Mini-Review

Molecular mechanism of Endosulfan action in mammals

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Endosulfan is a broad-spectrum organochlorine pesticide, speculated to be detrimental to human health in areas of active exposure. However, the molecular insights to its mechanism of action remain poorly understood. In two recent studies, our group investigated the physiological and molecular aspects of endosulfan action using *in vitro, ex vivo* and *in vivo* analyses. The results showed that apart from reducing fertility levels in male animals, Endosulfan induced DNA damage that triggers compromised DNA damage response leading to undesirable processing of broken DNA ends. In this review, pesticide use especially of Endosulfan in the Indian scenario is summarized and the importance of our findings, especially the rationalized use of pesticides in the future, is emphasized.

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Pesticides constitute a heterogeneous category of molecules specifically designed for the control of pests, weeds or plant diseases. They are designed to selectively affect the pest without causing any harm to non-target organisms including humans. However, achieving absolute specificity in biological systems is a persisting challenge. Pesticides, by virtue of their chemical nature, have been considered potential mutagens. Although studies on the acute toxicity of pesticides are extensively available in the literature, their molecular mechanism of action is a poorly explored area. The genotoxic potential of pesticides is the primary factor for long-term effects such as carcinogenic and reproductive toxicology. Various studies have revealed that many agrochemical molecules possess mutagenic properties and induce gene mutations, chromosomal alterations or DNA damage (Garrett *et al.* 1986; Dearfield *et al.* 1993). Globally, people are inevitably exposed to pesticides, and their metabolites, through environmental contamination or occupational use.

A positive association between occupational exposure to complex pesticide mixtures and the presence of chromosomal aberrations (CA), sister-chromatid exchanges (SCE) and micronuclei (MN) formation has been detected in many studies (Pastor *et al.* 2001; Singh *et al.* 2011). One of the most common ways by which these pesticide molecules induce DNA damage is via the production of reactive oxygen species (ROS) (Bagchi *et al.* 1995). It has been shown that a variety of pesticides induce DNA damage and trigger various levels of repair activities in cell lines (Ahmed *et al.* 1977). These studies speculate that many of the molecules may be potentially harmful to humans despite their wide spread use in agriculture and relatively low acute toxicities in animal models and cell lines, because of their genotoxic potential, that can exert direct or indirect modifications to the DNA.

1. Pesticides and India

In India, use of pesticides started with DDT (dichlorodiphenyltrichloroethane). Despite being a country with 1.3 billion people, the use of pesticides in India is only 0.5 kg/ha, vis-à-vis countries with smaller populations such as Japan (12 kg/ha) (Abhilash and Singh 2009). The worldwide use of pesticides is approximately 2 million tons per year, of which 24% is consumed in the USA, 45% in Europe and 25% in the rest of the world. India accounts for 3.75% of total global consumption with 25% of the total agricultural land coverage. Among different classes of chemicals, organochlorine pesticides constitute ~10% of the total consumption in India as of 2010 (figure 1A, B).

Keywords. DNA damage; double-strand break; genomic instability; infertility; MMEJ; NHEJ; pesticides

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(A)

Name	Usage in metric tons	Class of chemical
Endosulfan	2061	Organochlorine
Acephate	1213	Organophosphate
Dichlorvos	1011	Organophosphate
Malathion	1170	Organophosphate
Monocrotophos	1383	Organophosphate
Quinalphos	1474	Organophosphate
Carbendazim	1154	Carbamate
Mancozeb	1847	Carbamate
Butachlor	1291	Acetanilide

(B)

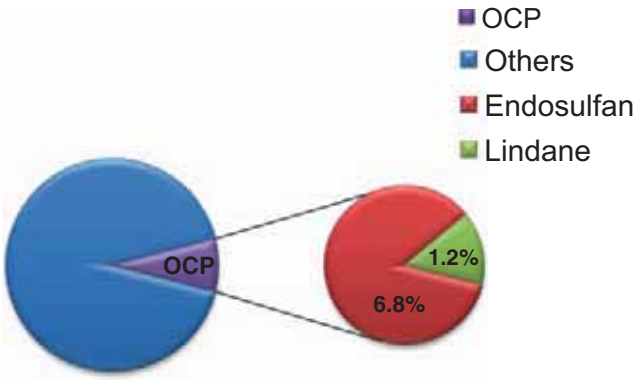

Figure 1. Endosulfan and other major pesticides consumption in India. (**A**) Table listing most commonly used pesticides in India and their chemical class. Usage shown is for the period 2009–2010. (**B**) Share of Endosulfan in total pesticide consumption in India (2009–2010) (OCP, organochlorine pesticides) (data based on information from Department of Agriculture, Government of India).

