Act.

19. It is respectfully submitted that Precautionary Principle replaces the Assimilative Capacity Principle. That earlier the Concept was based on the 'assimilative capacity' rule as revealed from Principle 6 of the Stockholm Declaration of the U.N.Conference on Human Environment, 1972. The said principle assumed that science could provide policy-makers- with the information and means necessary to avoid encroaching upon the capacity of the environment to assimilate impacts and it presumed that relevant technical expertise would be available when environmental harm was predicted and there would be sufficient time to act in order to avoid such harm. However, in the 11th Principle of the U.N. General Assembly Resolution on World Charter for Nature, 1982, the emphasis shifted to the 'Precautionary Principle', and this was reiterated in the Rio Conference of 1992 in its Principle 15 which reads as follows:

"Principle 15: In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage; lack of full scientific certainty shall not be

used as a reason for proposing cost-effective measures to prevent environmental degradation."

20. In other words, inadequacies of science is the real basis that has led to the Precautionary Principle of 1982. It is based on the theory that it is better to err on the side of caution and prevent environmental harm which may indeed become irreversible. ***The principle of precaution involves the anticipation of environmental harm and taking measures to avoid it or to choose the least environmentally harmful activity.*** It is based on Scientific uncertainty. Environmental protection should not only aim at protecting health, property and economic interest but also protect the environment for its own sake. Precautionary duties must not only be triggered by the suspicion of concrete danger but also by (justified) concern or risk potential.

21. That the balance of convenience is in favour of the applicant and the ends of justice shall suffer if the relief as prayed is not granted.

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A Study on the Distribution of Health Complications among the Endosulfan Pesticide Affected Victims of Karnataka

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KEYWORDS

Endosulfan, Disorders, Disabilities, Health Complications

ABSTRACT

The world has faced numerous severe harmful and deadly disasters for several decades. These are hazardous and destructive chemical substances that resulted in severe complications and damage to humans, animals, and the environment. Before the ban on it by the Supreme Court of India, Endosulfan was extensively used as a major pesticide in the agricultural and allied sectors as an insecticide to control the harmful insects and mites in agricultural fields. Endosulfan has been extensively sprayed in the government-owned cashew plantations of Dakshina Kannada, Udupi, and Karwar districts by Karnataka Cashew Development Corporation Ltd., Karnataka for almost two decades. In April 2011 the Stockholm Convention recommended the global ban on the manufacture and use of Endosulfan. WHO categorizes Endosulfan as a moderately hazardous chemical substance and it was banned due to adverse impacts and complications triggered on the health of humans, animals, and the environment. All over the world, several studies have established a connection between aerial spraying of Endosulfan pesticides and the growing health complications and disorders in human populations from toxic exposure. The present study aims to know the nature and prevalence of disabilities and health complications among the Endosulfan affected victims in the selected villages of Karnataka state, India.

Introduction

Endosulfan is an organochlorine insecticide, chemically belonging to the cyclodiene group (Min *et al.* 2012). Around the globe, it is extensively used as a major crop protecting pesticide in the agricultural and its allied sectors as an insecticide to control harmful insects (Li *et al.* 2009; Greeshma, *et al.* 2017; Verma, *et al.* 2011; Savvy Soumya, *et al.* 2017; Patocka, *et al.* 2016). Endosulfan is registered for use in the agricultural fields on a varied variety of fruits, cereals, oilseeds, vegetables, ornamental shrubs, vines, ornamentals, cashew, cotton, coffee, tea, paddy, potato as a pesticide, and also used as a wood preservative (Savvy, Soumya, 2017). Normally, Endosulfan does not soluble in saturated hydrocarbons such as hexane (WHO, 1980) and does not dissolve in water, but dissolves readily in xylene, chloroform, and kerosene and is easily soluble in most organic solvents and readily adheres to clay particles and persists in the soil particles and water for several years (Anonymous, 2000; Patocka, *et al.* 2016). In April 2011, the Stockholm convention has recommended a global ban on the production and use of Endosulfan in agricultural fields due to the dangerous tragedies triggered by it. World Health Organisation (WHO) categorizes Endosulfan as a moderately hazardous chemical substance (class II) and was banned due to adverse impacts and complications triggered on the health of humans, animals, and the environment (WHO, 2002; Irshad, *et al.* 2015). The toxic remains of the Endosulfan residues that persist in the environment are exposed to people through contaminated air, soil, water, and food. It is easily absorbed by the stomach, lungs, and the skin. Exposure may occur through breathing, eating, drinking, and skin

contact (Anonymous, 2000; Savvy Soumya, 2017).

The deadly toxic properties generated by these chemical pesticides can cause severe complications in the immune system of humans at very low levels of exposure. In developing countries, these pesticides have impacted mostly people with low levels of protein diets (ATSDR, 1993). The poisonous properties generated by the harmful Endosulfan residues resulted in severe health complications for human beings. People were suffering from severe multiple disorders and deformities such as the changes in the sex hormones (Saiyed, *et al.* 2003), Hypothyroid problems (Anonymous, 2001). The clinical signs of severe exposure include salivation, hyperactivity, respiratory distress, tremors, hunching convulsions, nausea, unconsciousness, coma, permanent brain damage, and even death (WHO, 2004). DNA damage, mutation in the somatic cells, changes in sex chromosomes, endocrine disruption, reproductive health problems in both male and females, cancerous growth, skin problems, infertility, children's born with mental abnormalities and physical disfigurement and congenital birth defects, loss of immunity, neurological and mental diseases and high occurrences of neurological, congenital and reproductive abnormalities, blood cancer, endocrine disruption, CNS disorders, liver cancer, liver tissue injury, topical diseases, asthma, psychiatric disorders and suicidal tendencies, infertility and undescended testes among males, miscarriages, and hormonal irregularities among females, congenital anomalies, physical deformities, mental retardation, cerebral palsy, epilepsy, developmental delay in sexual maturity among the boys, hormone estrogen complication in females, reproductive and developmental damage in both, animals and humans, hydrocephalus, neurobehavioral disorders, a congenital malformation in both males and females, gynecological, reproductive complications in both male and female, new-born have suffered from abnormalities, seizure(fits), neurotoxicity, late sexual maturity, physical deformities, severe mental retardation, and other multiple abnormalities due to poisoning and even loss of family members were reported among the villagers residing in and around the plantations areas. The short-term toxic exposure has triggered long-term severe health complications among the people who are prone to deadly exposure (Dayakar *et al.* 2015; Andersen *et al.* 2002; Harikrishnan, 2004; Melangadi F., 2017; Patocka J. *et al.* 2016; NIOH, 2002).

All over the world, several studies have established a connection between aerial spraying of Endosulfan pesticides and the growing health complications and disorders in human populations from toxic exposure (Savvy Soumya *et al.* 2017). The chemical pesticide Endosulfan was aerielly sprayed widely on the government-owned cashew farms in the states of Kerala and Karnataka over two decades to control the tea mosquitoes that harmed the cashew fruits in the plantations. In the state of Kerala, the regular spraying of Endosulfan was started around 1981 by the Plantation Corporation of Kerala (PCK) in the division of Kasaragod and was sprayed two to three times a year in the 4696 hectares of cashew plantations of the Kasaragod district. The number of people affected is increasing drastically from the Endosulfan toxic exposure after the aerial spraying continuously for 20 years in the cashew plantations and about surrounding 15 villages half a million people were continuously exposed to exposure (Asha, *et al.* 2012).

In the year 2001, the Centre for Science and Environment conducted the Endosulfan tests to identify the prevalence of endosulfan toxic residues in the Endosulfan pesticide exposed village Padre in Kasaragod district in Kerala. After the examinations results showed the occurrence of poisonous consequences from Endosulfan pesticide. After the spraying of Endosulfan in the Kasaragod district of Kerala in 2011 about 9,000 individuals were affected by the deadly exposure, about 4,800 individuals were bedridden, and more than a thousand individuals have lost their lives and are resulted in the death of at least 4,000 people along with induced chronic health ailments in more than 10,000 inhabitants (Satheesh S., 2017). (Buckwald, *et al.* 2006).

According to Kerala, government authorities listed that there are 6,212 (assessment by the People 10,000) victims who are prone to deadly exposure (Arjun Raghunath, 2019). The Karnataka Cashew Development Corporation Ltd. (KCDC), an organization undertaken by the government of Karnataka

has aerielly sprayed 32,604 liters of highly toxic Endosulfan pesticide as an insecticide on the cashew plantations in the divisions of Puttur, Kumta, Kundapur, Moodbidri from the year 1980 to 2000 and in addition of 11,225 liters of Endosulfan was sprayed manually from the ground. After the trial spraying, more than 242 villages were exposed to the deadly toxic properties in the districts of Dakshina Kannada (3,762 persons), Udupi (1,557 persons), Karwar (1,793 persons) of Karnataka, and around 7,112 persons were severely got affected by the deadly exposure (Arjun Raghunath, 2019). In the Dakshina Kannada district, around 90 villages in five talukas cover more than a thousand hectares of cashew farms. For about two decades, KCDC has sprayed the Endosulfan pesticide on the 3,500 hectares of cashew plantation areas and about 69 villages have reported the use of Endosulfan insecticide (Dayakar, *et al.* 2015; Badiger, *et al.* 2019). Keeping the above background in mind, the present paper aims to assess the nature and prevalence of disabilities and health complications among Endosulfan pesticide-affected victims in the selected village of Karnataka state, India.

Material and Methods

The prevalence of disabilities and the health complications among Endosulfan pesticide-affected victims are ranging from most affected villages to least affected villages from toxic exposure to Endosulfan. The multistage sampling methods were used during sample data collection. Districts and Villages were selected based on the purposive sampling technique. The simple random technique was used for selecting the individual samples. Omnibus interview schedules were used for gathering information. A sample of 160 (65 males and 95 females) Endosulfan pesticide-affected victims of age 0-81+ years were collected from eight (8) harmful toxic exposed villages viz. Kokkada, Koyyur, Laila, Nidle, Patrame, Belaalu, Ujire, and Dharmasthala in the Belthangady taluk of Dakshina Kannada district of Karnataka, India. Age 0 represents the age of less than 1 year. For analysis of data, Statistical Package for Social Sciences (SPSS) 14.0 is used to know the frequency, percentage, chi-square, and cross-tabulation distribution. Traditional anthropological knowledge of fieldwork such as observation methods and informal interviews were utilized to understand the impact and nature of health complications.

Results and Discussion

A total of 160 (65 males and 95 females) samples were collected from eight village panchayats of Dakshina Kannada district depicting the frequency and percentages ranging from highest to lowest (Table-1).

