RESEARCH ARTICLE

Increased Micronucleus Frequency in Peripheral Blood Lymphocytes Contributes to Cancer Risk in the Methyl Isocyanate-Affected Population of Bhopal

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Abstract

   The Bhopal gas tragedy involving methyl isocyanate (MIC) is one of the most horrific industrial accidents in recent decades. We investigated the genotoxic effects of MIC in long-term survivors and their offspring born after the 1984 occurrence. There are a few cytogenetic reports showing genetic damage in the MIC-exposed survivors, but there is no information about the associated cancer risk. The same is true about offspring. For the first time, we here assessed the micronucleus (MN) frequency using cytokinesis-blocked micronucleus (CBMN) assay to predict cancer risk in the MIC-affected population of Bhopal. A total of 92 healthy volunteers (46 MIC-affected and 46 controls) from Bhopal and various regions of India were studied taking gender and age into consideration. Binucleated lymphocytes with micronuclei (BNMN), total number of micronuclei in lymphocytes (MNL), and nuclear division index (NDI) frequencies and their relationship to age, gender and several lifestyle variabilities (smoking, alcohol consumption and tobacco-chewing) were investigated. Our observations showed relatively higher BNMN and MNL (P<0.05) in the MIC-affected than in the controls. Exposed females (EF) exhibited significantly higher BNMN and MNL (P<0.01) than their unexposed counterparts. Similarly, female offspring of the exposed (FOE) also suffered higher BNMN and MNL (P<0.05) than in controls. A significant reduction in NDI (P<0.05) was found only in EF. The affected group of non-smokers and non-alcoholics featured a higher frequency of BNMN and MNL than the control group of non-smokers and non-alcoholics (P<0.01). Similarly, the affected group of tobacco chewers showed significantly higher BNMN and MNL (P<0.001) than the non-chewers. Amongst the affected, smoking and alcohol consumption were not associated with statistically significant differences in BNMN, MNL and NDI. Nevertheless, tobacco-chewing had a preponderant effect with respect to MNL. A reasonable correlation between MNL and lifestyle habits (smoking, alcohol consumption and tobacco-chewing) was observed only in the controls. Our results suggest that EF and FOE are more susceptible to cancer development, as compared to EM and MOE. The genotoxic outcome detected in FOE reflects their parental exposure to MIC. Briefly, the observed cytogenetic damage to the MIC-affected could contribute to cancer risk, especially in the EF and FOE.

Keywords: Methyl isocyanate exposure - long-term survivors and offspring - genotoxic effects - Bhopal - India

Introduction

   Methyl isocyanate ($CH_3N=C=O$) (MIC; CAS No. 624-83-9) is a common monoisocyanate that is widely used as a precursor in carbamate pesticide production (Bucher, 1987; ATSDR, 2002). Humans exposed to MIC occupationally or accidently may run an elevated risk of developing respiratory disorders including asthma-like allergy and inflammation, airway hyper responsiveness, reactive airway dysfunction syndrome (RADS) (HSFS,

2002; Dhara and Dhara, 2002; Bessac et al., 2009) and eye infection (Andersson et al., 1990). Potential hazards of MIC include immunotoxicity (Luster, 1986; Karol et al., 1987), genotoxicity (Tice et al., 1986; Conner et al., 1987; Shelby et al., 1987), reproductive and developmental toxicity (Schwetz et al., 1987). But, very little is known about the carcinogenicity of MIC in animals (Gassert et al., 1986; Ennever and Rosenkranz, 1987; Bucher and Uraih, 1989). Accidental or occupational hazard of MIC in humans with respect to cancer is yet-unknown.

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Besides, the information regarding cancer risk associated with MIC-exposure also remains scanty. In contrast, emerging *in vitro* evidences suggest genomic instability by MIC analogs in lung (Panwar and Mishra, 2011), liver (Panwar et al., 2011) and ovary epithelial cells (Raghuram et al., 2010) that may undergo oncogenic transformation. Whether exposure to MIC induces a site-specific cancer is still a point of much controversy (Senthilkumar et al., 2012). The bottleneck faced by the scientific community in this regard is mainly due to attempts on extrapolation of the data on the animals to human studies (Senthilkumar, 2012).

At Bhopal, India, on 3rd December 1984 some 40 metric tons of MIC were accidentally released from Union Carbide India Limited (UCIL) plant. This catastrophic episode took a toll of 3600 human lives instantly and about 2,00,000 inhabitants were exposed to MIC to the varying degrees. Amongst the survivors, 50,000 were expected to survive with long-term effects (Dhara and Kriebel, 1993; Varma and Guest, 1993). In 1985, Indian Council of Medical Research (ICMR) launched a series of long-term clinical studies, followed-up till 1994 (10 years) and then declared that the effects of MIC are short-term and that will not persist in the exposed population (ICMR, 2004). Since 1994, substantial debate suggested that both the exposed survivors and their offspring continue to suffer from the yet-unknown long-term effects (Sarangi et al., 2010). Recently, we have also observed higher prevalence of atypical lymphocytes in the peripheral blood of MIC-exposed parents and their offspring born post-exposure (Senthilkumar et al., 2013).

