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Effect on Antioxidants and Chromosomal Aberrations in the Offspring of Methyl Isocyanate Exposed Population of Bhopal

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Abstract

Bhopal gas tragedy was one of the worst industrial disasters that produced short-term as well as long-term health effects in the exposed population. The present study is an attempt to know about the genomic damage as well as the level of antioxidant caused by Methyl Isocyanate in the offspring of the exposed population and to know about the frequency of chromosomal aberrations in them. Offspring's of the Methyl Isocyanate Exposed individuals living in the vicinity of 1.5 km from the Union Carbide's pesticide plant were selected randomly from the study. Equal number of age (20 years – 50 years), sex (male / female) and locality matched non-exposed individuals were also enrolled as controls. All the subjects were non-smokers and not employed at any type of chemical industry or a radiation department. Cytogenetic analysis was performed by standard protocol of lymphocyte culture as per as Moorhead *et al.* Chromosomal aberrations were observed. The antioxidant level of Vitamin E and Blood Glutathione was also recorded. Student's *t*-test was performed to test the significance difference between means of chromosomal aberrations. The means were considered to be statistically significant at 1% level of probability. It was observed that the level of antioxidants was highly significant in the MIC Exposed and MIC Exposed F_2 Generation Population as compared to MIC Non-Exposed Population. But, significant difference between MIC Exposed and MIC Exposed F_2 Generation Population was observed. The mean percentage of normal metaphase in the Exposed group was significantly higher ($p < 0.0001$) as compared to Non-Exposed. Besides this, chromosomal aberrations (dicentrics and double minutes) were recorded at mitotic metaphase and were statistically elevated in the MIC Exposed Population. The present study indicates that the genomic instability still persists at a higher intensity among the Bhopal Gas Survivors. The higher incidence of chromosomal aberrations may act as the intermediate processes in the pathway of the progression of any genetic disorders and cancers. Hence, our study suggests that the Exposed Populations and their Offspring's is more vulnerable and gas affected.

Keywords
Chromosomal aberrations, methyl isocyanate, Bhopal gas tragedy, long-term genotoxicity, exposed population

Introduction

Bhopal gas tragedy of 1984 was the world worst industrial disaster that had occurred at a pesticide plant resulted in the immediate death of approximately 3800 people and causing significant and premature death of many thousands more [1 – 4]. Primary causes of deaths were choking, reflexogenic circulatory collapse and pulmonary oedema. Finding during autopsies revealed that there are not only changes in the lungs but also cerebral oedema, tubular necrosis of the kidney, fatty degeneration of the liver and necrotizing enteritis was damaged [5]. Studies have been carried out so far, reporting the genotoxicity and mutagenicity of methyl isocyanate in the exposed population [6]. Various studies have shown that these survivors experience a higher incidence of health problems such as febrile illness, respiratory, neurological, psychiatric, and ophthalmic symptoms [7]. Besides genotoxicity, antioxidant studies have been carried out to understand the physiological role of antioxidant is to prevent from cellular damage as the consequence of chemical reactions involving free radicals. Vitamin E belongs to the family of α- β-, γ-, δ- tocopherols and corresponding tocotrienols. α- tocopherol is an important lipid-soluble vitamin helps to prevent oxidative stress. Its function as an antioxidant in the glutathione peroxidase pathway [8] and it protects cell membranes from oxidation by reacting with lipid radicals produced in lipid peroxidation chain reaction [9]. Other known antioxidants, Glutathione (GSH), a gamma glutamyl cysteinyl glycine - tripeptide containing glycine, glutamate and cysteine functions as erythrocyte membrane integrity andis important in keeping enzymes in active state especially glutathione family [10]. Glutathione act as scavenger of free radicals, efficient scavenger to quench superoxide (O_2^-) and hydroxyl radicals (OH). It functions as a non-toxic reservoir of cysteine and proteins, thus maintaining the essential thiols at a reduced state [11]. Indian Council of Medical Research (ICMR), New Delhi was one of the foremost organizations to initiate clinical research on the exposed population of Bhopal. It presented its technical report of Bhopal Gas Survivors on the "Health effects of the toxic gas leak from the methyl isocyanate plant in Bhopal". According to ICMR, 2004 a large section of exposed population still remains chronically ill associated with diseases such as respiratory, gastrointestinal, reproductive, neurological and other systems. Two months after the exposure a study was conducted on humoral and cell mediated immunity in the exposed population of Bhopal gas tragedy. It was revealed that the cell mediated immunity was suppressed with toxic methyl isocyanate gas persist for several months after the exposure [12].