It is alarming that more than 70% of the total formulations used in agriculture in India are banned or restricted in the western and eastern parts of the world (Subramanian *et al.* 2007). Uncontrolled use of pesticides having hazardous side effects, which are banned elsewhere in the world, might impose serious health hazards on the Indian population and this calls for scientific interventions in order to understand the mechanisms of toxicity.

2. Endosulfan

Organochlorine pesticides (OCPs) are one of the major classes of pesticides, with high transport potential, known to cause toxic and various health effects on non-target organisms including humans (Sanpera *et al.* 2002; Ali *et al.* 2014). Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide), a cyclodiene OCP consisting of α and β isomers (3:1 ratio), is of concern as an environmental pollutant (Usenko *et al.* 2007) with speculated adverse effects on human health (Saiyed *et al.* 2003).

Endosulfan is a class II (moderately toxic) pesticide according to the World Health Organization (WHO) and is listed in the Stockholm convention as a persistent organic pollutant (POP) because of its environmental impacts (*http://chm.pops.int/default.aspx*). Owing to its extensive use in last 50 years, Endosulfan is still one of the most commonly detected pesticides (OCPs) in surface water and air in USA (Siddique *et al.* 2003; Sun *et al.* 2006). Such an environmental persistence of Endosulfan is attributed to its lipophilic nature, stable metabolic by-products and high transport potential across the food chain. Despite the ban of Endosulfan in some countries, it is still preferred and used in many Asian countries including China and India, jeopardising the health of over 2.5 billion people (Wesseling *et al.* 2005), and is of a global concern due to increasing trade of agricultural products.

Endosulfan is known to exert adverse health effects, such as reproductive defects, neurobehavioural disorders, and

endocrine and immunological toxicity (Mrema *et al.* 2013). The mechanism of action of Endosulfan in causing these ill effects could be diverse (genomic or nongenomic) and context-dependent. Broadly, these mechanisms include mitochondrial dysfunction, induction of oxidative stress and modulation of stress responsive signal transduction pathways. For example, Endosulfan was shown to block GABA mediated chlorine channel operation, impairing cerebral cortex functions, leading to neurotoxicity (Kamijima and Casida 2000). The mitochondrial uncoupling activity, ROS generation and oxidative stress by Endosulfan are well studied in cell lines (Jia and Misra 2007). This oxidative stress could be considered as a trigger for Caspase 3 and NFkβ activation, thus leading to apoptosis, resulting in cytotoxicity. Apoptotic effects of Endosulfan were also monitored in leukaemic cell lines and germ cells (Sinha *et al.* 1999; Sohn *et al.* 2004). Besides, Endosulfan was shown to interfere with androgen and estrogen receptors. However, the consequence of such bindings *in vivo* remains to be investigated (Bretveld *et al.* 2006). One of the most important aspects of Endosulfan poisoning in mammals includes testicular toxicity, although comprehensive studies linking toxicity to reproductive abnormalities and its molecular mechanism have remained elusive until recently (Mrema *et al.* 2013).

3. Endosulfan in India

Endosulfan is the most commonly used pesticide in India (Department of Agriculture, Government of India, 2009–2010) (figure 1) (Abhilash and Singh 2009). During 1999–2000, about 81,000 metric tons of endosulfan was manufactured in the country (Saiyed *et al.* 2003). The molecule was linked with various health hazards such as mental and physical disabilities, reproductive failure, hormonal imbalance, skin rashes, concurrent abortions, etc., in certain regions where it has been used for long time (Saiyed *et al.* 2003). These include Kasargod district in Kerala and Malanad regions in Karnataka, where Endosulfan was used extensively for more than two decades, via the aerial spraying method.