Epidemiological studies from Bhopal, India show diverse cancer pattern amongst the MIC-affected population. A case-control investigation observed a relative risk of lung, oropharynx and oral cavity cancers in the MIC-exposed males between 1987 and 1992 (Dikshit and Kanhere, 1999). A hospital-based study reported breast cancer incidence in MIC-exposed females between 1994 and 2002 (Ganesh et al., 2005). Recently, in a five year (2006 to 2011) cross-sectional study we reported higher prevalence of lung and breast cancer (Senthilkumar et al., 2011) among the MIC-exposed long-term survivors and their offspring. Results from these epidemiological studies indicate that the cancer rate in MIC-affected population has doubled during the last 10 years. This prompted us to biomonitor the population for cytogenetic damage using micronucleus (MN) assay that suggests heightened cancer risk.

Few studies have evaluated the cytogenetic damage in the MIC-exposed population. Increase in chromosomal aberrations (CAs) (Saxena et al., 1988; Goswami et al., 1986, 1990) and sister chromatid exchanges (Ghosh et al., 1990) were observed in the exposed population two decades ago. There was no follow-up biomonitoring after these findings. More recently, we reported a significant increase of chromosome-type and chromatid-type aberrations in the MIC-exposed survivors (Malla et al., 2011). All the earlier cytogenetic studies overlooked the offspring (born post-catastrophe) of the MIC-exposed survivors and their imminent cancer risk. Moreover, a wide range of significant factors including lifestyle and habits were not taken into consideration while studying the effects of MIC exposure on the cytogenetic make up earlier. We have endeavoured to biomonitor and study several aspects including smoking, alcohol consumption and tobacco-chewing that may indeed throw fresh light on the cytogenetic damage in relation to cancer risk.

In biomonitoring studies, MN assay in the peripheral blood lymphocytes (PBL) is extensively used as a valuable genotoxic end-point to measure the cytogenetic damage (Fenech and Morley, 1985a; Fenech et al., 1999; Pastor et al., 2001; Bolognesi et al., 2011; Albertini et al., 2000). MN arises from acentric chromosome fragments or whole chromosomes that are not incorporated in the main daughter nuclei during nuclear division (Figure 1C). MN is a functional indicator of chromosomal damage (chromosome breakage and loss) (Fenech, 2000; Bonassi et al., 2007). Frequency of MN in human peripheral lymphocytes may be identified using cytokinesis-block method in the cells that have completed one nuclear division following treatment with cytochalasin-B (Cyt-B). The detection of MN is facilitated by the presence of binucleated or multinucleated appearance (Carter, 1967; Fenech, 1993; 2000; Liao et al., 2014) (Figure 1B, D, and F).

As compared to other cytogenetic techniques, the cytokinesis-blocked micronucleus (CBMN) assay is a rapid and simple technique to detect the cytogenetic damage in the populations exposed to radiation, pesticides and genotoxic chemicals (Bolognesi et al., 1993; 2002; Scarpato et al., 1996; Da Silva Augusto et al., 1997; Joksic et al., 1997; Meng and Zhang, 1997; Calvert et al., 1998; Gómez-Arroyo et al., 2000; Pastor et al., 2001; Costa et al., 2006; Bhalli et al., 2006; Kumar et al., 2011; Sellappa et al., 2010; 2011 a, b; Balamuralikrishnan et al., 2012; Erdem et al., 2012; Donmez-Altuntas and Bitgen, 2012; Gajski et al., 2013; Rodrigues et al., 2014; Yang et al., 2014). An increased MN frequency in the circulating lymphocytes indicates increased cancer risk for those exposed to genotoxic agents (Znaor et al., 2003; Bonassi et al., 2007; 2011). The CBMN assay is increasingly used these days to assess the cancer risk associated to genotoxic exposures (El-Zein et al., 2011) and genomic instability of several diseases (Wu et al., 2012; Abdullah and Orta, 2012; Wang et al., 2013; Sitaraman et al., 2014; George et al., 2014; Soares et al., 2014; Main et al., 2015).

For the first time we evaluated the MN frequency using CBMN assay in the MIC-affected (long-term survivors and their offspring) population of Bhopal. Distribution of subjects with respect to smoking, alcohol consumption and tobacco-chewing was also taken into account while studying its effect on MN frequency.

Materials and Methods

Study design

The study was designed according to the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC) criteria (Hook, 1982). All participating subjects were healthy volunteers categorized in two groups: MIC-exposed long-term survivors and their offspring (affected) and the unexposed

(control). The subjects with genetic disorders, history of malignancy or any continuing disease, exposure to therapeutic or diagnostic radioactive elements, chemotherapy, vaccination, occupationally involved in asbestos, petroleum, chemical and agrochemicals, automobile, and wood industries were excluded from the investigation radar.

