CHAPTER 4

RESULTS AND DISCUSSION

4.1 *IN VITRO* DOSE SELECTION TESTS

4.1.1 Ames Tests

The doses for the current study were selected based on the comprehension of a dose range study using the test substance (Endosulfan) alone as well as supplementing it with metabolic activator - S9 mix (table:1,1.1 and1.2). The essentials of the same are outlined as follows:

- There was complete lawn inhibition (absence of lawn) at the dose of 5µl/plate (i.e. 1750µg/plate). Therefore, the dose 5µl/plate (i.e. 1750µg/plate) was not considered for main study.
- Only moderate lawn inhibition could be observed in the plates which were not supplemented with metabolic activator. However, lawn was absent at the highest applicable dose for both the strains (TA 98 and TA 100) tested.
- Nevertheless the plates supplemented with metabolic activator showed a strain specific response wherein the lawn was normal in TA 98 while completely absent in TA 100 at the dose of 0.5µl/plate.
- Dose dependent reductions in number of revertant colonies were apparent in both the strains challenged with or without metabolic activator.

Based on the above results, for the presence and absence of metabolic activation groups, doses of 4, 14, 47, 158 and 525 µg/plate were selected for the main experiment.

4.1.2 Cell Gene Mutation Test

Results of precipitation

No visible sign of precipitation was observed after treatment. However, an observation under inverted microscope revealed the presence of globules in the groups treated with highest concentrations.

Results of confluency

There was no noticeable difference in confluency of the cell in any of the groups studied

except for the highest dose group, where a patchy distribution of monolayer was observed.

pH

Change in pH was not recorded at any of the doses since all the measured pH values were within the biological pH range of 7.4 ± 0.2.

Cytotoxicity

Dose dependent decrease in percentage (%) Relative Cloning Efficiency (%RCE) was observed which was 100, 95.2, 65.3 and 33.1 in control (0.0µg/ml), low (0.175µg/ml), mid (0.35µg/ml) and high (0.525 µg/ml) doses respectively for the absence of metabolic activation (table: 5, 5.1, fig: 31 & 32). The above results of % RCE, are in agreement with the reported requirement of survival frequency (70 - 90% for low dose, 40-65% for mid dose and 10-35% for high dose) for low, mid and high doses (Gupta, 1984). However, percentage Relative Cloning Efficiency was not observed for the presence of metabolic activation during dose selection assay due to atypical response.

4.1.3 Chromosomal Aberration Test

Dose selection study

A short-term exposure test for cytotoxicity was performed for dose selection wherein morphological (confluency) and mitotic index studies were performed.

Morphological observations under inverted microscope

All the cells were dead at 45.57 and 11.39µg/ml and more than $1/4^{th}$ of the cell population was observed detached (died) in 2.848µg/ml treatment group. Slides could not be prepared at these dose levels due to observed high toxicity in the groups subjected to test substance with or without metabolic activator.

Mitotic Index

In the absence of metabolic activation

Mean mitotic indices of 4.1556, 2.5691, 2.0175, 1.56315 and 1.1559 were observed for the doses of distilled water, DMSO and 0.045, 0.178 and 0.712µg/ml of Endosulfan respectively. Moreover, a dose dependent reduction (21.47%, 39.16% and 55.01%) was noticed in the test substance treated groups. The results were significant for the highest ($p \leq 0.05$) and control values (table: 7.1). A regression equation of $y = -18.271x + 116.77$, $R^2 = 0.9952$, $r = 0.99$, is obtained which shows that a 50% reduction in mitotic index can be expected at a dose of 0.572µg/ml. The above regression also points to a significant dose dependent decrease in mitotic index (fig: 43).

In the presence of metabolic activation

The observed mean mitotic indices were 2.05415, 2.5691, 2.036, 2.447 and 1.65685 for the same dose levels (of distilled water, DMSO and 0.045, 0.178 and 0.712µg/ml of Endosulfan) mentioned above for the absence of metabolic activation. This finding was contradictory to the mitotic index observed in the absence of metabolic activation and shows no significant inhibition of mitotic index in the groups supplemented with mitotic activator. The percent reductions were 20.75%, 4.75%, 35.51% respectively for low, mid and high doses (table: 7.2). A regression equation ($y = -9.0528x + 107.38$, $R^2 = 0.523$, $r = 0.0.7231$) is derived which shows that a 50% reduction can be obtained at 1.109µg/ml concentration (fig: 44).

Three doses *viz.*, 0.175, 0.350 and 0.525 were selected based on the results of confluency and mitotic index of both the presence and absence of metabolic activation groups for main study (refer table: 7.1 and 7.2). A higher dose for the S9 mix group was not selected to maintain uniform dose levels in both the cases. Therefore, the cells in the main study were not exposed to a near cytotoxic dose. Moreover, the selection of these doses was also based on the results of gene mutation experiments to make homogeneity in the selected doses.

In the present study however, the Mitotic Index (MI) was not much useful in dose selection. The mitotic index is 'the ratio' of the number of cells seen in mitosis to the total number of cells present. Only in an ideal 'steady-state' population, there is a simple relationship between MI and mitotic duration. Such populations are seldom realized, especially when the cell population is disturbed by clastogenic treatments. Simple interpretation of MI is therefore not always possible and MI is not therefore a useful measure of 'mitotic rate'. Treatment, for instance, which affect the differential length of interphase and mitosis can result in either an increase or decrease in MI (Lovell, *et al.*, 1989).

4.2 IN VITRO MAIN STUDY

4.2.1 Ames test

4.2.1.1 Growth

All the Salmonella tester strains (TA 1537, TA 1535, TA 98, TA 100 and TA 102) showed optimum growth before treatment in both the methods of treatment. The optical density was in the range of 0.42-0.51 (table: 3)

4.2.1.2 Lawn Inhibition Test and Reduction in Number of Revertant Colonies

Strain specific and treatment specific toxicities were observed which are as follows:

Plate incorporation method (in the absence of metabolic activation)

Mild lawn inhibitions were observed in the strain TA 1537 and strain TA 1535 at the highest concentration *viz.*, 525µg/plate. However, in strain TA 100, moderate to complete lawn inhibition were observed at the concentrations of 158 and 525µg/plate (fig: 55 to 58). All the other strains showed normal lawn at all the tested dose levels (table: 2, 2.1, 2.2, 2.3, 2.4, and 2.5).