Immunologic, mutagenic and genotoxic effects observed in the exposed population show that MIC reacts with enzymes involved in DNA replication and repair. This may be the cause of some of the pregnancies. It was found that 43.8% of pregnant womenresiding near the Union Carbide pesticide plant did not result in the birth of live child [13]. Various studies have been carried out so far, reporting the genotoxicity and mutagenicity of toxic MIC gas in the exposed population. Chromosomal aberrations have been found significantly higher in the exposed groups as compared to the controls [14]. Higher frequency of chromosomal anomalies was seen in the MIC exposed group and a yearly gradual increase of different types of cancers in the Bhopal gas survivors has also been reported [15].

Keeping in view the toxicity of the gas, it is very necessary to determine whether the methyl isocyanate has produced any long term genetic damage. Few cytogenetic studies were also undertaken to assess genetic damage in the gas exposed population. However, no studies are available on biochemical changes of toxic gas exposed population. Therefore, this study was undertaken to evaluate cytogenetic damage through chromosomal aberrations and biochemical changes among gas exposed survivors after 39 years of their exposure to the methyl isoyanate toxic gases.

Materials and Methods

The study was approved by the Human Institutional Ethical Committee **(IEC No. 1672 / 225 / 2013)** of Jawaharlal Nehru Cancer Hospital & Research Centre, Idgah Hills, Bhopal, Madhya Pradesh and written informed consent was taken from each participant before commencing the study. The human subjects were selected randomly, living in the vicinity of 1.5 km from the pesticide plant and were divided into three groups (G1-MIC non-exposed, G2-MIC exposed and G3-MIC exposed F_2 generation population). Each group

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consisting 25 males and females equally matched with same age, sex, and locality. MIC non-exposed individuals were served as a control. Selection of subjects were based on the criteria set and subject must hold a Gas Victim official card, or any other authenticated evidence issued by the Government authority which epic the physical presence of his / her on the site of occurrence during the time of exposure. Also, any other evidence which shows hospitalization on or within 48 hours of the episode. Further, a person must not have a habit of smoking or tobacco chewing or working in any type of chemical or petroleum industry with no family history of any genetic disorder.

The antioxidant activity of Vitamin E level was determined by Jargar *et al.*, [16]. Serum samples were collected from the survivor of MIC Gas Exposure and Non-MIC individuals who were treated as control. 750 µl of ethanol (aldehyde free) and 750 µl serum were added in centrifuge tubes marked as sample. The blank was prepared by adding 750 µl of ethanol (aldehyde free) and 750 µl of distilled water which were considered as control. All the tubes were covered tightly and shaken vigorously for 30 seconds. Then 750 µl of xylene was added. All the tubes were again covered and shaken vigorously for 30 seconds and then centrifuged for 10 minutes at 3000 rpm (revolution per minute). 500 µl Xylene layer was transferred in small sized test tubes. In each test tube 500 µl of 2, 2' – bipyridyl solution was added followed by 100 µl of ferric chloride solution and waited for 2 minutes. The absorbance was measured at 492 nm.

Heparinised blood samples were collected from both MIC non-exposed and MIC exposed population for determining another antioxidant activity level of Glutathione spectrophotometrically Beutler *et al.*, [17]. 70.2 ml of blood was collected and dissolved in 1.8 ml double distilled water. 3 ml of precipitating solution was added and allowed to stand for 5 minutes so that a precipitate is formed. It was filtered with Whattman's filter paper. 2 ml of filtrate was added to 8 ml phosphate buffer. 1 ml 5, 5' – dithio-bis – (2 – nitrobenzoic acid) (DTNB) was added to the above solution as it acts as a stabilizing component given the very unstable nature of GSH in plasma and absorbance was noted at 412 nm.

Lymphocyte Culture and Chromosome Aberration Assay

Lymphocyte cultures were set according to Moorhead *et al.* [18]. Heparinised venous blood samples were collected in the heparinised vacutainers from both the exposed subjects as well as the controls after an informed consent and aseptically transferred into a sterile culture bottles. The chromosomal anomalies were assessed on the metaphase plates of 72-hour standard lymphocyte culture. The culture medium was used 5 – 8 ml of RPMI – 1640 (Gibco), supplemented with L – glutamine, 10% fetal bovine serum (Himedia labs, India) and antibiotics: Penicillin – Streptomycin solution (Invitrogen, USA). Phytohaemagglutinin (Gibco) was used as a mitogen. The lymphocyte cultures were incubated in a CO_2 incubator for 72 hours in order to determine the level of structural chromosomal aberrations.