Table-1: Village wise distribution of samples

Villages	Size of the sample	% to the total sample
Kokkada	38	23.8
Koyyur	29	18.1
Laila	20	12.5
Nidle	18	11.3
Patrame	18	11.3
Belaalu	16	10.0
Ujire	16	10.0
Dharmasthala	05	03.5
Total	160	100.00

Out of 160 samples, 38 were collected from Kokkada village with 23.8% of the total sample size. From Koyyur, 29 samples with 18.1%, 20 samples from Laila with 12.5%, 18 samples from Nidle with 11.3%, 18 samples from Patrame with 11.3%, 16 samples from Belaalu with 10%, 16 samples from Ujire with 10%, and 5 samples from Dharmasthala with 3.5% were collected for the study. Among all Kokkada

village is the most affected village and Dharmasthala is the least affected by the Endosulfan pesticide.

Fig.1: Gender wise distribution of samples

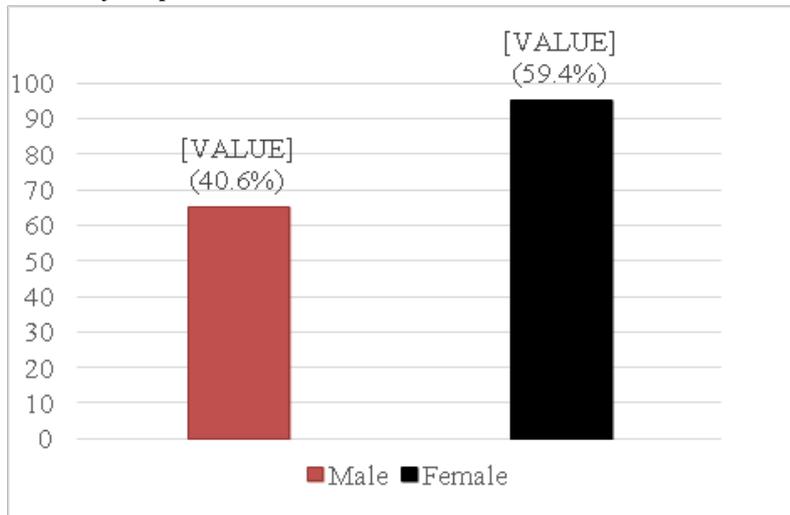


Fig.1 depicts the gender-wise distribution of the Endosulfan pesticide-affected victims. Out of the total 160 samples, 65 were males with 40.6 percent and 95 females with 59.4 percent.

Fig.2: Age-wise distribution of samples

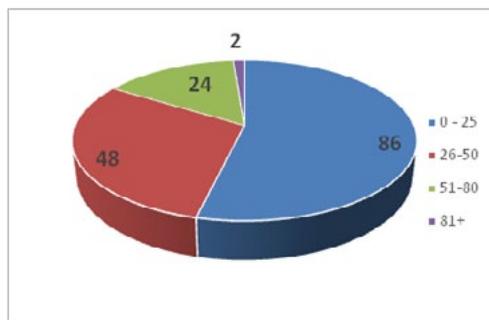


Fig.2 shows the age-wise distribution of Endosulfan pesticide-affected victims in the selected eight villages. The sample was distributed ranges from less than a year (0) to 81 and above aged group. Out of the total of 160 samples, 86 were grouped under the 0-25 aged group of 53.8 percent forming the major sample in size. There are 48 samples collected from the age group of 26-50 years. It forms the percentage of 30. Among the 51-80 age group, 24 samples were collected covering 15%. Only 2 samples were listed out under the 81+ age group with 1.3%.

Table-2: Age group-wise distribution of patients against consultation of doctors for treatment

Treatment wise specialists	Age Group				Total
	0-25	26-50	51-80	81+	
General Medicine	6	6	2	0	14
Psychiatry	51	19	4	0	74
ENT	6	1	2	1	10
Gynecology	2	13	7	0	22
Pediatric	7	0	0	0	7
General Surgeon	0	1	2	0	3

Dermatology	0	0	1	0	1
Orthopedic	1	4	1	0	6
Neurology	10	3	2	0	15
Endocrinology	0	1	0	0	1
Ophthalmology	1	0	3	1	5
Pediatric Cardiologist	2	0	0	0	2
Total	86	48	24	2	160

$$\chi^2 = 88.45, df = 33 \text{ and } p = 0.000$$

The pesticide-affected victims are suffering numerous health problems. The majority of them are having Psychological, Gynecological, and Neurological illnesses compared to diseases. Many patients are continuously taking treatment for different complications. Table-2 shows the Age-wise distribution of patients against treatment by the specialized doctors. 51 persons from the age group of 0-25, 19 persons from the age group of 26-50, and 4 persons from the age group of 51-80 are consulting the doctors for regular treatment for Psychological illnesses. 2 persons from the age group of 0-25, 13 persons from the age group of 26-50, and 7 persons from the age group of 51-80 are availing treatment for Gynecological related disorders. For Neurological problems, 10 persons from the age group of 0-25, 3 persons from the age group of 26-50, and 2 persons from the age group of 51-80 are suffering from disorders. The chi-square value shows that there is a significant age difference in consultation of doctors for treatment among Endosulfan victims ($\chi^2 = 88.45$, $df = 33$, and $p = 0.0001$).

Table-3: Gender wise distribution of patients against Speciality Attended

Specialty Attended	Gender		Total
	Male	Female	
General Medicine	7	7	14
Psychiatry	31	43	74
ENT	6	4	10
Gynaecology	0	22	22
Paediatric	3	4	7
General Surgeon	2	1	3
Dermatology	0	1	1
Orthopaedic	3	3	6
Neurology	9	6	15
Endocrinology	0	1	1
Ophthalmology	3	2	5
Paediatric Cardiologist	1	1	2
Total	65	95	160

$$\chi^2 = 22.79, df = 11 \text{ and } p = 0.019.$$

Table-3 shows the Gender wise distribution of patients against Speciality attended for the treatments. The majority of the males and females are suffering from psychological illnesses and consulted a Psychiatrist for the treatment, total of 74 persons were getting treated out of 160, of which 31 were male and 43 were female victims. The 22 of the females are suffering from the problems associated with

fertility and consulted Gynecologists for the treatment. Some are suffering from Neurological illnesses and 15 victims of endosulfan pesticide consulted Neurological specialists for the treatment out of which 9 males and 6 females. It has been observed that a greater number of females were consulting the doctors for various health complications. There is a significant gender difference in availing of the treatment was found among Endosulfan victims ($\chi^2= 22.79$, $df = 11$, and $p < 0.05$).

Table-4: Gender wise distribution of samples with a Disability percentage.

Gender	Disability %				Total
	0-25	26-60	61-80	81-100	
Male	6	14	34	11	65
Female	34	11	40	10	95
Total	40	25	74	21	160

$$\chi^2 = 15.41, df = 3 \text{ and } p = 0.001$$

Gender wise distribution of samples with different percentages of Disability and ranges from 0-100% (Table-4). Out of 40, 6 males and 34 females fall under the category of 0-25%, whereas in this category the number of female victims is very high compared to the other gender ($34 > 6$). But in the category of 26-60% disability, 25 victims fall in this category out of which 14 males and 11 Females. A total of 74 victims fall under the percentage category of 61-80%, of which 34 are males and 40 are females. This category has the highest frequency of showing the disability in endosulfan pesticide victims regardless of gender. In the category of 81-100%, a total of 21 victims were assigned to this category out of which the males were 11 and the female were 10. Table-4 clear that the disability percentage against the gender was found statistically differ in Endosulfan victims ($\chi^2= 15.41$, $df = 3$, and $p < 0.01$).

Fig-3: Age-wise distribution of samples with a disability percentage

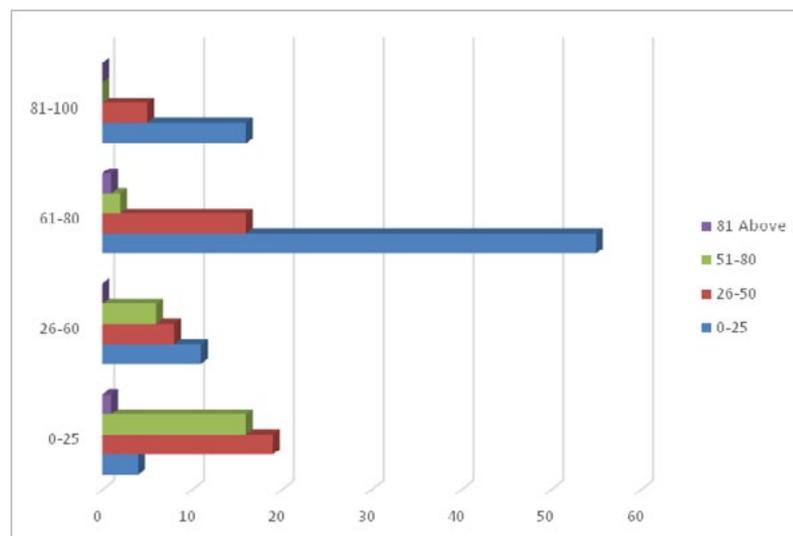


Fig.3 shows the Age-wise distribution of samples with different percentages of Disability and ranges from 0 to 81 and above with different percentages of disability. In the 0-25 age group 4 persons fall under the percentage category of 0-25%, 11 persons fall under the percentage category of 26-60%, 55 persons fall under the percentage category of 61-80%, and 16 persons fall under percentage category of 81-100%. In the 26-50 age group 19 persons fall under the percentage category of 0-25%, 8 persons fall under the percentage category of 26-60%, 16 persons fall under the percentage category of 61-80%, and 5 persons fall under percentage category of 81-100%. In the 51-80 age group 16 persons fall under the percentage category of 0-25%, 6 persons fall under the percentage category of 26-60%, 2 persons fall

under the percentage category of 61-80%, and 0 persons fall under percentage category of 81-100%. In the 81-100 age group 1 person falls under the percentage category of 0-25%, 0 person falls under the percentage category of 26-60%, 1 person falls under the percentage category of 61-80%, and 0 person falls under percentage category of 81-100%. Therefore, it was found that more percentage of people succumb to disability under the age group of 0-25.