Reduction in number of revertant colonies, were observed only in strain TA 100, rest all the strains exhibited normal colony counts (fig: 54).

Plate incorporation method (in the presence of metabolic activation)

Mild lawn inhibitions were observed in the strains TA 1537 and Strain TA 1535 at the highest concentration of 525µg/plate. While the strain TA 102 has shown excessive toxicity and lawn inhibition has started from 0.014 to 525µg/plate in dose dependent manner. The dose 0.014 has given moderate and complete lawn inhibition. All other strains showed normal lawn at all the tested dose levels (table: 2.6, 2.7 and 2.10).

Reduction in number of revertant colonies was observed only in strain TA 102 subjected to doses 0.047 and 158. However, 525µg/plate dose group showed zero colony counts. The results of both lawn inhibition and reduction in colony counts point to severe toxicity in strain TA 102 and moderate toxicity in the strain TA 100 (table: 2.8 and 2.10). Strain TA 98 has shown normal lawn in all the dose groups (table: 2.9).

4.2.1.2 Lawn Inhibition Test and Reduction in Number of Revertant Colonies

Strain specific and treatment specific toxicities were observed which are as follows:

Pre incubation method (in the absence of metabolic activation)

Strain TA 1537 and strain TA 1535 treated with the highest concentration exhibited mild lawn inhibition (table: 2.6). However, moderate lawn inhibitions were observed in TA 100 at the concentrations of 158 and 525µg/plate. All other strains exhibited normal lawn at all the tested dose levels (table: 4.4).

Further, no reduction in the number of revertant colonies was observed in any of the strains studied (table: 4.3 and 4.5).

Pre incubation method (in the presence of metabolic activation)

Mild lawn inhibitions were observed in the strain TA 1537 and strain TA 100 at the highest concentration of 525µg/plate while the strain TA 1535 showed a dose dependent toxicity from 158 to 525µg/plate. The dose 158 and 525µg/plate evoked moderate and complete lawn inhibition. Rest all the strains showed normal lawn at all the tested dose levels (table: 4.6, 4.7 and 4.9).

An obvious reduction in number of revertant colonies was observed only in strain TA 1535 at the dose of 158 and 525µg/plate. The dose 525µg/plate resulted in zero colony counts (table: 4.7).

Both lawn inhibition and reduction in colony counts in strains TA 1535 and TA 100 indicate toxicity to test substance (table: 4.7 and 4.9).

From the above results it could be inferred that Endosulfan induces strain dependent toxicity. The toxicity is also found to be depended on metabolic activation. The metabolic end product generated during oxidative transformation seems to be toxic to the bacterial strain TA 102 which is sensitive to oxidative mutations. However, in the absence of metabolic activation, toxicity gets reduced and mutagenicity gets triggered and shows positive response in strain TA 102 indicated by 3 fold increase in revertant frequency.

4.2.1.3 Revertant Frequency

Plate incorporation method

TA 1537 (-S9)

An increase in revertant colonies per plate was observed in the strain TA 1537. The doses of 4, 14, 47, 158 and 525 µg/plate evoked 1.56, 1.11, 2.28, 2.72, and 1.28 fold increase in revertant colonies over the concurrent control (fig: 11). The result indicates moderate increase in mutation frequency (revertant frequency) and also absence of mutagenicity for the group (table: 2.1). However, the increase in mutant frequency was not consistent as indicated by the low r value ($y = 2.1429x + 4.7857$, $r = 0.13$).

TA 1535 (-S9)

A definite increase ($y = 1.5893x + 7.6429$, $r = 0.82$) in revertant colonies per plate was observed in the strain TA 1535 since 0.45, 0.79, 0.76, 1.34, 1.55 time increase in revertant frequency was noticed in the dose groups over the concurrent control (table: 2.3, fig: 12). The current observation indicates moderate increase in mutation frequency (revertant

frequency) and absence of mutagenicity for the test substance in the absence of metabolic activation.

TA 98 (-S9)

A slight increase (y = 1.2321x + 13.143, r = 0.79) in revertant colonies per plate was observed in the strain TA 98. The observed levels of increase were 0.65, 0.85, 0.60, 1.10 and 1.13 times for the chosen doses over the respective control (table: 2.2, fig: 13). The current observation indicates absence of mutagenicity for the test group.

TA 100 (-S9)

This strain showed decrease (y = -17.125x + 173.64, r = 0.84) in revertant frequency in all the tested dose groups and became toxic to the highest dose (525 µg/plate) tested (table: 2.4). The strain subjected to treatment exhibited 0.72, 0.84, 0.55, 0.53 and 0.27 time increase in revertant frequency compared to that of control (fig: 14). The observed nonspecific response points to absence of mutagenicity for the group.

TA 102 (-S9)

A statistically significant increase (y = 90.839x + 103, r = 0.98) in revertant colonies per plate was observed in the strain TA 102. A dose dependent increase of 1.00, 1.29, 1.31, 1.37 and 3.43 time revertant frequency was apparent for studied strain (table:2.5, fig: 15). The higher r value indicates significant increase in mutation frequency (revertant frequency) and mutagenicity of Endosulfan in the absence of metabolic activation for TA 102.

TA 1537 (+S9)

A nonspecific increase in revertant colonies per plate was observed in the strain TA 1537. The recorded increase in revertant frequency in the treatment groups compared to that of control was as follows 1.10, 1.15, 1.00, 0.95 and 1.75 (table: 2.6, fig: 16). The values indicate aberrant increase in mutation frequency (revertant frequency) and absence of mutagenicity for TA 1537 subjected to various doses of Endosulfan together with S9.

TA 1535 (+S9)

The strain TA 1535 when treated with serial concentration of test substance showed an increase in the frequency of revertant colonies compared to that of control (table: 2.7, fig: 17). However, a negative correlation between the frequencies of colony reversion to increase in concentration of Endosulfan was quite apparent in this group. The result therefore, indicates absence of mutagenicity in the test system using TA 1535.

TA 98 (+S9)

The doses of 4, 14, 47, 158 and 525 µg/plate evoked 0.80, 0.99, 0.64, 1.04 and 0.68 fold increase in revertant frequency compared to that of concurrent control (table: 2.9, fig: 18). The comparable mutation frequencies (revertant frequency) for varied doses indicate absence of mutagenicity in this group.