Although Endosulfan's association with health defects in humans was speculated in the areas of its active use, comprehensive molecular studies to understand the mechanism of action resulting in genomic instability, DNA damage or ill health were limited until recently.

4. Dissecting the mechanism of Endosulfan action

Recent *in vitro, ex vivo* and *in vivo* studies from our group have revealed the molecular and physiological effect of Endosulfan (figure 2) (Sebastian and Raghavan 2015a, b, 2016). Comparing the experimentally validated bioavailability in mice and the known concentrations of Endosulfan reported in accidental and occupational exposure in humans, we resorted to physiologically relevant sub-lethal concentrations of Endosulfan in the investigation.

While studying the physiology of Endosulfan-exposed animals, we observed high degree of atrophy and tubular necrosis in mouse testis, with many seminiferous tubules having fully or partially defoliated spermatogonial mother cells and spermatids (Sebastian and Raghavan 2015a, b, 2016). TUNEL assay and FACS analyses of testicular cells at various time points after Endosulfan treatment revealed testicular cell death. Further, clinical analyses of epididymal sperms confirmed that testicular cell death resulted in reduction of quantitative and qualitative aspects of sperms. This led to reduced fertility in the treated animals by ~30%. We also observed that the combined treatment in males and females did not increase the infertility levels compared to treatment in males alone. This suggests a male-specific action of Endosulfan (Sebastian and Raghavan 2015a, b, 2016).

In continuation to the physiological aspects, we studied the molecular mechanism of Endosulfan action. Endosulfan is found to be a ROS producer (but a non-DNA intercalator) and induced DNA damage in an ROS-dependent manner (Sebastian and Raghavan 2015a, b, 2016). It is also interesting that our experiments detected an elevated level of ROS in epididymal sperms of exposed animals which may be correlated to reduced sperm chromatin integrity observed immediately after the exposure to Endosulfan (Sebastian and Raghavan, 2015a, b, 2016). Immunohistochemistry, immunofluorescence and Western blot analyses revealed Endosulfan-mediated DNA damage in the lungs and testes of exposed animals. Following DNA damage, a specific pattern of expression of DNA-repair-associated proteins such as 53BP1, KU70, KU80, CtIP and LIG3 was observed in the testes (Sebastian and Raghavan 2015a, b, 2016). Consistent to the DNA damage pattern and TUNEL-positive cells, most of the expression was found to be in spermatogonial mother cells, sertoli cells and primary spermatocytes.

Quantitative analyses of DNA repair efficiency using *in vitro* nonhomologous DNA end joining (NHEJ) assay using cell-free extracts of these tissues showed that the DNA end joining pattern is altered. Extensive sequencing analyses showed increased length and frequency of deletions catalysed by lungs and testes upon Endosulfan treatment, with several of them favouring microhomology. Further, a dedicated microhomology-mediated end joining assay demonstrated that Endosulfan indeed favoured microhomology during DNA end joining (Sebastian and Raghavan 2015a, b, 2016).

Unlike classical NHEJ (c-NHEJ), microhomology-mediated end joining (MMEJ) is considered error-prone and deleterious (Simsek and Jasin 2011; Deriano and Roth 2013). Besides its role in genomic instability in nuclear DNA, MMEJ has been implicated as the principal mediator during DSB repair associated with mitochondrial DNA lesions (Tadi *et al.* 2015). The potential of Endosulfan to induce DNA damage in the background of compromised repair or repair fidelity further intensifies the threshold of