Table-5: Gender-wise prevalence of Health Problems/ Diagnosis

Diagnosis/Health complications	Male	%	Female	%	Total	%
Asthma	1	1.54	0	0.00	1	0.63
Epilepsy	4	6.15	3	3.16	7	4.38
Pterygium	0	0.00	1	1.05	1	0.63
Glaucoma	1	1.54	0	0.00	1	0.63
Bilateral Corneal Opacity	0	0.00	1	1.05	1	0.63
Primary Infertility	0	0.00	11	11.58	11	6.88
Bipolar Affective Disorder	0	0.00	1	1.05	1	0.63
Mild Hearing Loss	1	1.54	1	1.05	2	1.25
Infertility	0	0.00	7	7.37	7	4.38
Congenital Heart Disease	1	1.54	1	1.05	2	1.25
Bilateral Hearing Loss	3	4.62	3	3.16	6	3.75
Limb Spondylosis	0	0.00	2	2.11	2	1.25
Primary Sterility	1	1.54	0	0.00	1	0.63
Blind Both Eyes	1	1.54	0	0.00	1	0.63
Carcinoma Ovary	0	0.00	3	3.16	3	1.88
Acute Glomerulonephritis	1	1.54	0	0.00	1	0.63
Mass: Supra Renal Gland	1	1.54	0	0.00	1	0.63
Prurigo Nodularis	0	0.00	1	1.05	1	0.63
Lumbar Spondylosis	1	1.54	1	1.05	2	1.25
Seizure Disorder	3	4.62	1	1.05	4	2.50
Duodenal Ulcer	1	1.54	1	1.05	2	1.25
Hyperactive Airway Disease	0	0.00	1	1.05	1	0.63
Thyroid Swelling	0	0.00	1	1.05	1	0.63
Premature Ovarian failure	0	0.00	1	1.05	1	0.63
Haemo Fibroma	0	0.00	1	1.05	1	0.63
Depression	1	1.54	0	0.00	1	0.63
Global Development Delay	1	1.54	1	1.05	2	1.25
I TP	1	1.54	0	0.00	1	0.63
Schizophrenia	4	6.15	2	2.11	6	3.75
Deformed Right Elbow	1	1.54	1	1.05	2	1.25
Mild Mental Retardation	13	20.00	13	13.68	26	16.25
Monoperasis	1	1.54	0	0.00	1	0.63
Moderate Mental Retardation	7	10.77	22	23.16	29	18.13

Deaf-Mute	2	3.08	0	0.00	2	1.25
Cerebral Palsy	8	12.31	5	5.26	13	8.13
Retinal Detachment Right Eye	1	1.54	0	0.00	1	0.63
Severe Mental Retardation	3	4.62	6	6.32	9	5.63
Down's Syndrome	1	1.54	0	0.00	1	0.63
Chronic Kidney Disease	1	1.54	0	0.00	1	0.63
Buccal Mucosa Carcinoma with SNHL	0	0.00	3	3.16	3	1.88
Total	65	100.00	95	100.00	160	100.00

$$\chi^2 = 55.59, df = 39 \text{ and } p = 0.041$$

Table-5 shows the Gender-wise prevalence of health problems among the Endosulfan pesticide affected male and female victim's samples with percentage. Many of the Endosulfan pesticide-affected victims are suffering from the severe health complications such as Epilepsy, Moderate Mental Retardation, Mild Mental Retardation, Severe Mental Retardation, Seizure Disorder, Cerebral Palsy, Epileptic Seizures (Kutluhan et al., 2003), Bilateral Hearing Loss, Schizophrenia, Buccal Mucosa Carcinoma with SNHL, etc. The results found that out of 95 female victims most of them were suffering from the health complications related to Infertility (7 with 7.37%) and Severe Mental Retardation (6 with 6.32%), Cerebral Palsy (5 with 5.26%), Moderate Mental Retardation (22 with 23.16%), Mild Mental Retardation (13 with 13.68%), Carcinoma Ovary (3 with 3.16%), Buccal Mucosa Carcinoma with SNHL (3 with 3.16%). The statistical value shows that there are significant gender differences in the diagnosed disorders among the affected victims ($\chi^2 = 55.59$, $df = 39$, and $p < 0.05$).

Conclusion

Based on the findings, Pesticides are hazardous destructive toxic chemical substances which are resulted in severe complications and damage to humans, animals, and the environment. The affected victims are suffering numerous health problems. The majority of the Endosulfan pesticide-affected victims are suffering from Psychological, Gynecological, and Neurological illnesses, etc. The majority of female victims are suffering from problems related to Infertility and Mental illnesses. It was found that more percentage of affected victims fell under the age group of 0-25. The chi-square analysis revealed that age-wise, gender-wise, speciality-wise, disability-wise and disorder-wise distribution of Endosulfan affected victims were statistically changed ($p < 0.05$). Therefore, it is required to conduct further studies to understand the veracity of disorders and treatment patterns reported in the Endosulfan affected areas of Karnataka. Concerned authorities need to take further progress to develop permanent rehabilitation centres and health care facilities by introducing specific schemes and programs.

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ANNEXURE
A1/7

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Exposure to endosulfan can cause long term effects on general biology, including the reproductive system of mice

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Increased infertility in humans is attributed to the increased use of environmental chemicals in the last several decades. Various studies have identified pesticides as one of the causes of reproductive toxicity. In a previous study, infertility was observed in male mice due to testicular atrophy and decreased sperm count when a sublethal dose of endosulfan (3 mg/kg) with a serum concentration of 23 µg/L was used. However, the serum concentration of endosulfan was much higher (up to 500 µg/L) in people living in endosulfan-exposed areas compared to the one used in the investigation. To mimic the situation in an experimental setup, mice were exposed to 5 mg/kg body weight of endosulfan, and reproductive toxicity and long-term impact on the general biology of animals were examined. HPLC analysis revealed a serum concentration of ~50 µg/L of endosulfan after 24 h endosulfan exposure affected the normal physiology of mice. Histopathological studies suggest a persistent, severe effect on reproductive organs where vacuole degeneration of basal germinal epithelial cells and degradation of the interstitial matrix were observed in testes. Ovaries showed a reduction in the number of mature Graafian follicles. At the same time, mild vacuolation in liver hepatocytes and changes in the architecture of the lungs were observed. Endosulfan exposure induced DNA damage and mutations in germ cells at the molecular level. Interestingly, even after 8 months of endosulfan exposure, we observed increased DNA breaks in reproductive tissues. An increased DNA Ligase III expression was also observed, consistent with reported elevated levels of MMEJ-mediated repair. Further, we observed the generation of tumors in a few of the treated mice with time. Thus, the study not only explores the changes in the general biology of the mice upon exposure to endosulfan but also describes the molecular mechanism of its long-term effects.

KEYWORDS

endosulfan, genomic stability, infertility, spermatogenesis, oogenesis, reproductive toxicity, double-strand break, DNA repair

Introduction

Pesticides are applied in the environment to increase agricultural yield by controlling pests. Currently, ~2 million tonnes of pesticides are utilized around the globe each year, which has been estimated to increase to 3.5 million tonnes by 2020 (Eddleston et al., 2002; Zhang, 2018; Sharma et al., 2019). Organochlorine pesticides like DDT (Dichlorodiphenyltrichloroethane) and BHC (Benzene hexachloride) are one of the earliest chemical pesticides used around the world (Simonich and Hites, 1995; Pimentel, 1996; Vijgen et al., 2011). Due to their persistence and long-range transport, they were replaced with the less persistent organophosphate and carbamate pesticides (Goff and Giraud, 2019). Nevertheless, the residue of organochlorine pesticides is still detected in the environment in many countries where it is not at all used. Moreover, developing countries, including several Asian countries, still use these pesticides (Iwata et al., 1993; Senior, 2009; Gill et al., 2020). Thus, investigating the health and environmental effect of these pesticides are still relevant and necessary.

Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methanol-2, 4,3-benzodioxathiepin 3-oxide) is one of the major cyclodiene pesticides, which is in continuous use since the 1950s. Although banned in several western countries by the late 2010s (Vandenberg et al., 2020), endosulfan (ES) is still used in developing countries. The WHO classified it as Class II (moderately hazardous), while US EPA and EU have classified it as Class I (highly acutely toxic) (ATSDR, 2000a; Atsdr, 2000b; World Health, O., and International Programme on Chemical, S, 2010).

Commercially, ES is a mixture of two isomeric forms, α - and β -Endosulfan, in a ratio of 3:1 (Carrera et al., 2002; Singh et al., 2014). In mammalian systems, it is metabolized into the most persistent and toxic metabolite, endosulfan sulfate, and endosulfan diol, which is further metabolized into endosulfan ether, hydroxy ether, and lactone (Kataoka and Takagi, 2013). Endosulfan and its related isomers were listed as persistent organic pollutants due to their high toxicity, bioaccumulation, long residual period in soil, and long-distance transportation under the Stockholm Convention held in May 2011 (UNEP, 2011). It is persistent in the environment, taking decades to biodegrade. Endosulfan is frequently detected in river water in Africa, Europe, and Asian countries, including India (Dalvie et al., 2003; Hellar-Kihampa et al., 2013; Loha et al., 2020; Montuori et al., 2020). A study showed that a concentration of α - and β - ES ranged from non-detectable limit to 35.21 $\mu\text{g/L}$ and 37.56 $\mu\text{g/L}$, respectively, in Tapi river water in India (Sarker et al., 2021). The mean concentration of ES in the river Ganga in India was ~36 ng/L (Khuman and Chakraborty, 2019). Some of its initial degradation products are structurally similar to parent

endosulfan isomers and share the same toxic effects (Dorough et al., 1978).

A previous study from our laboratory has shown that exposure to ES in mice (3 mg/kg body weight) induces an increase in DNA damage, triggers compromised DNA damage response, and promoted the error-prone microhomology-mediated end-joining (MMEJ) pathway of DNA repair in the male reproductive organ (Sebastian and Raghavan, 2016). ES-induced DNA damage was highest in the lungs and testes when analyzed through immunohistochemistry, immunofluorescence, and Western blot analysis (Sebastian and Raghavan, 2015a; Sebastian and Raghavan, 2015b; Sebastian and Raghavan, 2016; Sebastian and Raghavan, 2017). TUNEL assay revealed DNA damage in spermatogonia mother cells, Sertoli cells, and primary spermatocytes in the same study. The potential of ES to induce DNA damage and compromised DNA repair mechanisms in germ cells is critical for fertility and the stable propagation of species. In the same study, when a sublethal dose of ES was used with a serum concentration of 23 $\mu\text{g/L}$ after 24 h, male mice (33%) infertility was observed due to testicular atrophy. It decreased sperm count and increased mortality (Sebastian and Raghavan, 2015b). However, the serum concentration of ES is much higher in people living in highly exposed areas, with a concentration range of 8.85–547.6 $\mu\text{g/L}$ ES in the affected human population, which can be 700 $\mu\text{g/L}$ after 2 h of exposure (Mnif et al., 2011; Sebastian and Raghavan, 2015b).