TA 100 (+S9)

The revertant frequency of 0.57, 1.24, 0.27, 1.34 and 1.25 fold over the respective control observed in the test substance dosed group indicates non-significant increase in mutation frequency (revertant frequency) and hence, absence of mutagenicity for TA 100 treated with Endosulfan and S9 supplement (table:2.8, fig: 19).

TA 102 (+S9)

The dose groups 4, 14, and 47µg/plate exhibited 0.99, 0.30 and 0.07 fold increase in the frequency of revertant colony in TA 102 (table: 2.10, fig: 20). The decrease in revertant frequencies with increase in dose could be due to excessive toxicity in the presence of metabolic activation.

Positive Control

The *Salmonella* tester strains TA 1537, TA 1535, TA 98, TA 100 and TA 102 showed 76.67, 10.07, 67.5 and 6.21 times increase in revertant frequency when challenged with known mutagen in the absence of metabolic activation. However, in the presence of metabolic activator (S9) the frequency of revertant colony got changed to 4.65, 72.7, 26.97, 14.28 and 2.36 respectively for the *Salmonella* tester strains TA 1537, TA 1535, TA 98, TA 100 and TA 102 (table: 2.1, 2.2, 2.3, 2.4 and 2.5).

The results of positive control show sensitivity of tester strains towards its specific mutagen. The increase in frequency of revertant mutation in the positive control groups compared to vehicle control also signifies that the strains used in the assay are capable to detect mutations and proves efficacy and validity of the test performed.

Pre incubation method

TA 1537 (-S9)

In this strain an increase in the frequency of revertant colony to the tune of 1.94, 1.63, 2.00, 2.94 and 1.56 fold was observed when treated with test substance at a dose level of 4, 14, 47, 158 and 525µg/plate (table:4.1). However, regression analysis ($y = 1.6607x + 7.0714$, $r = 0.66$), shows that there is no difference in colony counts when compared to control (fig: 21).

TA 1535 (-S9)

The observed increase in values of revertant colonies per plate was 2.98, 1.96, 2.53, 1.71 and 1.00 times in the respective treatment groups compared to control group (table: 4.2). However, no statistically significant difference in revertant colonies per plate (y = 10.799Ln(x) + 34.063, r = 0.33) was observed in any of the treatment group compared to the concurrent control (fig: 22).

TA 98 (-S9)

Compared to respective control an increase of 0.75, 2.14, 0.94, 0.84 and 1.00 fold, was observed in TA 98 when treated with sublethal doses of Endosulfan (table:4.3). There is however, no significant increase in revertant colonies per plate for the selected doses in TA 98 as evident from the regression equation y = -1.3713Ln (x) + 37.599, r = 0.06 (fig: 23).

TA 100 (-S9)

TA 100 when subjected to the test substance in the range of 4, 14, 47, 158 and 525 µg/plate induced 0.94, 1.08, 1.05, 0.84 and 0.87 fold increase in the frequency of revertant colony (table: 4.4). Though, this strain shows slight decrease (y = -7.4948Ln(x) + 125.77, r = 04) in the frequency of revertant colony with increase in dose it is not significant statistically (fig: 24).

TA 102 (-S9)

A non-significant decrease (y = -41.656Ln(x) + 467.66, r = 0.68) in revertant colonies with increase in dose was observed in the strain TA 102 (fig: 25). The strain showed 1.01, 1.01, 0.95, 0.88 and 0.76 times increase in the frequency of revertant colony in the dosed groups compared to concurrent control (table: 4.5).

TA 1537 (+S9)

The strain TA 1537 treated with selected doses of Endosulfan registered 1.77, 1.18, 0.86, 0.36 and 0.42 increase in frequency of revertant mutation over the respective control (table: 4.6,). Further analysis however, revealed that there exist no significant correlation between the dose and the frequency of revertant mutation (fig: 26).

TA 1535 (+S9)

The increase in revertant colonies per plate observed in the strain TA 1535 was in the tune of 0.85, 0.98, 1.39, 1.24 and 0.00 times compared to that of control (table: 4.7, fig: 27). Regression analysis showed a dose dependent decrease in frequency of revertant mutation (y = -4.8051Ln(x) + 25.352, r = 0.35) possibly due to toxicity in the high doses groups.

TA 98 (+S9)

The observed increase in the frequency of revertant mutation in the treated groups was 0.95, 1.05, 0.93, 1.11 and 0.38 when compared to that of control (table: 4.8, fig: 29). The result indicates aberrant response to increase in doses of test substance by the strain TA 98 in the presence of metabolic activator ($y = -1.296\ln(x) + 35.078$, $r = 0.09$).

TA 100 (+S9)

The strain subjected to increase in concentration of test substance showed 1.33, 1.23, 1.49, 1.53 and 1.57 fold increase in revertant mutation compared to that of control (table: 4.9, fig: 28). The current result indicates mild increase ($y = 38.695\ln(x) + 103.45$, $r = 0.93$) in mutation frequency (revertant frequency) and absence of mutagenicity for the test substance in the presence of S9.

TA 102 (+S9)

The strain TA 102 when treated with Endosulfan together with metabolic activator registered 1.09, 1.09, 0.90, 0.67 and 0.58 fold increase in revertant colony compared to that of control (table: 4.10, fig: 30). The observed dose dependent decrease in revertant frequencies ($y = -70.956\ln(x) + 474.27$, $r = 0.57$), could be due to excessive toxicity in the presence of metabolic activation at the highest given doses.

A careful scan through the available literature revealed that Endosulfan has been reported toxic to yeast and is also designated a potent mutagen without metabolic activation (Yadav *et. al.*, 1982). Moreover, Endosulfan is known to induce reverse mutations and mitotic gene conversion and also increase the percentage of aberrant colonies in *Saccharomyces cerevisiae*. But it did not induce mitotic cross-overs (Yadav *et. al.*, 1982). The above observations indicate that Endosulfan is capable of inducing chromosome breakage and loss. Endosulfan also induced cytotoxic activity (significant increase in the number of crossover colonies) in the yeast strain *Saccharomyces cerevisiae* T2 (deficient in repair system) but not in *Saccharomyces cerevisiae* T1 (L'Vova, 1984).

However, mutagenic activity was not reported for Endosulfan when applied to various strains of the *Salmonella typhimurium viz.*, TA97a, TA98, TA1535, TA1537, TA1538 without metabolic activation (Moriya *et.al.*,1983, Pedenekar *et.al.*,1987) or for *Escherichia coli* without metabolic activation (Moriya *et.al.*,1983). Endosulfan also tested negative in the *Salmonella* mutagenicity test with or without metabolic activation (Dorough *et.al.*,1978).