At 70 hours 0 – 5 ml of 0.02% aqueous colchicines solution (Sisco, Bombay) was added as the pre-treatment agent. After 72 hours of incubation the cell suspensions were poured into labelled centrifuge tubes and were centrifuged for 10 minutes at 1000 rpm. The supernatant was expelled out. Hypotonic solution (0.56% KCl or 0.75M KCl) was added to the pellet of cell at the bottom of the tube which was suspended by the gentle flushing and cyclomixed. The centrifuge tubes were kept at 37^{0}C for 45 minutes. The cell suspension was centrifuged again carefully at 900 – 1000 rpm for 30 minutes. The supernatant was removed and 4 ml of freshly prepared pre-chilled Cornoy's fixative was added to the pellet while mixing on cyclomixer. The tubes were allowed to stand overnight at room temperature and washed with freshly prepared pre-chilled Cornoy's fixative repeatedly for 3 – 4 times. The cells were gently flushed with a Pasture pipette. To avoid cell loss care was taken to see that the cell suspension did not reach a higher level than the stem of the pipette.

Preparation of Slides

The air drying techniques of Rothfels and Siminovitch, 1958, [19] was employed. Clean, grease free slides were chilled. The cell suspension was gently flushed and 2 – 3 drops were dropped from a height of about

6 inches. The excess fixative was allowed to run off by tilting the slides which were gently heated to help in the spreading of chromosomes. Slides were examined under ordinary light microscope with condenser lowered and illumination reduced. Depending upon the number of cell obtained, the suspension was concentrated by centrifugation and reducing of fixative.

A minimum of four slides were prepared from each culture suspension. The remaining was stored at -10^{0}C. One slide from each culture was sustained in Giemsa to study blastoid transformation, the remaining slides stored to study banding.

Chromosome preparation were stained with 1% Giemsa working solution for 5 – 8 minutes and the extra stain was removed by single rinse of the slides with double distilled water as followed Seabright protocol 1917 [20].

All the slides were observed under 10 X objective of Olympus BX 60 (Phase Contrast) microscope to locate the metaphase in them and for the observation of minute chromosomal aberrations 100 X (Oil Immersion) objective were used. Hundred well spread metaphase were analyzed per sample and chromosomal aberrations were recorded in a standard format and classified according to the International Nomenclature (ISCN, 1995) [21]. The values for cytogenetic parameters such as percentage of normal metaphase, frequency of chromosomal aberrations such as dicentrics and double minutes were recorded.

Statistical Analysis

Statistical analysis was done with the help of GraphPad PRISM (Ver.4) software. Student's *t*- test was performed to test the significance of difference between the means of chromosomal aberrations recorded. The means were considered to be statistically significant at 1% level of probability.

Results

Table 1: The mean values for antioxidant parameters

Characteristics	Vitamin E	Blood Glutathione
Group 1 MIC Non-Exposed Population	12.0 ± 0.36	23.5 ± 0.87
Group 2 MIC Exposed Population	1.77 ± 0.46$^{@}$	16.0 ± 1.45$^{@}$
Group 3 MIC Exposed F_2 Generation Population	5.1 ± 0.31$^{@\#}$	12.0 ± 0.36$^{@\#}$

$^{@}$ Significant when compared with Group 1

$^{\#}$ No Significant when compared with Group 2

The present study includes 25 males and 25 females. Table 1 depicts the mean values antioxidant parameters according to the study variable. It can be observed that Group 2 and Group 3 of Vitamin E and Blood Glutathione were highly significant than Group 1. On comparison with Group 2 and Group 3 it was observed no significant changes.

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Table 2: The mean values for cytogenetic parameters according to the study variable

Characteristics	Normal metaphases	Double Minutes	Dicentrics
Group 1 MIC Non-Exposed Population	99.2 ± 0.45	0.10 ± 0.05	0.10 ± 0.05
Group 2 MIC Exposed Population	92.7 ± 1.14[@]	0.36 ± 0.06[@]	0.48 ± 0.09[@]
Group 3 MIC Exposed F_2 Generation Population	90.6 ± 1.67[@#]	0.32 ± 0.08[@#]	0.45 ± 0.11[@#]

[@] Significant when compared with Group 1
[#] No Significant when compared with Group 2

The values for cytogenetic parameters such as percentage of normal metaphase, frequency of chromosomal aberrations such as dicentrics and double minutes were recorded.

Table 2 shows the distribution of cytogenetic parameters according to different groups. The one-way ANOVA showed that there was a declining trend in the mean value for all cytogenetic parameters according to the group. When the MIC Exposed Population (Group 2) and MIC Exposed F_2 Generation Population (Group 3) were compared, there was highly significant difference in values of normal metaphases as compared with Non MIC Exposed Population (Group 1). Further, no significant changes were observed when compared to MIC Exposed Population Group 2 and Group 3.