ES poisoning increases the incidence of neurological complications and congenital and reproductive abnormalities among the affected people (James and Emmanuel, 2021). However, the effect of ES on the female reproductive system was not investigated previously. Epidemiological studies show that farmers exposed to ES were more prone to developing tumors (Zahm and Ward, 1998) and multiple myeloma (Khuder and Mutgi, 1997). Similarly, people exposed to ES were reported to have an increased risk of developing various cancers in their life (Melangadi, 2017). Although the immediate effects of pesticides are studied extensively, their delayed actions are not explored at the molecular level. There is a knowledge gap between pesticide exposure and its long-term effects on humans. Therefore, to investigate the long-term impact of ES in an exposed human population, we have used a mice model system, considering the relevant concentrations of ES in the exposed areas. In the present study, we report that exposure to ES leads to significantly compromised fertility in both male and female mice. Further, we find a long-term persistent effect on the histopathology of reproductive tissues, liver and lung. Persistent elevated levels of DNA damage leading to compromised DNA repair were also observed. Further, we report an increase in the incidence of tumor development in the mice exposed to ES in their later life.

Materials and methods

Chemicals, reagents, and antibodies

Endosulfan (pestanal) was purchased from Millipore-Sigma (Catalogue No. 32015). Other chemicals and reagents were obtained from Millipore-Sigma (St. Louis, MO, United States) or Sisco Research Laboratories Ltd. (SRL), India. Antibodies were procured from Santa Cruz Biotechnologies (United States) and BD Biosciences (United States).

Animals

All animal experiments were carried out with the approval of the animal ethical committee of the Indian Institute of Science (IISc), Bangalore, India (CAF/Ethics/228/2013; CAF/Ethics/793/2020). Balb/c mice of 4–6 weeks old weighing 20 g–25 g were purchased from Central Animal Facility (CAF), IISc. The animals were housed in polypropylene cages and provided a standard pellet diet (21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorous, 3.4% glucose, 2% vitamin, and 55% nitrogen-free extract (carbohydrates) and purified water *ad libitum*. The mice were maintained under controlled temperature and humidity conditions with a 12 h light/dark cycle.

Administration of endosulfan in mice and hematological analysis

ES was dissolved in 0.05% methylcellulose, and mice were orally fed using a gastric gavage (Sebastian and Raghavan, 2015b). The treatment consisted of a dose of 5 mg/kg on every alternative day for 10 doses.

After 31 days of ES exposure (5 mg/kg, 10 doses), two animals from the treated and control groups were sacrificed using CO₂ asphyxiation. Blood was collected in EDTA-coated vials by heart puncture. Analysis of blood samples was performed at Rohana Veterinary Diagnostic Lab, Bangalore, India. Red blood cells (RBC), white blood cells (WBC), platelet counts (PLT), packed cell volume (PCV), hemoglobin (HGB), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), lymphocytes, neutrophils, eosinophils, and monocytes were analyzed as described before (Sharma et al., 2020; Sharma et al., 2021).

Serum biochemistry

After 31 days of ES exposure (5 mg/kg, 10 doses), two animals from the treated and control groups were sacrificed

using CO₂ asphyxiation. Blood was collected in tubes without anticoagulant and centrifuged (900 ×g, 10 min) for serum extraction. Serum samples were analyzed at Rohana Veterinary Diagnostic Lab, Bangalore, India. Liver and kidney function tests such as serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), albumin, total protein, bilirubin, blood urea nitrogen (BUN), creatinine, uric acid, and phosphorous were analyzed as described earlier (Sebastian and Raghavan, 2015b; Vartak et al., 2016; Sharma S. et al., 2020).

Impact of endosulfan exposure on fertility

Mating experiments in Balb/c mice were performed as described before (Chiruvella et al., 2012; Sebastian and Raghavan, 2015b). After 7 days post-completion of ES treatment, 1:2 (male: female) mating was set up in separate cages for 10 days or two oestrus cycles. At the end of the 10th day, males and females were separated, and the females were observed for pregnancy in the following days. A male was considered infertile if all two females in the cage failed to get impregnated. Four groups of animals were maintained for mating; 1) treated male and untreated female; 2) treated female and untreated male; 3) treated male and treated female; 4) control group comprising untreated male and untreated female ($n = 5/\text{group}$ for male and $n = 10/\text{group}$ for female). The experiments were repeated three independent times on separate occasions.

Histopathological evaluation

Mice organs (brain, lungs, liver, ovary, testes, intestine, and spleen) collected from the animals after 8 months of ES treatment were processed as per standard protocol (Srivastava et al., 2012; Sharma et al., 2013; Gopalakrishnan et al., 2021) and embedded in paraffin. Microtome sectioning was done using rotary microtome with a section thickness of 5 μm (Leica Biosystems, Germany) and stained with hematoxylin and eosin. Images were captured using a bright field microscope (Carl Zeiss, Oberkochen, Germany). Tissues from two mice were subjected to histopathological analysis a minimum of two independent times. The veterinarians analyzed histopathological results at Central Animal Facility, IISc, Bangalore.

Immunofluorescence analysis

Immunofluorescence studies were performed as described before (Chiruvella et al., 2012; Kumari et al., 2019). Paraformaldehyde fixed paraffin embedded tissues sectioned at

5 μm thickness were de-paraffinized and rehydrated in a series of alcohol gradients. Antigen retrieval was done in 10 mM sodium-citrate buffer (pH 6.0), followed by blocking in PBST containing 1% BSA and 10% FBS. Primary antibody [53BP1 (SC 22760) dilution 1:100 and Ligase III (BD 611876) dilution 1:500]; incubation was carried out overnight at 4°C. Slides were washed and incubated with appropriate Alexa Fluor conjugated secondary antibody for 3 h at room temperature. After washing the slide, it was mounted with DAPI-DABCO, and images were taken in a confocal microscope (Olympus, FLUOVIEW FV3000, Japan) and processed by Olympus software. The experiments were repeated independently with multiple technical repeats on separate occasions.

Pharmacokinetics of endosulfan

ES was administered orally to Balb/c mice (5 mg/kg, one dose). Animals were sacrificed at 4, 8, 10, 12, 24, and 48 h post-administration, deproteinization was done using acetonitrile, and the sera were used for HPLC analysis (Shimadzu, Kyoto, Japan) (Srivastava et al., 2012; Sebastian and Raghavan, 2015b; Sharma et al., 2021). Liquid Chromatography (LC) was carried out using a C18 analytical column (Shimadzu). The mobile phase consisted of an isocratic mixture of acetonitrile and water at 70:30 v/v. Spectra were acquired at 214 nm. The injection volume was 20 μl (automatic), and the flow rate used was 0.5 ml/min. The standard solution of ES was prepared by diluting the stock to 1, 5, and 10 μM of the compound in the serum. Standard calibration curves were plotted using peak area against the concentration and retention time of the compound, were also determined. Plasma concentrations of the treated groups were extrapolated from standard curves. Pharmacokinetic parameters were analyzed using LabSolutions software (Shimadzu, Japan). The values obtained were plotted with GraphPad Prism (ver5.1) software, where “C” is the predicted concentration, and “t” is time. Data were analyzed using nonlinear regression analysis. Maximum drug plasma concentration (C_{max}) and time to reach maximum concentration T_{max} were determined by the area under the curve versus the time curve. The clearance rate (CL) was calculated by the rate of drug elimination in plasma (Vartak et al., 2016; Sharma et al., 2021).

Quantification and statistical analysis

All quantifications were performed using GraphPad Prism software, and statistical significance (mean \pm SEM) was calculated. All statistical analyses were performed in GraphPad Prism software using one-tailed unpaired student's *t*-tests. Error bars have been shown depicting mean \pm SEM (ns: not significant, **p* < 0.05, ***p* < 0.005, ****p* < 0.0001). Colocalization analysis was done using JaCoP in ImageJ

software. The values were plotted in GraphPad Prism 5.0, and the significance was calculated using the same.

Result

The bioavailability of endosulfan increases with an increased dose

The appropriate concentration of ES for analysis of physiological changes in mice model system, which can be correlated with humans, is important for the investigation of its effect on human health as exposure to it is reported to cause abnormalities and congenital disabilities in areas of extensive use (Satheesh, 2017; James and Emmanuel, 2021). Since the people living in ES-exposed areas showed a serum concentration of up to 550 $\mu\text{g/L}$ of the pesticide, our study selected a concentration that can mimic the situation. Mice were treated with 5 mg/kg body weight of endosulfan based on the previous investigation (Sebastian and Raghavan, 2015b) (Figure 1A). HPLC analysis was performed to ascertain the serum concentration of ES in mice by collecting serum samples at different time points (4, 8, 10, 12, 24, and 48 h) after oral ingestion (Figure 1B). For standard, serum samples from control mice were collected and spiked with different concentrations of ES. Our results show that the serum concentration of ES after 24 h was \sim 50 $\mu\text{g/L}$ compared to the previously observed 23 $\mu\text{g/L}$ when 3 mg/kg of ES was used (Sebastian and Raghavan, 2015b) (Figure 1B).

HPLC analysis post oral dose of ES revealed that the maximum concentration of ES (C_{max}) was 237 $\mu\text{g/L}$ and the time to reach C_{max} , i.e., T_{max} , was 8 h (Figure 1C). We observed the clearance rate (CL) of 5.14 ml/min/kg in mice serum based on the elimination rate in plasma (Figure 1C). It was eliminated from the blood at the rate of 5.14 ml/min/kg. We did not detect ES in serum after 48 h of oral ingestion.

Exposure to endosulfan affects the normal physiology of mice

Evaluation of biochemical changes in organs is essential to understand the effects of xenobiotics. Therefore, we were interested in evaluating the impact of exposure when 5 mg/kg (10 doses) of ES was used. To do this, we performed liver and kidney function tests after 31 days of ES treatment (Figure 2A). Results showed no significant difference between the control and treated mice in the level of SGPT, total protein, albumin, bilirubin, and ALP (Figure 2B). However, analysis of kidney function (creatinine, phosphorous, uric acid, and BUN levels) showed a significant decrease in BUN levels indicating liver and kidney injury (Figure 2C). This result suggests that ES affects the function of the kidney and liver.