The present experimental results are in agreement with the above findings wherein Endosulfan has been tested non mutagenic in the strains of *Salmonella typhimurium* TA98, TA1535, TA1537 except for TA102 when applied without S9. Further, the current study indicates that in the presence of metabolic activation the compound (Endosulfan 35% EC) get metabolized and becomes non mutagenic to the strain TA102. Dubois and coworkers (1996) opined that Endosulfan could induce the formation of DNA adducts in both fetal rat hepatocytes and Hep G2 (human liver hepatoblastoma) cells this activity strongly correlated with high induction of CYP3A gene expression and hence, metabolic activation.

4.2.2 Cell Gene Mutation Test

4.2.2.1 Cloning Efficiency

In the current study the % absolute cloning efficiency observed were 105.50%, 83.00%, 56.00%, 82.33%, 7.50% and 64.50 % for the dosage of NC (0.00) VC (0.00), 0.175, 0.35, 0.525µg/ml and PC (10 µl EMS) respectively in the absence of metabolic activation system (table:5.2).

Percent relative cloning efficiency were 127.11%, 100.00%, 67.47%, 99.20%, 9.04% and 77.71 for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525µg/ml and PC (12µg B(a)P /ml of culture) respectively in the absence of metabolic activation system (table: 5.2). The absolute cloning efficiency was significantly decreased in 0.525µg/ml of culture, indicates possible cytotoxicity at the high dose level.

The absolute cloning efficiency observed in the present study were 74.75%, 75.00%, 89.50%, 44.83%, 53.25% and 67.75% for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525µg/ml and PC (10µl of EMS/ ml of culture) respectively in the presence of metabolic activation system (table: 5.3).

The currently observed % relative cloning efficiency were 99.67%, 100.00%-del, 119.33%, 59.78%, 71.00% and 90.33% for doses of 0.175, 0.35 and 0.525 µg/ml, respectively in the presence of metabolic activation system (table: 5.3). However, no decrease in relative cloning efficiency was recorded in the presence of metabolic activation. The result indicates absence of cytotoxicity for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525 and PC (12µg of B (a) P) /ml of culture) µg/ml respectively in the presence of metabolic activation system (table: 5.3).

CHO cells have been previously reported to be particularly sensitive to the toxic effects of an S9 mix (Kirkland *et al.*, 1989). Clastogenic effects of an S9 mix have been detected in CHO cells, but not in human lymphocytes. This may be due to an S9 mediated generation of reactive oxygen species in the cells, which might be inactivated by blood components in the lymphocyte cultures (Kirkland *et al.*, 1989). Clastogenic activity of S9 has also been reported in mouse lymphoma cells (Cifone *et al.*, 1987).

4.2.2.2 Mutation Frequency

A comparison with the concurrent control value revealed no dose related increase in mutation frequency in the treatment groups of the present study (table: 6.1 and 6.2, Fig. 33 to 42). However, Endosulfan was reported positive in forward mutation assay of mouse lymphoma cell line (MLTK thymidine kinase assay) by McGregor *et.al*, (1988) in the absence of metabolic activation.

The observed negative response in mutation frequency might be due to ineffectiveness of CHO-HGPRT assay for mutation. There are cases where the cell lines are sensitive for detecting clastogenicity but are poor to detect mutagenicity of the compound (Moore *et al.*, 1989). The CHO HGPRT assay system gives little or no evidence of genotoxicity (Moore *et al.*, 1989).

The inability of CHO HGPRT assay to detect increased mutation frequency as observed by aberrant cell frequency (chromosomal aberration) obtained in same CHO cell line (refer section 4.2.1.3 and 4.2.3.2) and a positive response obtained in Ames test in strain TA 102 is hard to comprehend. However, it may be explained by the prevailing hypothesis that "CHO *hgprt* locus is unable to evaluate the clastogenic component of a genotoxic compound due to hemizygous nature of the *hgprt* locus which permits recovery primarily of mutations of a single gene" (Evans *et. al.,* 1986; Hsie *et al.,* 1986, Stankowaski and Hise, 1986; Yandell *et al.*, 1986, Little *et al.*, 1987; Moore *et al.*, 1987).

A test of long term exposure (i.e. 24 or 48 hour exposure) was not performed in the present experimental protocol and hence, it could be possible that one might have missed out mutant cells in the above mentioned experiment. Therefore, a long term assay needs to be performed in order to make any affirmative conclusion. Further, it is also necessary to perform the assay with more sensitive methods such as mouse lymphoma thymidine kinase ($TK^{-/+}$). However, these were beyond the scope of the current protocol and will be incorporated in the future plan of work.

An increase in cloning efficiency observed in the mid dose group indicates mitogenic nature of the test compound or cellular disintegration or loss of cell-cell contacts at that dose level. Further, in the present study a different result was obtained in the presence of metabolic activation group as compared to that of test substance alone treated group. A mild increase in cloning (both absolute and relative) efficiency in low dose group indicates growth promoting (mitogenic) nature of the metabolites of Endosulfan at low dose level.

4.2.3 Chromosomal Aberration Test

Short Term Exposure

4.2.3.1 Mitotic Index

The mitotic index observed were 4.6729%, 5.0325%, 8.1487%, 6.7447%, 5.2835% and 4.5820% respectively for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525 µg/ml and PC (0.5 µg of Mytomycin-C / ml of culture) in the absence of metabolic activation system (table: 8.1). A dose related reduction in mitotic index was not observed which indicates that the compound tested is not affecting the cells at their G0, S, G2 and M phases of cell division but affects the viability by some other mechanism.

The mean mitotic index in the presence of metabolic activation were 10.7850%, 9.3101%, 14.8305%, 9.8161%, 9.0589% and 6.6962% respectively for the dosage of NC (0.00), VC (0.00), 0.175, 0.35, 0.525µg/ml and PC (25µg of Cyclophosphamide/ml of culture). A dose dependent increase ($p \leq 0.01$) was observed in 0.175µg of endosulphan/ml of culture (table: 8.2). However, the mild increase in mitotic index in low dose group indicates to a mild mitogenic nature of the test compound at the given dose level. This could be because the compound or its metabolic end product might be acting as stimulating factor for cells under exposure. There is an isolated report that Endosulfan is inducing cell cycle kinetics in two different mammalian cells in culture (Sebastien *et al.*, 2007). An increase in mitotic index predicts aneugenic response of the test agent under investigation.