The frequency of double minutes and dicentrics were highly significant in MIC Exposed Population (Group 2) and MIC Exposed F_2 Generation Population (Group 3) as compared to Non MIC Exposed Population (Group 1) while no significant changes were observed when compared to Group 2 and Group 3.

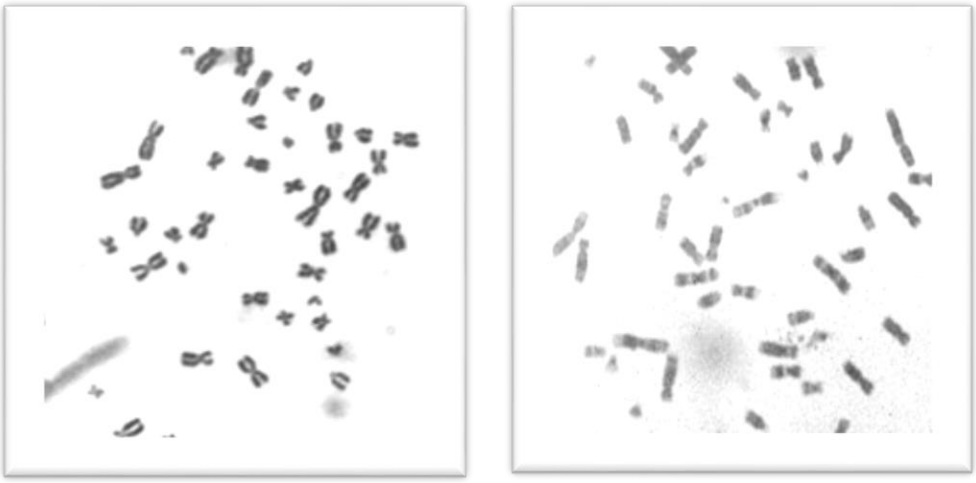

Figure 1: Normal Metaphases of Non-Exposed Population

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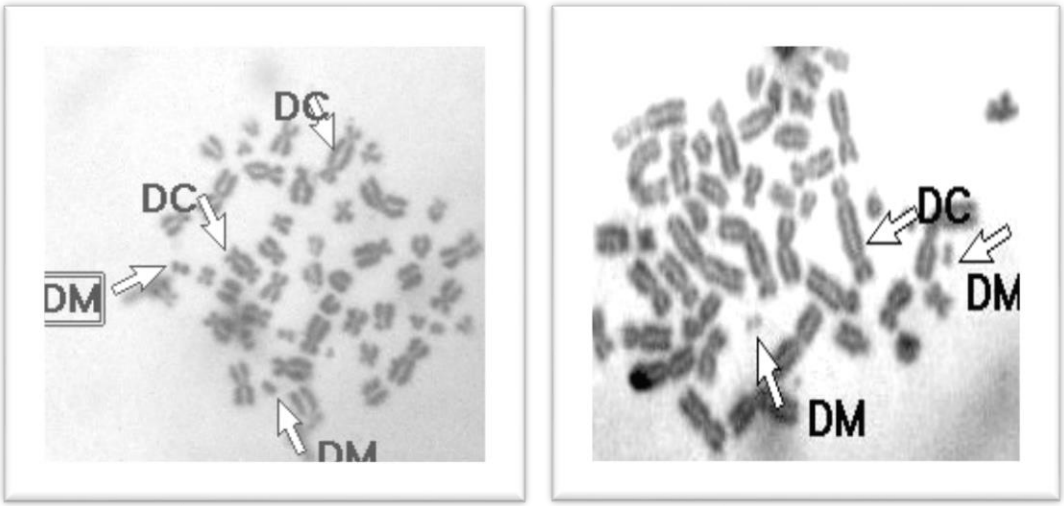

Figure 2: Metaphase showing Double Minutes and Dicentrics in Exposed Population

Discussion

Based on the observation Vitamin E has an ameliorative effect in determining its specific molecular function and these are related to its chain breaking antioxidant function that prevents the propagation of free radical reactions in human beings [22 – 27]. It is located in cell membranes where it interrupts lipid per oxidation and plays an important role in modulating intracellular signaling pathway that relay on reactive oxygen species. It directly quenches ROS including superoxide radical, OH and O_2. It is an important compound implicated in recycling of α-tocopherol radical. This removes the free radical intermediates and prevents the oxidation reaction from continuing. The oxidized α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol. Because of this the human body has several mechanisms to counteract the damage caused by free radicals; this is either by enzymes like glutathione peroxidase, superoxide dismutase and catalase or by antioxidants and therefore, the levels found to less among the Gas Victims than the Non-Exposed Population. The frequency of human Vitamin E deficiency as a result of α- TTP defects is unknown. The low level of antioxidants or inhibition of the antioxidant enzymes causes oxidative stress and damage or kill cells [28 – 29].