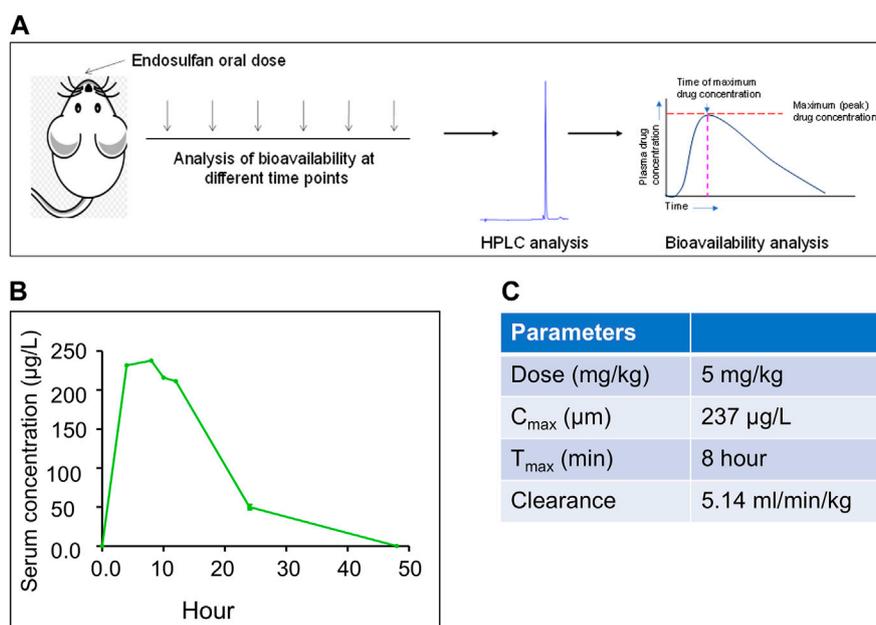


FIGURE 1

Evaluation of bioavailability of ES in mice when exposed to 5 mg/kg body weight of ES. (A) Schematic of the experimental strategy. HPLC analysis was performed using serum samples collected at different time points 4, 8, 10, 12, 24, and 48 h after oral ingestion of ES (5 mg/kg body weight, $n = 3$). (B) *In vivo* bioavailability analysis of ES in the serum of Balb/c mice at 4, 8, 10, 12, 24, and 48 h after exposure with ES (5 mg/kg body weight). Three animals were sacrificed for each time point, sera were collected and subjected to HPLC analysis at 214 nm wavelength. (C) Pharmacokinetics of ES in mice serum. The area under the curve (AUC) was calculated to obtain the maximum circulating concentration (C_{max}) and maximum time (T_{max}) to reach C_{max} .

Hematological parameters are routinely used to indicate the physiological or sublethal stress responses to endogenous and exogenous changes. To analyze any biochemical changes, we assessed different blood parameters in ES-exposed mice (5 mg/kg) after 31 days compared to the control mice (Figure 2D). The quantitative analysis showed a significant increase in total leukocyte counts and neutrophils percentage, possibly due to inflammation but a significant decrease in lymphocyte percentage compared with the controls, indicating that the mice were under stress (Figure 2D). Although an increase in the monocyte population was observed, it was not significant. Blood parameters like platelet counts, RBC, MCHC, MCV, Eosinophils, MCH, and PCV remained unchanged. These results indicate that ES alters the hematological and biochemical parameters in mice.

Endosulfan induces the reproductive toxicity

Mating experiments (1:2 ratio, male: female) were set up the post-one week of the last dose of ES treatment (5 mg/kg, 10 doses) in the mice. The mating was set up in four different groups, Group 1, where ES-exposed male mice were mated with normal female mice; Group 2, where ES-exposed female mice

were mated with normal male mice; Group 3, where both male and female mice were exposed to ES and Group 4 was the untreated control group (Figure 3A).

Male mice were considered infertile if they could not impregnate any female mice. In contrast, female mice were considered infertile if it was not able to get pregnant even after the mating period of 10 days. The percentage of infertility in Group 1 (only male treated) was 55%, while in Group 2 (only female treated), it was 62%. The most severe effect was observed when both parents were treated (67% infertility). However, no infertility was observed when untreated mice were subjected to mating (Figure 3B). Therefore, a significant increase in infertility was observed in the present study compared to the previous report (Sebastian and Raghavan, 2015a).

Endosulfan affects the reproductive organs of mice even after several months of exposure

Although the acute effect of pesticides is studied in detail, its long-term effects are not followed up systematically. When we evaluated the mice for long-term effects of pesticide exposure, we did not find any difference in food and water intake between

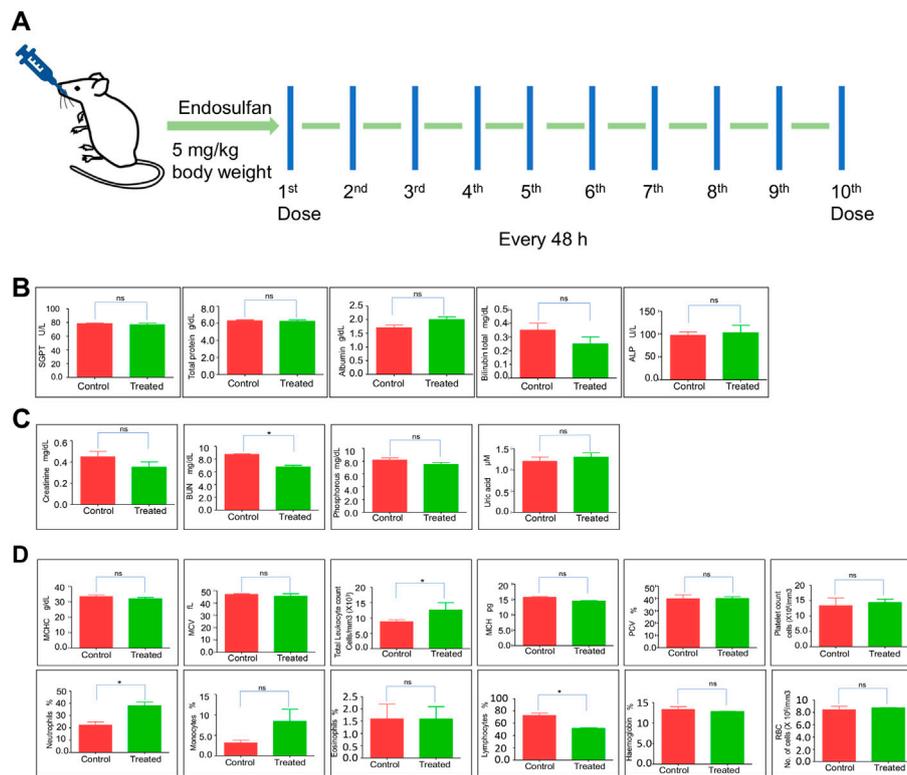


FIGURE 2 Evaluation of physiological effects of ES in mice. **(A)** Schematic representation of ES-treatment in mice. Mice were exposed to 5 mg/kg body weight of ES through oral gavage every alternate day for 10 doses. **(B,C)** Liver and kidney function test of ES-treated mice. Bar graphs indicating enzymatic activities reflective of liver and kidney function at 31st day after ES treatment (5 mg/kg, $n = 2$). **(D)** Blood parameter analysis following oral administration of ES in mice after 31st day ($n = 2$). Analysis of red blood cells (RBC), white blood cells (WBC), haemoglobin (HGB), platelets, MCHC, MCV, MCH, PCV, neutrophils, monocytes, eosinophils, and lymphocytes counted among control and treated after 31 days of administration. Error bars denote mean \pm SEM (ns: not significant, $*p < 0.05$).

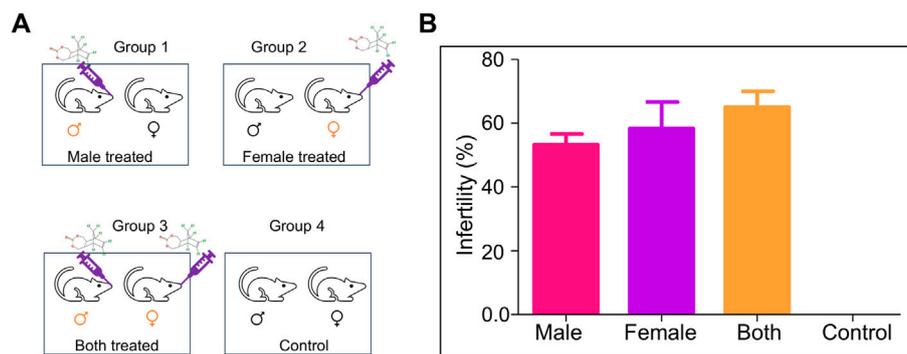
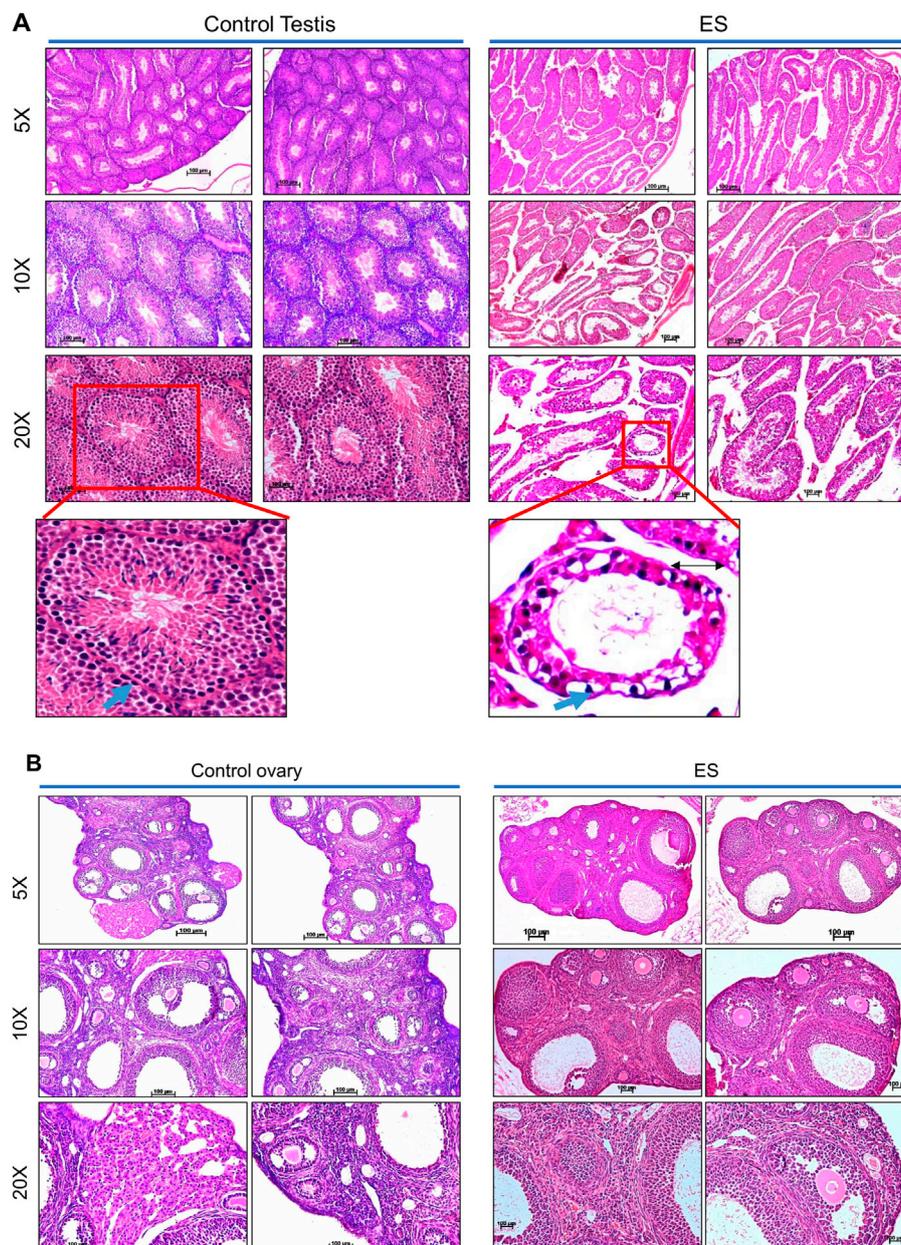