4.2.3.2 Percent Aberrant Cells

Structural Chromosomal Aberration

The structural chromosomal aberrations observed in the present study include acentric chromosome, translocation, fragment, deletions, dicentrics, multicentrics ring chromosomes (fig: 59 to 63).

4 -hour -S9 CAM

A comparable result was observed between S9 and vehicle control indicating that DMSO itself at 1% exposure level did not induce any chromosomal aberration. A comparable result

was also observed between vehicle control and low dose group, indicates low level of aberration at this (0.175µg/ml) dose level (table: 8.3 fig: 44 & 45). However, test chemical induces cytotoxicity at the higher dose level and hence, reduces the population of cells in metaphase.

Dose dependent increase in % aberrant cells was observed in the present study. A polynomial trend was observed between the groups with a regression formula of y = 0.1646x2 + 0.1816x + 1.1831 and r value of 0.76. This shows a strong relationship of a dose response with a polynomial trend. However, a test for significance was not performed as standard deviation was found more between the groups.

A two-fold increase in the aberrant cell frequency indicates clastogenic nature of the chemical in question at the doses of 0.35µg/ml and higher. The above results indicate a biologically significant response as the aberrant cell frequency showed two-fold increase in mid and high dose groups. This result is considered positive as per the criteria mentioned by Sofuni *et. al.,* (1990) and Galloway *et. al.*, (1984).

Numerical Aberration

An increase in occurrence of polyploidy was observed in all the tested dose levels, indicates that colchicine at the extended exposure (> 2 hours) duration affects mitosis and increases occurrence in polyploidy (table: 8.5, 8.6; fig: 64).

4 - hour +S9 CAM

A linear trend in dose dependency was apparent between the groups as evidenced by the regression formula y = 1.89x + 2.02 and r value of 0.98 (table: 8.4; fig: 46 & 47). The results however, were further not analyzed statistically for a test of hypothesis as standard deviation was more between the groups. The present finding gain credence from the observations of Sobti *et al.,* (1983) who reported chromosomal anomalies in cultured human lymphoid cells exposed to Endosulfan with and without metabolic activation.

In continuation to the above-mentioned study for short-term exposure, a long-term study was planned to assess the clastogenic nature of the compound for longer exposure in the following experiment.

Long Term Exposure (Trial –I)

Without metabolic activation (72 hour)

A dose dependent increase in aberration was observed between the groups with a

regression formula of y = 2.174x + 1.052 and r value of 0.97 (table: 9.1 & 9.2, fig: 48 & 49). The equation shows a strong relationship of a dose response with a linear trend. A significant ($p \leq 0.05$) increase in % aberrant cells was observed in all the groups except 0.350µg/ml.

Long Term Exposure (Trial –II)

Without metabolic activation (7 days)

Dose induced chromosomal aberrations was confirmed by this set of experiment (table:10, 10.1). In summary, Genotoxicity studies of Endosulfan have provided evidence that the test compound is mutagenic and clastogenic.

4.3 IN VIVO DOSE SELECTION STUDY

4.3.1 Mortality

Mortality was found to be 100% for doses 100mg/kg body weight and 40 mg/kg body weight while 75% and 25% mortality were observed at the doses of 20 and 10 mg/kg body weight respectively.

The detail of endpoint (mortality) observed in each group was as follows:

100 mg/kg body weight

All the animals were found dead after treatment in this dose group.

40 mg/kg body weight

Complete mortality was observed both in male and female mice in this group.

20 mg/kg body weight

Mortalities were observed in male (2 out of 3) mice only.

10 mg/kg body weight

One animal died at 10mg/kg body weight.

From the above dose range study a safer dose of 8 mg/kg body weight was selected as high dose for the main studies.

4.3.2 Clinical Symptoms

The symptoms of toxicity were more severe within one hour (40-60 minutes) of treatment. The animal died after one hour, if there is severe toxicity.

The observed morphological symptoms of toxicity were: Exaggerated response to sound stimuli, clonic tonic convulsion of fore and hind limbs, Catalapsy, Salivation, Lacrimation, Convulsion, Writhing and Dilation of pupil.

The gross pathological symptoms of toxicity observed were mottling in liver, lung and kidney of dead animals in both the sexes. These symptoms were co-examined by a trained veterinary pathologist for confirmation.

Further, based on the reference of Khan and Sinha (1993) a dose of 3 mg/Kg body weight Endosulfan (35% EC) was provided for 7 days using oral intubation in *Mus musculus*.

4.4 IN VIVO MAIN STUDY

4.4.1 Clinical Symptoms

4.3.2.1 Morphological

The morphological symptoms observed include abdominal breathing and convulsions, catalapsy and death (table: 11).

The most prominent signs of acute overexposure to Endosulfan in both humans and animals are hyperactivity, tremors, decreased respiration, dyspnea, salivation, and tonic-clonic convulsions. Five cases of acute lethal poisoning in humans resulting from accidental or intentional ingestion of Thiodan® were reported in an earlier study by Terziev *et al.* (1974).

Control group, low dose group

Control (0.0 mg/kg b.wt) and Low dose (4.0 mg/kg b.wt.) group animals showed no adverse clinical symptoms of toxicity.

High dose group

The animals exhibited overt signs of toxicity within 30-120 minutes of the treatment, which disappeared gradually after 2 hours of treatment. Symptoms of toxicity were first observed only in the high dose (8.0mg/kg b. wt.) group male animals. These symptoms include hyperactivity, tremor and abdominal breathing.

Three male animals died due to toxicity and showed severe tremors, writhing and salivation. The animals died at different intervals of the treatment *viz.*, 19th, 20th and 21st day

of the treatment. However, even though few female animals exhibited hyperactivity and mild tremors they survived course of experiment.

On day 26th post-treatment, female animals exhibited hyperactivity, salivation and severe tremor. However, on 27th day of treatment the animals exhibited severe tremor, salivation, and lethargy. Out of the total one female animal died within two hours of treatment. This animal showed tremor, writhing, excessive salivation and finally vomited blood before it died. All other animals got recovered after treatment and were found to be normal at the time of sacrifice.