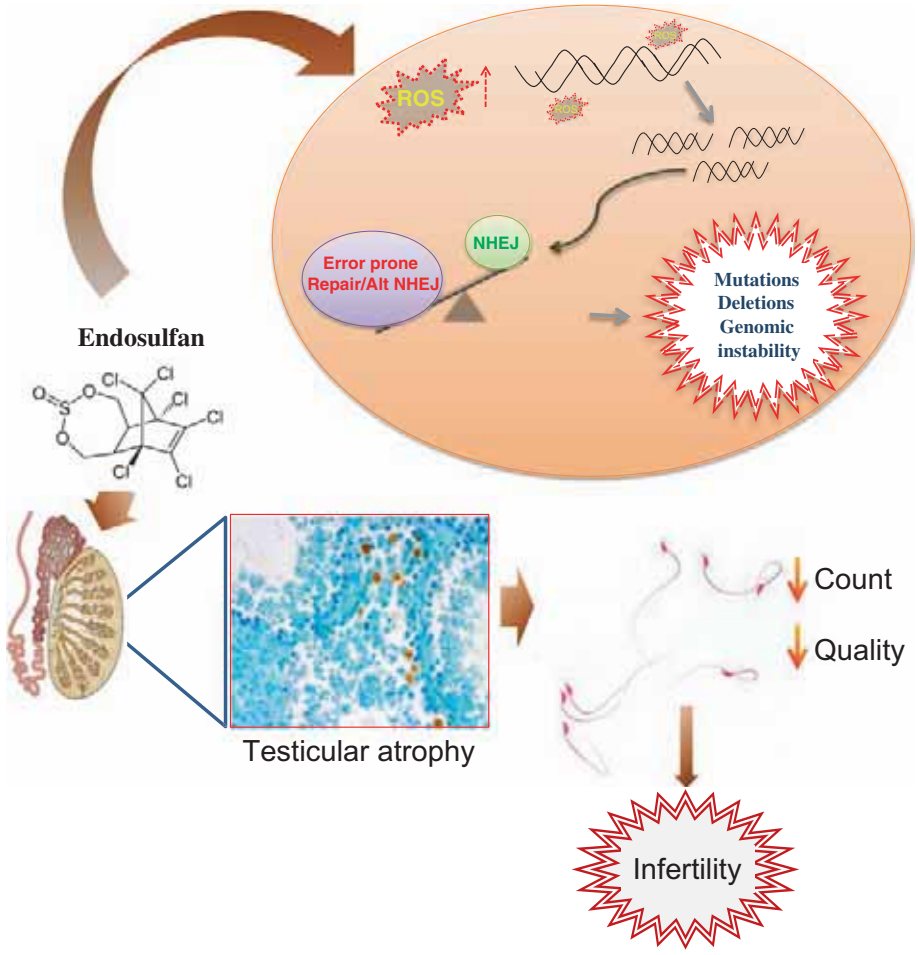

Figure 2. Model illustrating the molecular and physiological mechanisms of action of Endosulfan. Endosulfan induces ROS-mediated DNA damage in cells, which triggers a DNA damage response (DDR). While the classical NHEJ pathway is altered and up-regulated, deletion and mutation prone MMEJ is favoured, most likely due to differential protein expression. This further accentuates the genomic instability of Endosulfan-exposed cells and tissues, which could result in disease conditions in organisms. At a physiological level, Endosulfan exposure predominantly affects testes. It induces testicular atrophy, depleting cell populations in the seminiferous tubule and affecting spermatogenesis. This further results in qualitative and quantitative reduction in epididymal sperms, culminating in male infertility.

genomic instability, and when present in germ-tissue-like testes, could induce long-lasting ill effects (figure 2). The collective effect of these molecular and physiological events in the progeny is being investigated. Although the current study used physiologically relevant concentration of Endosulfan, it may not reflect the outcome of chronic exposure, where persistent presence of the molecule could trigger additional molecular and physiological changes.

Our recent studies prove very relevant to the Indian scenario, given the increasing concerns about pesticides and their health hazards and an absence of understanding of the same (Enserink *et al.* 2013; Kohler and Triebskorn 2013; Mascarelli 2013; Stokstd and Grullon 2013). This is also an important aspect globally due to the world trade of agrarian produce and the absence of, or poor-quality check, measures in many countries (Stokstd and Grullon 2013). Future studies along the similar lines of other pesticides under suspicion would help us regulate the use of pesticides and innovate new molecules with reduced off-target effects and environmental toxicity.

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