FIGURE 3 Evaluation of the effect of ES on fertility in mice. **(A)** Schematic is showing mating groups following exposure to endosulfan (5 mg/kg body weight); Group 1 (male treated), Group 2 (female treated), Group 3 (male and female treated), and Group 4 (untreated control). **(B)** Bar graphs show the difference in fertility levels when ES was given only to males ($n = 5$), only to females ($n = 10$), or to both males and females ($n = 15$). Mating was in the ratio of 1:2 of male to female. Experiments were repeated 3 times. Error bars denote mean \pm SEM.

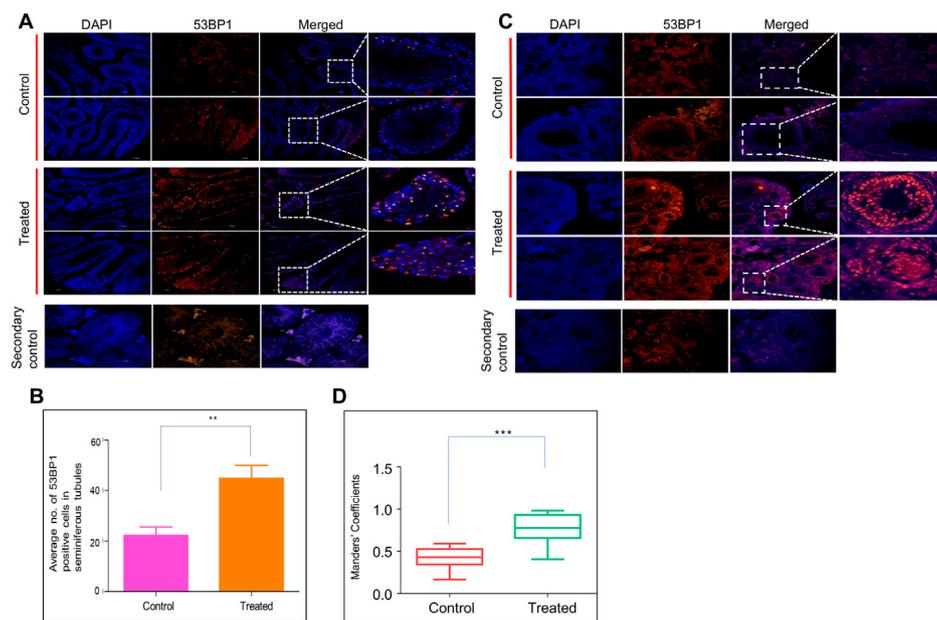
**FIGURE 4**

Histopathological examination of reproductive organs from ES-exposed mice. **(A)** Histopathology of testis of mice following ES administration. Control indicates testis tissue from mice with no treatment, and ES represents tissue from ES-treated mice (5 mg/kg, 10 doses; $n = 2$; Magnification: $\times 5$, $\times 10$, and $\times 20$). To evaluate long-lasting effects of exposure to ES, tissue samples were collected after 8 months following exposure. Nuclear components, including heterochromatin and nucleoli were stained by haematoxylin as deep blue or purple while eosin-stained cytoplasmic components like collagen, elastic fibers, muscle fibres and red blood cells. The blue arrows indicate the spermatogonia cells in basal epithelial layer, black arrow in ES indicates the interstitial space between the seminiferous tubules. **(B)** Histopathology of the ovary of mice following ES administration (5 mg/kg, 10 doses) after 8 months of exposure. The experiments were repeated two independent time for each tissue. Control indicates ovary tissue from mice with no ES treatment and ES represents tissue from ES exposed mice (Magnification: $\times 5$, $\times 10$, and $\times 20$; Scale bar: 100 μm).

control and ES-exposed mice. Besides, their social behaviour remained unchanged.

Histopathological analysis was performed to understand the direct and long-lasting effect of ES on the reproductive organs of

exposed mice (Figure 4). After 8 months of ES treatment, analysis of the testes revealed vacuolar degeneration of basal epithelial cells and increased interstitial spaces due to the shrinkage of seminiferous tubules compared to control mice (Figure 4A).

**FIGURE 5**

Evaluation of ES induced DNA breaks in mice reproductive organs. To evaluate the long-term effects of ES, mice ($n = 2$) were exposed to ES (5 mg/kg, 10 doses), and tissue samples were collected after 8 months and examined for 53BP1 foci, a hallmark of DSBs. **(A)** Immunofluorescence for 53BP1 expression in testis of ES treated mice. Control indicates sections of testes from untreated control mice, while treated refers to sections from testes of ES-exposed mice. **(B)** Bar graph indicates the average number of 53BP1 positive cells in control and treated mice seminiferous tubules. **(C)** Immunofluorescence of 53BP1 expression in ovary of control and ES treated mice. Control indicates ovary sections from untreated mice, whereas ES is sections from treated mice. The experiments were repeated two independent times. **(D)** Bar graph indicates number of 53BP1 positive cells in control and treated mice nucleus calculated as Mander's coefficient on ImageJ (ns: not significant, $**p < 0.005$). In panel **(B)** at least 25 seminiferous tubules and in panel **(D)** at least 20 fields each were analyzed.

Many seminiferous tubules were affected, which had depleted spermatogonia mother cells in ES-exposed animals. A visible difference was also observed in the sperm population in the lumen of seminiferous tubules in treated mice compared to controls (Figure 4A).

Similar to the histopathology of mice testes, decreased follicular activity and an increased number of corpus luteum were observed in the ovary of treated female mice even after 8 months of ES exposure (Figure 4B). An increase in infertility in females correlates with decreased follicular activity in ES-exposed mice ovary. Thus, our results reveal the persistent, long-lasting effect of ES exposure on the male and female reproductive system.

Endosulfan exposure leads to persistent DNA breaks in the reproductive organs of mice

Since the histological investigation of reproductive organs implicated the persistent effect of ES, we evaluated the reproductive tissues for long-term persistent DNA damage. DNA damage was investigated by immunofluorescence

staining of 53BP1 (p53-binding protein 1) in both testes and ovaries in ES-exposed mice (Figure 5). 53BP1 is a well-known DNA damage response factor, and it is recruited at the site of DNA damage (Gupta et al., 2014). 53BP1 staining on testis sections from ES-exposed mice showed significantly elevated expression in seminiferous tubules of testes when compared to controls, suggesting the generation of DNA double-strand breaks (DSBs) (Figures 5A,B). While the 53BP1 foci were restricted only to the spermatogonial mother cells surrounding the basal epithelial layer of the seminiferous tubules in the control mice testis, a significantly elevated number of 53BP1 foci were seen in spermatocytes and spermatids in the case of treated mice testis. These results implicated the persistent DNA damage in testicular cells when exposed to ES.

Further, a significant increase in 53BP1 foci was observed in the ovary of ES-exposed mice compared to controls (Figures 5C,D). Specifically, 53BP1 foci were seen in primordial follicles and the granulosa cells of the primary follicles in treated mice. Considering that granulosa cells are involved in the maturation of the entire follicle and may play a crucial role in ovarian physiology, damage to these cells affects the proper maturation of the oocytes resulting in infertility (Amsterdam and Rotmensch, 1987).

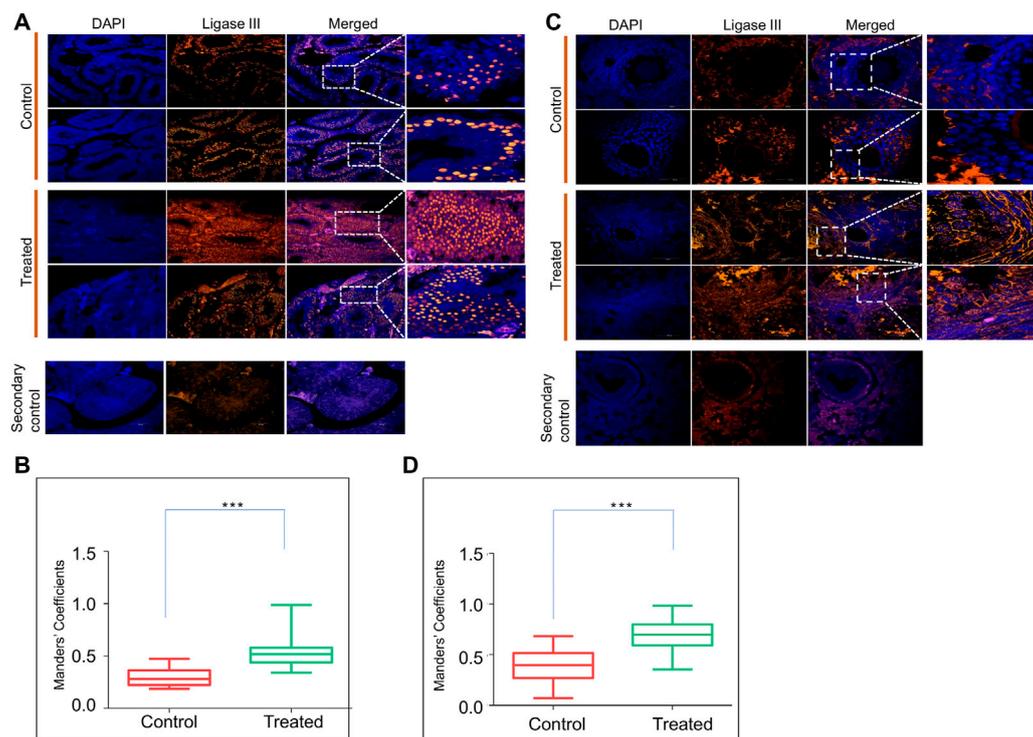


FIGURE 6

Evaluation of ES induced DNA repair through Ligase III in reproductive organs. **(A)** Immunofluorescence of DNA Ligase III expression in testes of ES-treated mice (5 mg/kg, 10 doses, $n = 2$). Tissue samples were collected after 8 months of exposure to ES. Control indicates sections of testes from untreated mice, whereas, ES is sections from treated mice. The nucleus is counterstained with DAPI. The merged image shows localisation of DNA Ligase III positive cells. **(B)** Bar graph shows localization of DNA Ligase III positive cells in control and treated mice testis calculated as Mander's coefficient on ImageJ. **(C)** Evaluation of ES induced DNA Ligase III in mice ovary. Immunofluorescence staining of DNA Ligase III expression in ovary of control and ES treated mice. **(D)** Bar graph indicates localization of DNA Ligase III positive cells in control and treated mice ovary calculated as Mander's coefficient on ImageJ. In panels **(B,D)** at least 20 fields each were analyzed from two independent batches (ns: not significant, $*p < 0.05$, $**p < 0.005$, $***p < 0.0001$).