The above results indicate that:

1. Male animals are more sensitive (sex related toxicity) to the test substance in question.
2. Endosulfan at the given dose evoked acute and accumulative toxic effects (which was much prominent in female animals though) and
3. Hence, justifies 8.0 mg/kg b. wt as the highest applicable dose for a 28 day repeated dose study.

Signs of acute lethal Endosulfan poisoning in animals are similar to those observed in humans and include hyper excitability, dyspnea, decreased respiration, and fine tremors followed by tonic-clonic convulsions. Oral LD_{50} (median lethal dose) values for technical-grade Endosulfan vary depending on species, sex, formulation tested, and nutritional status of the animal being exposed (Gupta and Gupta 1979; WHO 1984). With regard to species sensitivity, mice appear to be quite sensitive to Endosulfan lethal effects, with a reported LD_{50} value of 7.36 mg/kg in males (Gupta *et al.* 1981) and 2 out of 10 male mice dying after administration of 7.5 mg technical Endosulfan/kg in the diet for 7 days (Wilson and LeBlanc 1998). The sensitivity to mortality in male mice compared to female is also supported by NCI (1978).

The two isomers of Endosulfan (α and β) also reported to have different LD_{50} values in rats. The α-isomer is more toxic than the β-isomer in female rats, with an oral LD_{50} value of 76 mg/kg b. wt. versus an LD_{50} value of 240 mg/kg b. wt. for β-Endosulfan (Maier-Bode 1968; Hoechst 1975, 1990). The same difference was reported in female albino mice, the lethal dose for α-Endosulfan being 11 mg/kg b. wt. versus 36 mg/kg b. wt. for β-Endosulfan (Dorough *et al.* 1978). The lethal dose for Endosulfan sulfate in mice was comparable to that of the α-isomer, 8 mg/kg b. wt. (Dorough *et al.* 1978). Also, Hoechst (1966a, 1966b) had reported an LD_{50} of 14 mg/kg b. wt. for α-Endosulfan and 17 mg/kg b. wt. for β-Endosulfan in female mice.

Further, increased mortality was observed in both male rats (at doses of 20.4 mg/kg/day and above) and male mice (at doses of 0.46 mg/kg/day and above) in a 2-year bioassay conducted by the National Cancer Institute (NCI 1978). The authors attributed the excessive mortality in the male rats to treatment-related toxic nephropathy. The high mortality in male mice was possibly due to fighting since no other treatment related cause for the deaths could be determined. But in present case mortality is treatment related and manifested by clinical symptoms. Survival in females of both species was unaffected by Endosulfan (NCI 1978). However, survival was significantly decreased in female rats that consumed 5 mg/kg/day for 2 years (FMC 1959b), and in female mice that consumed approximately 2.9 mg technical grade Endosulfan/kg/day for 2 years (Hoechst 1988b; Hack *et al.* 1995). In these studies, survival in male rats was not affected at 5 mg/kg/day for 2 years (FMC 1959b) and survival in male mice was not affected at 2.51 mg/kg/day for 2 years (Hoechst 1988b).

4.3.2.2 Gross pathological observation

Immediately after death the mice, were dissected out and examined by specialized pathologists at and during different phases of experiments. The organs examined were liver, kidney, lungs, testes, gastrointestinal tract (oesophagus, stomach, ileum etc.) as presumed target organs.

The overall gross symptoms, observed include mottlings in liver, lung and kidney as overt symptoms of toxicity in both the sexes (table:12).

Case reports of human poisonings and studies in animals indicate that during acute oral exposure to lethal or near-lethal amounts of Endosulfan, involvement of a large number of organ systems (respiratory, cardiovascular, gastrointestinal, hematological, hepatic and renal) is observed. However, during long-term exposure, the liver and kidney appear to be the primary systemic target organs (Hack *et al.* 1995).

4.3.2.3 Histopathological observation

In the absence of any prominent symptoms in the target organs, detailed histopathological examination of these tissues were not carried out. The present notion gains credence from the observations of Dikshith and coworkers (1988). They in a 30 repeated dermal dose study of Endosulfan up to 62.5mg/kg/day (males) and 32 mg/kg/day (females) in rat reported no effect on organ weight or histoarchitecture.

4.4.2 Body weight

Dose related changes in body weight were not observed both in male and female animals (table: 13.1 and 13.2). This indicates Endosulfan toxicity can not be judged by change in animal body weight. The present findings also corroborates with the findings of Wilson and LeBlanc (1988) wherein no significant change in mice body weight was observed for food treated with technical grade Endosulfan at 15mg/kg in the food for 7 days.

In another finding, body weight gain was significantly reduced in male but not in female mice in the diet supplemented with 2.5mg Endosulfan/kg/day for 24 months (Hoechst 1988b; Hack *et al.* 1995). Neither food nor water consumption was significantly altered in mice or rats administered with technical grade Endosulfan in the diet for 24 months (Hack *et al.* 1995). The present finding for 28 days also supports indirectly the above report as there is no significant difference in body weight.

4.4.3 HAEMATOLOGY

Male Animals

WHITE BLOOD CELLS (WBC)

A slight increase in WBC count was obtained in all the groups as compared with the reference ranges (table: 14.1, 14.2). However, the data was statistically not significant when compared to that of concurrent control values, except an increase in HCT and MCHC at $p \leq 0.05$ which is of no any biological significance.

RED BLOOD CELLS (RBC)

There observed no differences in the RBC count in all the treatment and control groups. Further, the data is also comparable to reference range (table: 14.1 and 14.2).

HAEMOGLOBIN (Hb)

There were no differences in the haemoglobin count in all the groups (table: 14.1 and 14.2). This result is also comparable to its reference range and it is not significant.

HEMATOCRIT (HCT)

Here too the values were comparable to reference ranges and though a statistically significant increase ($p \leq 0.05$) was observed in high dose group, the result obtained is biologically insignificant (table: 14.1 and 14.2).

MEAN CORPUSCULAR VOLUME (MCV)

The Mean Corpuscular Volume (MCV) values were comparable to reference ranges, and

among themselves. The results obtained are biologically and statistically insignificant (table: 14.1 and 14.2). A negative value was observed in the print generated by instrument being used. This is a calculation based result which should be considered positive instead negative values, it has been reported as per the instrumental print.

MEAN CORPUSCULAR HAEMOGLOBIN (MCH)

MCH is also a calculation type of result which is negative in the instrumental print; its values are also comparable to reference ranges and among themselves (table: 14.1 and 14.2). Hence, it is not significant.