The reduced GSH is a natural antioxidant which played a vital role in glutathione metabolism and also, involved in cellular and molecular function. GSH-dependant enzymes utilize GSH as a substrate for their metabolic functions and donates electron from reduced GSH and forms oxidized glutathione disulphide (GSSG). Accumulation of glutathione disulphide (GSSG) leads to oxidative stress and cytotoxicity. Similarly, acceptance of hydrogen from reduced GSH forms GSSG which decreases the reduced GSH level in the human body resulting in cell death. The normal metabolism of glutathione is fully dependent on reduced GSH. Glutathione reductase is a specific enzyme that catalyses glutathione disulphide back to reduced GSH. Glutathione reductase is known as a factory of GSSG reduction, and it decreases the level of glutathione in the blood. Hence, this causes oxidative stress. It is evidenced that in this study the level of glutathione was found to be decreased in the MIC exposed population than MIC non-exposed population.

The alteration in the level of glutathione found both in inter- or intra- cellular surfaces which causes oxidative stress may be due to Glutathione reductase and it is considered to be a critical biomarker for GSH / GSSG homeostasis to enhance the cell viability and stability against inter- or intra-cellular stressor [30]. Other studies have also suggested that glutathione is an important factor in protecting organisms against toxicity and diseases.

The higher incidence of chromosomal aberrations in the exposed group than the non-exposed is in accord with the findings of Ghosh *et al.* [14]. Preliminary studies conducted by Goswami [32] revealed 71.4% of

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chromosomal aberrations affected by the Bhopal Gas Survivors. In the present study 100% chromosomal anomalies was observed in the MIC Exposed individuals which are the indication of genomic instability at a higher intensity. Increased level of chromosomal aberrations in the MIC exposed population due to various toxic chemicals and radiation is an evidence of genotoxic exposure and early biological effects on DNA. Remarkably higher percentage of chromosomal aberrations in exposed individuals due to the exposure of toxic gas released at the time of Bhopal Gas Tragedy.

Conclusion

There is overwhelming evidence that oxidative stress occurs in cells as a consequence of normal physiological processes and environmental interactions and that the complex web of antioxidant defence systems plays a key role in protecting against oxidative damage. A more complete understanding of the biochemical events occurring at a cellular level to influence oxidative damage is required to guide future therapeutic advances.

Chromosomal aberrations often induced by environmental exposure represent a large proportion of genetic disorders which is being transmitted through germ line mutations. Higher incidence of chromosomal abnormalities was found at the time of pre-natal and post-natal stages in the exposed population that validates chromosomal instability. These chromosomal aberrations may act as the intermediate processes in the pathway of the progression of any genetic disorder like cancer. The incidence of chromosomal aberrations like dicentrics and double minutes still persists even after a long period indicating clastogenic effects due to the exposure. This emerged as noteworthy cytogenetic endpoints that link between MIC exposure and possibility of risk of disease. This study highlights the genotoxic effect of MIC gas exposure to the MIC Exposed and MIC Exposed F_2 Generation Population suggesting the urgent need for health surveillance and risk management to reduce the genetic damage in Bhopal MIC affected population.

The long term effect of MIC needs to be studied to see the level of genomic damage caused in the generations of MIC exposed population. Therefore, this study was an attempt to screen the genetic condition of MIC exposed Bhopal population and their offspring's. Though the risk of cancer and diseases related to kidney, respiratory have been increased aftermath of the exposure. However, it cannot be directly linked to MIC as there are multiple confounding factors such as living environment, living style, occupational exposure, dietary factors and inherent genetic conditions.

Hence, our present study suggests that 1984 Bhopal Gas Tragedy produced long-term genotoxic effect that persists even after three decades. In fact, the aftermath of such findings would aid in shaping strategies for preventive management of future industrial disasters and filter the risk that mankind faces from chemicals and other environmental hazards.

Thus, the study concludes that the toxic methyl isocyanate is more vulnerable to the genetic diseases and disorders which may leads to delayed milestones in pedantic and must be counselled for dietary and lifestyle changes to minimize the risk of developing any kind of genetic disorder. The toxic MIC exposed population is being more vulnerable to the genetic diseases and disorders; a long-term effect of MIC needs to be studied to find the level of genomic damage caused in the generations of MIC exposed population.

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