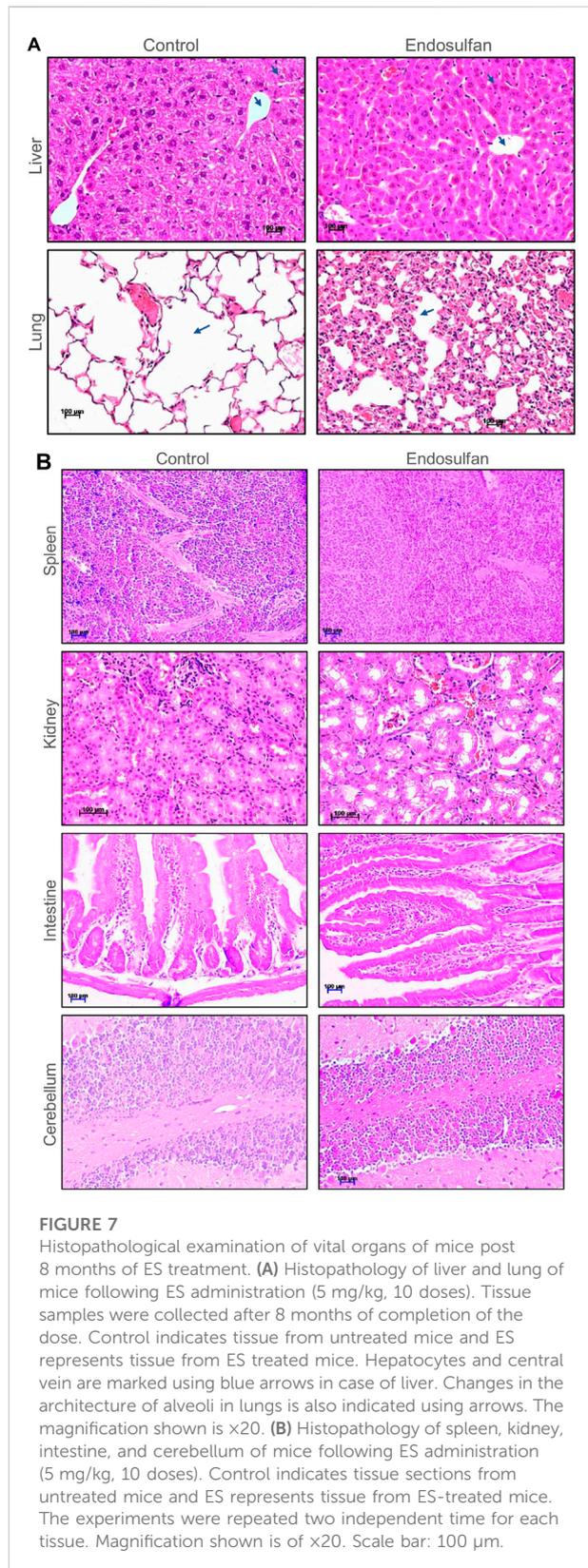
Exposure to endosulfan results in overexpression of DNA ligase III in reproductive organs

Previously, we observed that ES induces error-prone DNA repair through MMEJ in which DNA ligase III is one of the most critical enzymes responsible for the final sealing of breaks (Wang et al., 2005; Sharma et al., 2015; Sebastian and Raghavan, 2016). Therefore, we examined the expression of DNA Ligase III in the reproductive tissues by immunofluorescence staining on the testis and ovary sections after 8 months of ES exposure (Figure 6). In the ES-exposed mice testes, the expression of DNA Ligase III was significantly higher than that of the control (Figures 6A,B). A similar analysis of the ovary section revealed elevated expression of DNA Ligase III in ES-exposed mice (Figures 6C,D). Interestingly, DNA Ligase III expression was observed highest in granulosa cells, as was the case of 53BP1 (Figures 5C,D), which indicated the induction of the alternate error-prone DNA repair pathway. Thus, our results indicate that

the persistent ES-induced DNA double-strand breaks in the reproductive organs of exposed mice led to the elevated expression of DNA Ligase III.

Endosulfan induces damage in the lungs and liver of mice but not in other organs

We were interested in testing the persistence and direct effect of ES on the vital organs of mice after 8 months of its exposure (5 mg/kg, 10 doses). The histopathological analysis of the lungs, liver, kidney, intestine, cerebellum, and spleen was performed on ES-exposed mice after 8 months of the treatment. Results showed changes in the architecture of alveoli, which was more congested in the lungs of treated mice than in control (Figure 7A). Mild vacuolation in hepatocytes was observed in ES-treated mice liver, indicating irritant in the blood system (Figure 7A). We did not find any remarkable difference in the histology of the spleen, kidney, intestine, and cerebellum of the treated mice when



compared with the control (Figure 7B). Thus, histopathological analysis revealed that ES-exposure had a persistent effect on the liver, lungs, and reproductive organs.

Endosulfan induces tumorigenesis in mice

Pesticides are considered one of the major factors contributing to increased tumor incidence, although the direct link is still not well established (Anwar, 1997; Dich et al., 1997; Bharathi et al., 2013). In the present study, we observed the development of tumours in ES exposed (5 mg/kg, 10 doses) mice after 3 and 16 months of completion of the dose (Figures 8, 9). Abnormal growth was observed in the lungs of two ES-exposed male mice (Figure 8A, top and bottom panel). Histopathology of the suspected tumour tissues revealed undifferentiated cells (Figure 8B, top panel). Further, infiltrating cells in the bronchiole of the lung were also observed (Figure 8B, bottom panel, marked with a red arrow). To confirm the cancerous nature of the tissues, immunohistochemical analysis was performed with anti Ki67, a cell proliferation marker, and p53, the tumour suppressor gene, in ES-exposed mice. Results revealed the expression of Ki67 and p53 in the lung, confirming that the abnormal growth was indeed cancerous (Figure 8C). We also observed the tumour development in the neck region of one of the ES-exposed female mice 3 months after the dosing (5 mg/kg, 10 doses) (Figure 9A). Further, histopathological analysis of these tissues revealed the presence of undifferentiated cells (Figure 9B). Sheets of neoplastic cells which did not have well-defined borders were also visible. The cells were uniform in size and shape and were mononucleated. Thus, our observations show compelling evidence that ES has the potential to cause long-term harmful effects on organisms, including tumorigenesis.

Discussion

A preponderance of epidemiology studies shows that pesticides have a health burden on non-target species like humans due to their intrinsic toxicity and limited species selectivity. Widespread pesticide use contaminates the food chain and causes mutagenic activation or inactivation of the ingested chemicals (Pednekar et al., 1987; Bolognesi, 2003; Mostafalou and Abdollahi, 2013; Kapeleka et al., 2021). In the present study, we have used mice as a model system to study the chronic effects of pesticides once exposed when they are 4–8 weeks old. Therefore, the present study can link pesticide exposure and its long-term/persistent impact on humans and provide a molecular basis for its persistent effects.