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC)

The values obtained were comparable to reference ranges and among themselves (table:14.1 and 14.2). Though, a statistically significant result is obtained, the data is biologically not significant.

PLATELETS (PLT)

An increased average platelet count was observed compared to the reference ranges (table: 14.1 and 14.2). However, there was no difference in the value among all the groups studied including the control and therefore, this could not be considered as increased platelet count.

Female Animals

WHITE BLOOD CELLS (WBC)

A slight increase in mean WBC count was observed in all the groups as compared to the references values (table: 14.3 and 14.4). However, the data is statistically and biologically not significant though a mild decrease was noticed in the treated groups compared to control.

RED BLOOD CELLS (RBC)

There observed no difference in the mean RBC count among the groups (table: 14.3 and 14.4). Also, the data is comparable to the reference range though it is statistically significant ($p \leq 0.05$).

HAEMOGLOBIN (Hb)

There are no differences in the haemoglobin count in any of the group (table: 14.3 and 14.4). The observed values are also comparable to its reference range and it is not significant.

HEMATOCRIT (HCT)

Here again the values are comparable to reference ranges (table: 14.3 and 14.4) and the result obtained is biologically and statistically insignificant.

MEAN CORPUSCULAR VOLUME (MCV)

The Mean Corpuscular Volume (MCV) values were akin to its reference ranges, and among themselves (table: 14.3 and 14.4). The results obtained are statistically significant ($p \leq 0.05$) and biologically insignificant. A negative value was observed in the print generated by instrument being used; this is an algorithm based result and should be considered positive instead of negative readings.

MEAN CORPUSCULAR HAEMOGLOBIN (MCH)

MCH is also a calculation based result which is negative in the instrumental print; its values are also comparable to the reference ranges and among themselves (table: 14.3 and 14.4). Hence, it is not significant.

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC)

The values obtained are comparable to its reference ranges and among themselves (table14.3 and 14.4). The data is biologically and statistically not significant.

PLATELETS (PLT)

An increased mean platelet count was observed in the present study compared to the reference ranges (table: 14.3 and 14.4). However, there is not much difference in the values of PLT amongst the groups studied including control and hence, can not be considered as increased platelet counts.

There is no historical data available on Endosulfan induced haematological changes in mice. Mixed results have been reported from studies examining haematological effects of dermal exposure to Endosulfan in rats. Although decreased haemoglobin was observed in male rats following daily application of doses of Endosulfan of 18.75 mg/kg b. wt. for 30 days (Diikshith *et al.,* 1988), similar results have not been observed in female rats or in male rats at similar doses in other studies. For example, no haematological parameters were adversely affected following exposure of females to doses of 32 mg/kg/day for 30 days (Diikshith *et al.,* 1988). In addition, no adverse effects on routine haematological parameters were observed following exposure of rats for 30 days for 6 hours per day, 5 days per week to doses of Endosulfan ranging from 12 to 192 mg/kg/day (males) and from 3 to 48 mg/kg/day for females (Hoechst 1985d). Similarly negative results were obtained in a

comparable 30 day rat study using slightly lower Endosulfan doses (Hoechst 1985c). The above difference in different results of these studies is unclear but may have been related to differences in the age of the rats or the application protocol.

4.4.4.1 Differential Leucocyte Count

One day after exposure

Comparable results were obtained in animals of both the sexes (table: 15.1, 15.2,15.3 and 15.4).

One week after exposure

A dose dependent decrease in lymphocytes was observed in male animals (table: 16.1 and 16.2). The decrease was statistically significant ($p \leq 0.05$) in the high dose group whereas, a dose dependent increase was observed in neutrophils. This increase was statistically significant ($p \leq 0.05$) in the high dose group and may be due to the relative decrease of lymphocytes. This difference may be related to exposure with Endosulfan but are very low and cannot be interpreted as biologically significant increase. No other changes were observed in male animals after one week of repetitive exposure.

The observed changes were subtle in case of female animals and hence, cannot be considered as biologically significant. Therefore, it can be inferred from the present study that there is no difference, in differential leucocyte counts in both the sexes after one week of exposure.

The results of the earlier studies also support the current results. Sub-acute and chronic-duration studies using Endosulfan revealed no effects on hematological parameters or on routine gross and histopathological examination of bone marrow and the spleen in mice or dogs (FMC 1959a, 1967; Hoechst 1984b, 1988b, 1989c).

4.4.5 BIOCHEMICAL OBSERVATIONS

Biochemical tests were performed to find changes in glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), blood urea nitrogen (BUN) and Cholinesterase (CHE) both in male and female animals.

Male Animals

A slight increase in cholinesterase was found in the treated animals which however, was statistically and biologically not significant, this might be due to Endosulfan induced

neurogenic response (table: 17.1 and 17.2, fig: 50). All the other parameters mentioned in section 4.4.5 were within normal range and hence not significant.

Female Animals

A statistically significant decrease ($p \leq 0.05$) in alanine aminotransferase (ALT) and creatinine (CRE) was observed which is well within the biological range hence, changes are computed insignificant. All other parameters were normal (table 17.3 and 17.4, fig: 51).

Repeated dose studies have generally not shown adaptive or adverse effects on the liver. Routine gross and microscopic pathology has not revealed adverse hepatic effects in mice exposed to 2.51 mg/kg/day (males) or 2.86 mg/kg/day (females) for 2 years (Hoechst 1988b; Hack *et al.* 1995), in rats exposed to 5 mg/kg/day (females) or 2.9 mg/kg/day (males) for 2 years (Hack *et al.* 1995; FMC 1959a; Hoechst 1989a), or in dogs exposed to 1 mg/kg/day for 2 years (FMC 1967). Serum alkaline phosphatase was, however, elevated in dogs exposed to 0.67 mg/kg/day (males) or 0.6 mg/kg/day (females) for 1 year, suggesting adverse effects on the liver; however, no effects on liver weight, liver function, or microscopic pathology were observed (Hoechst 1989c).

4.4.6 BONE MARROW MICRONUCLEUS TEST

4.4.6.1 Ratio of Polychromatic to total Erythrocytes (P/E ratio) male and female

A mild suppression in P/E ratio was observed (table: 18). However, this suppression was statistically not significant. Moreover, this suppression indicates toxicity to bone marrow and was more prominent in female animals.

4.4.6.2 % MN PCE and % MN NCE

Male Animals

A dose dependent increase ($p \leq 0.01$) in %micronucleus frequency was observed both in polychromatic (%MN PCE) and normochromatic erythrocytes (%MN NCE) in male animals after 28 days repeated administration at the dose of 8 mg/Kg body per day (table,19 and 19.1, fig: 52). An increase in %micronucleus frequency in polychromatic (%MN PCE) was statistically not significant but biologically significant.

Female Animals

Female animals also showed a dose dependent increase ($p \leq 0.001$) in % micronucleus frequency both in polychromatic (%MN PCE) and normochromatic erythrocytes (%MN NCE) after 28 days repeated dosing at the dose of 8 mg/Kg body weight (table: 19.2; fig: 53; fig: 65).

PERIPHERAL BLOOD MICRONUCLEUS TEST

Peripheral blood was examined to evaluate bone marrow toxicity by comparing mature and immature red blood cell ratio in peripheral blood. For this, ratio of mature RBC among total was examined. The result reveled no much difference in the ratio of mature RBC among total in all the treated groups of both male and female animals (table: 21,21.1, 21.2 and 21.3).

Peripheral blood Micronucleus frequency

Micronuclei can also be scored in the polychromatic erythrocytes (PCE) in mouse peripheral blood (fig: 66). The significant advantage of the peripheral blood assay is less invasive blood sampling, which allows for repetitive sampling from the same animal over time for kinetic studies of micronucleus (MN) induction (MacGregor, 1980 and 1983). The peripheral blood micronucleus test was originally thought to be only applicable to the mouse because the mouse spleen is known to be incapable of removing micronucleated erythrocytes in the blood. However, recent studies in the rats also showed that newly formed micronucleated PCE (also referred to as reticulocytes) are also detectable in peripheral blood before they are removed from the spleen (Holden, 1997). Furthermore, the ability to score micronuclei in normochromatic erythrocyte (NCE), which accumulate in the peripheral blood, permits the evaluation of clastogenicity of a test article in the standard multiple dose toxicological studies. Indeed, retrospective evaluation of micronuclei in NCE of peripheral blood (fig: 67 & 68), have been performed in several mouse cancer bioassays in the National Toxicology programme (NTP) (Choy, 1997; MacGregor, 1990). Such studies provide examples that the micronucleus test can be incorporated into routine bioassays for the assessment of Genotoxicity under the same exposure conditions as in the animal bioassays.

1St Day of Exposure

The mean % MN frequency in RBC observed were 0.122, 0.123 and 0.117 in male and 0.116, 0.098 and 0.213 in female animals (table: 21.1). The female animals were shown statistically increase ($p \leq 0.05$) in percent micronucleated red blood cells.

1St Week of Exposure

The mean % MN frequency in RBC observed were 0.049, 0.191and 0.180 in male and 0.118, 0.145 and 0.313 in female animals (table: 21.2). Though the incidence of micronucleus was high in female animals of high dose group there were no difference in the 1st day and 1st week response.

2nd Week of Exposure

The mean % MN frequency in RBC observed were 0.117, 0.094, and 0.394 in male and 0.230, 0.169 and 0.216 in female animals (table: 21.3). An increase in peripheral blood micronucleus assay was observed in 2nd week of exposure in high dose male animals ($p \leq 0.05$) was observed. The above finding confirms the occurrence of increased micronucleus frequency in bone marrow cells followed by increase in peripheral blood.

4.4.7 SPERM MORPHOLOGY TEST

A dose dependent statistically significant ($p \leq 0.05$ for 4.0 mg/Kg body weight and $p \leq 0.01$ for 8.0 mg/Kg body weight, respectively) increase in percent abnormality of the sperm head was observed in the current study (table: 20). The observed abnormality include large head, small head, amorphous head, bending of head with no hook, double tail and coiling of tail (fig: 69 to 75). Further, the relative fold values were 2.2 and 7.64 to the doses of 4.0 and 8.0mg/Kg body weight respectively when compared to that of concurrent control group (table: 20.1). Both the above results indicate that the Endosulfan after 28 days consecutive exposure causes sperm abnormality.

Khan and Sinha (1996) observed significant reduction in sperm count of 35% (w/w) Endosulfan technical as active principle, and the remaining 65% (w/w) being solvents, emulsifiers and stabilizers. An increase in abnormal sperm count was observed by 14% at a dose of 3 mg/kg bodyweight/day for 35 consecutive days in Swiss albino mice.

The gross abnormalities in sperm head shape for Endosulfan were also observed and described as pin shaped, bottom-heavy (Soars *et. al.,* 1979), triangular, amorphous, rudimentary (Rastogi and Levin, 1987; Bhunya and Behera, 1987), hammer shaped, sickle shaped and flat based (Sinha and Prasad, 1990). Some sperms were characterized as having distinctly larger (twice the area) or smaller (half the area) head size as compared to normal, as documented by Seuanez *et.al.,*1977. Sperm with abnormal head number (twin-headed) were also recorded (Soars *et. al.,*1979; Sinha and Prasad, 1990).

Sinha and Saxena (1997) also found sperm abnormality in Druckery rats (3 weeks old) at the doses of 2.5, 5.0 and 10 mg/kg/day for 90 days with technical grade Endosulfan. A significant increase was observed in sperm head *viz.,* no hook, excessive hook, amorphous, pin and short head; and tail abnormalities were also observed as coiled flagellum, bent flagellum and bent flagellum tip. These findings are in agreement with the currently observed sperm abnormality in Endosulfan treated mice.

Lastly, the present study using a combination of *in vitro* and *invivo* test system to evaluate the toxicity, revealed beyond doubt that the test substance Endosulfan is a mutagen to the bacterial system however, even though it is found clastogenic, its mutagenic potency in mammalian system was equivocal. This warrant further confirmatory test like mouse lymphoma assay (MLA). Nevertheless, the results of the present study confirmed beyond doubt the aneugenic, clastogenic, and spermatotoxic potency of the test substance in question and hence, it is logical to surmise that Endosulfan might impart developmental anomalies in non target organisms including human. It is therefore, suggested that this pesticide needs to be recommended to be listed in Appendix III of Rotterdam Convention and ultimately convince the agrochemical industry to rapidly phase out the production of Endosulfan by suggesting appropriate alternate safe synthetic and/or biological pest control measures.