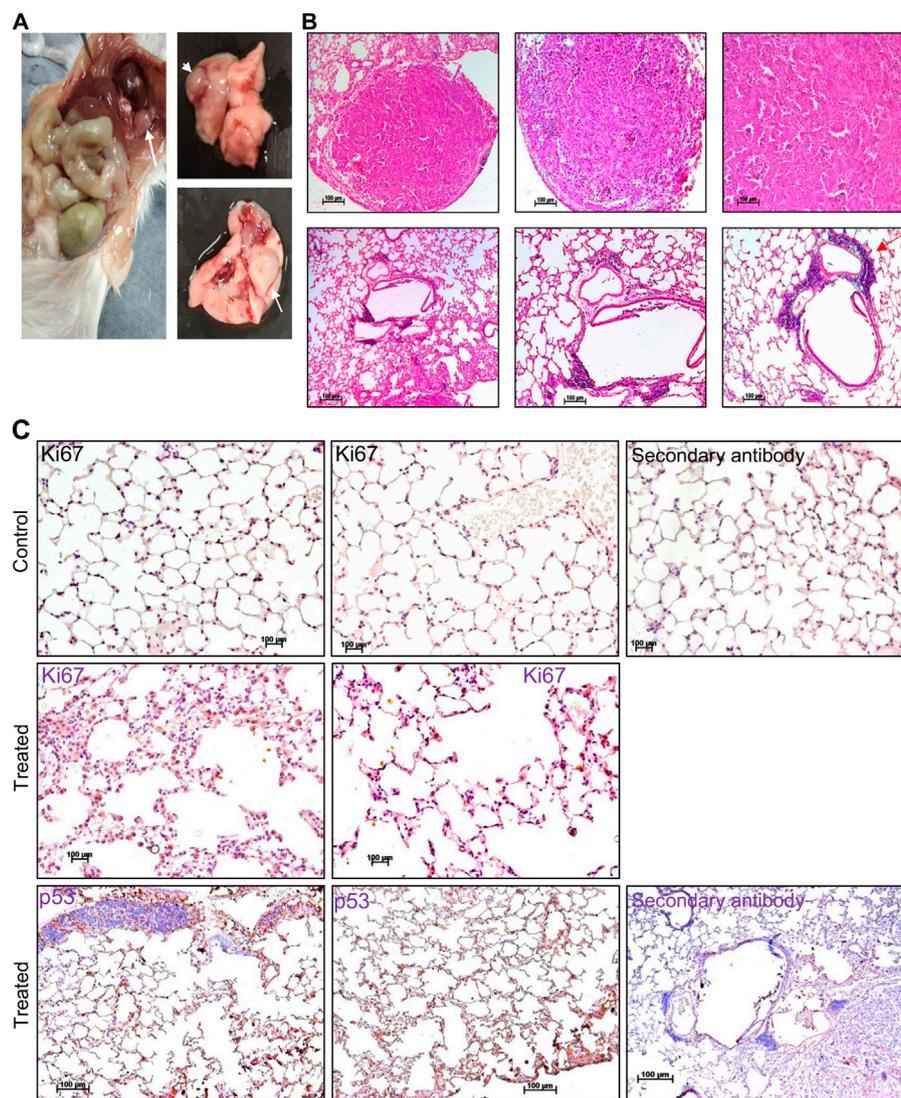


FIGURE 8

Histopathological examination of abnormal growth in lungs of mice post treatment with ES. (A) Abnormal growth was observed in the lungs following 16 months of ES exposure (5 mg/kg, 10 doses) in male mice. The white arrow indicates abnormal growth. (B) Histopathological analysis of abnormal growth in lungs from ES exposed male mice. The red arrow indicates the infiltrating cells (5 \times , 10 \times , and 20 \times). (C) Immunohistochemical analysis of abnormal growth in lungs of ES exposed mice. Immunohistochemical analysis of control mice lung with Ki67 (top panel) and treated mice lung with Ki67 and p53 (bottom panel) (20 \times). In panels (B,C) experiment was repeated three independent times from the tissues of same animal. Control indicates lung tissue from untreated mice and treated indicates ES-treated tissue from male mice. Scale bar: 100 μ m.

A distinct increase in infertility and increased reproductive issues have been associated with occupational or environmental exposure to pesticides during the last few decades (Oliva et al., 2001; Joffe, 2003). In the present study, a serum concentration of 50 μ g/L (24 h) was used, as this could mimic relevant concentration in such ES-exposed areas. Interestingly, we observed a significant increase in infertility in both male (30%–55%) and female (20%–62%) mice compared to the previous study in which serum concentration was 23 μ g/L (24 h) (Sebastian and Raghavan, 2015b). Several studies have

also reported that ES affects the reproductive tissues in different animal models (Sinha et al., 1995; Hiremath and Kaliwal, 2002b; Rey et al., 2009; Milesi et al., 2015). The observed increase in infertility in male and female mice was consistent with the epidemiological studies from the affected region (Jayadevan et al., 2005; Kanthila and Shenoy, 2012; Satheesh, 2017; Badiger et al., 2019). It has been shown that ES affects testicular functions due to its effects on reproductive hormones, leading to abnormal spermatozoa and decreased sperm count and sperm motility (Ozmen, 2011; Sebastian and

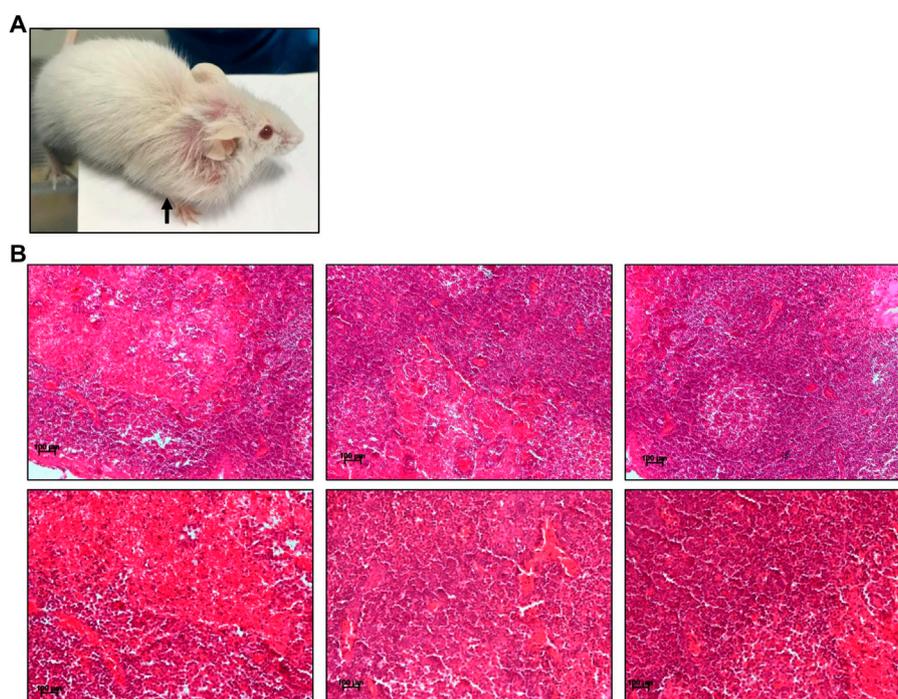


FIGURE 9

Histopathological examination of abnormal growth in ES-exposed mice post treatment. **(A)** Abnormal growth in neck region observed after 3 months of ES exposure indicated by black arrow in ES-exposed female mice (5 mg/kg, 10 doses). **(B)** Histology of abnormal tissue from the neck region of the ES-exposed female mice (x20). Experiment was repeated three independent times from the tissues of same animal. Scale bar: 100 μ m.

Raghavan, 2015a; Sengupta and Banerjee, 2013). Although there exists a limited amount of human data on the effect of ES, a cohort study by Saiyed et al. (2003) among school children (10–19 years old) showed delayed male sexual maturity and interference with male sex-hormone synthesis due to exposure to ES (Saiyed et al., 2003). ES is also shown to cause ovarian regression in females, alterations in hormone synthesis, follicular maturation, ovulation process, and ovarian cycle, which leads to an increase in infertility (Hiremath and Kaliwal, 2002a; Sharma R. K. et al., 2020).

The effect of ES on the reproductive system is linked to its hormonal-disrupting function, and such pesticides are often called endocrine-disrupting chemicals (EDCs). These EDCs significantly impact the reproductive system; their activity can be due to direct binding with hormone receptors owing to their conformational similarity with receptor-binding portions of natural steroid hormones (Mrema et al., 2013). Numerous studies have shown that ES behaves as an anti-androgen (Singh and Pandey, 1990; Wilson and LeBlanc, 1998; Murolo et al., 2001; Viswanath et al., 2010). We have also shown through docking studies and bioinformatic analysis that ES can bind to the ligand-binding site of the androgen receptor with considerable energy compared to its natural ligand dihydrotestosterone in the previous study (Sebastian and

Raghavan, 2015a). The reproductive toxicity of ES is not limited to mammals but also extended to various other animals like zebrafish (Han et al., 2011), crocodiles (Tavalieri et al., 2020), *C. elegans* (Du et al., 2015) and Newt (Park et al., 2001) indicating that it could affect most forms of life in the ecosystem.

Besides reproductive organs, the present study revealed that exposure to ES affected the lungs and liver in mice, which was also consistent with our previous results (Sebastian and Raghavan, 2015b). Studies from other animal models have also reported the liver, lungs, brain, and kidney as the major target organ for ES-exposure (Gupta and Chandra, 1977; Seth et al., 1986; Paul and Balasubramaniam, 1997; Caglar et al., 2003; Mor and Ozmen, 2003; Jia and Misra, 2007).

Although acute toxicity studies of pesticides are reported, their long-term effects are not well investigated, particularly in humans. Previously, immediate effects of ES were observed in the testes, lungs, and liver when the histochemical analysis was performed (Sebastian and Raghavan, 2015b). In the present study, when the long-term effect on the vital organs of mice was evaluated, we noted that such effects were persistent. We observed vacuolar degeneration of basal epithelial cells and increased interstitial spaces due to shrinkage of seminiferous tubules in mice testes even after

8 months of ES-exposure. A visible difference in the sperm population was also observed in the lumen of seminiferous tubules in the ES-exposed mice. A decrease in follicular activity in ES-exposed mice ovaries further suggests the pesticide's long-term effect on female reproductive tissues. A persistent effect was also seen in the lungs and liver of the ES-exposed mice. The architecture of the alveoli of the lungs was changed in ES-exposed mice. In the liver, mild vacuolation in hepatocytes was observed even after 8 months of ES exposure. The only available long-term study conducted in liver tissues of ES-treated mice, which was assessed after 6 months, demonstrated that exposure to ES caused liver tissue damage illustrated by hepatic/somatic index and increased levels of liver Lactate Dehydrogenase (Kurutaş et al., 2006). Further, our results, in conjunction with a previous study, revealed that ES-induced persistent DNA damage in testes and ovaries, altered DNA damage response, and possibly elevated levels of microhomology-mediated end joining (Sebastian and Raghavan, 2016).

A study by the International Agency for Cancer Research (IARC) has reported potential carcinogenicity of chemicals such as phenoxy acid herbicides, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), lindane, methoxychlor, toxaphene and several organophosphates in laboratory animals but epidemiological data on cancer risk in farmers are conflicting (Bolognesi, 2003). *In vitro* studies have reported the carcinogenic potential of ES (Flodström et al., 1988; Soto et al., 1994; Bulayeva and Watson, 2004; Zhu et al., 2008; Bedor et al., 2010). Besides the general public, farmers and industrial workers are exposed mainly to pesticides. Although there is controversy regarding the carcinogenicity of ES in the exposed region, we report the development of tumours in three different mice exposed to ES several months prior. Two male mice developed a lung tumour, while one female mouse developed a tumour near the neck region. Histopathology and immunohistochemistry studies indeed revealed the existence of cancer cells. Therefore, our study points toward the carcinogenic potential of ES. Further, this was consistent with our previous results, where ES induced an error-prone MMEJ in the reproductive and lung tissues leading to deletions and other genomic rearrangements (Sebastian and Raghavan, 2016). Thus, our study reveals the persistent effect of pesticides and indicates the need for a thorough investigation of the impact of pesticides on human health.

Although the present study is focused on one pesticide, these results can be extrapolated to other pesticides and may be helpful during the policymaking of the use and manufacture of such pesticides. Further, this study also underlines the need for detailed epidemiological and animal studies to understand the extent of pesticide poisoning to human health and the environment.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Animal ethical committee of the Indian Institute of Science (IISc), Bangalore, India (CAF/Ethics/228/2013; CAF/Ethics/793/2020).

Author contributions

Conceptualization, SR, AS, AK, SD, and BC; Methodology, AS, AK, SD, SK, and SR; Investigation, AS, AK, SD, and SK; Writing—original draft, AS and SR; Writing—review and editing, SR, AS, and BC; Funding acquisition, resources, supervision, SR.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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