Ethics statement

The study was conducted after ethical approval (Reference No. 390/ 225/ 2008) of the Institutional Review Board at Jawaharlal Nehru Cancer Hospital & Research Centre, Bhopal. The study was strictly in line with the ICMR ethical guidelines for biomedical research on human participants (ICMR, 2006) and principles of Declaration of Helsinki (DoH, 2000). All participants were given an explanation of the nature of the study and they could withdraw their participation at any time during the study according to Helsinki II declaration. Following their approval of the guidelines their written consents were obtained.

Inclusion and selection criteria

Interview of the participants was conducted to examine their suitability for the study. To avoid false-positive exposure status, a special screening and selection criterion was followed for the MIC-affected population. Gas victim smart card (GVSC) issued by Bhopal Memorial Hospital Trust to the MIC-exposed individuals were treated as evidence of their exposure to MIC. Prior to enrollment, GVSC or official compensation claims were screened and their reference number recorded for all participants exposed to MIC. Offsprings were enrolled after scrutinizing the GVSC of their parents.

Questionnaire

Each participant was personally interviewed using a questionnaire containing standard socio-demographic questions (age, gender, religion, ethnicity, residence, education, occupation, marital status, etc.) as well as medical (family and personal history including gynaecological problems and reproduction), lifestyle habits (physical activity, smoking, alcohol consumption, tobacco-chewing, etc.), dietary status (feeding habits). For the exposed group, specific questions related to immediate health effects during MIC-exposure were also recorded.

Study Population and subject recruitment

Between 2008 and 2012, a total of 92 healthy volunteers who fulfilled the criteria were registered for the study. The affected group comprised 46 MIC-exposed healthy long-term survivors and their offspring from the MIC gas-affected areas viz. chola, chanbad, nareal kheda, noor mahal, and durga nagar (1, 1.5, and < 5km distance from the UCIL factory, Bhopal). Gender and age-group matched 46 unexposed healthy individuals from Bhopal and various regions of India were selected as control. All the participants were of the Indian origin. Further, the MIC-affected and the controls were subdivided in to 8 sub-groups according to gender and age, such as, exposed females (EF), exposed males (EM), unexposed females (UEF), unexposed males (UEM) are ≥28-55 and female offspring of the exposed (FOE), female offspring of the unexposed (FOUE), male offspring of the exposed (MOE), male offspring of the unexposed (MOUE) are <28. With regard to the smoking habits, alcohol consumption and tobacco-chewing, individuals were classified as non-smokers, non-alcoholics, and non-tobacco chewers, who had never smoked, never consumed alcohol and never chewed tobacco. Smokers, alcoholics and tobacco chewers were currently addicted to the respective habits, therefore, the category of ex-smokers, ex-alcoholics, and ex-chewers was avoided in this study.

Sample collection

Peripheral blood (PB) samples were collected under sterile conditions and processed according to the recommendations described in the biomonitoring workgroup report (Bates et al., 2005). A certified trained medical laboratory technician (Certificate No: SCH-27/ Diplo/ 1447/2006) was recruited to draw 5mL PB samples by venipuncture with the use of sterile syringe, then transferred into heparinized vacutainer tubes (VAKU-8, HMD Healthcare, UK) and was mixed thoroughly. To avoid potential bias and to assure subject confidentiality, all the samples were coded and stored at 4°C, aseptically transported immediately to the laboratory and processed on the same day within 3 h of sampling.

Lymphocyte cultures for CBMN assay

Lymphocyte cultures were initiated immediately by adding 0.5 mL of the heparinized whole blood to 4.5mL RPMI 1640 medium enriched with 200 mM L-glutamine, 25mM HEPES buffer with 10% fetal bovine serum and 1.5% penicillin-streptomycin solution (Sera Laboratories International, UK; HiMedia Laboratories, India). Lymphocytes were stimulated by 1% phytohaemagglutinin (PHA-M) (Biological Industries, Israel; HiMedia Laboratories, India; GIBCO, USA) and incubated for 72 h at 37°C in the humidified atmosphere with 5% CO_2 (Hera cell CO_2 Incubator, Heraeus Instruments, Germany) at 37°C. After 44 h incubation, 6 μg/ml Cyt-B (Fenech, 2000) (Sigma, USA; HiMedia Laboratories, India) was added to block cytokinesis and cultures were reincubated for further 28 h. At 72 h post PHA-stimulation, the cultures were harvested through centrifugation at 1000 rpm for 8 min. Cells were treated hypotonically with 7 mL of 0.075M KCl at 4°C for 3 min, spun again and added 4 mL cornoy's fixative [methanol: acetic acid (3:1)] with gentle shake. This fixation procedure was repeated thrice by centrifugation at 500 rpm for 5 min and finally the cells were resuspended in the fixative and dropped on to prechilled slides. The slides were air-dried and treated for 10 min with giemsa stain (Biolab Diagnostics, India), as per the manufacturer's protocol. To reduce variability, all the coded slides were scored blindly by one scorer under the phase contrast trinocular (Olympus, Singapore) and image analyzer system (Motic B1 series, Version 2.0) (Motic Image Plus, Motic China Group Co. Ltd, China) microscopes.

The scoring criteria for binucleated lymphocytes with micronuclei (BNMN) and total number of micronuclei

in lymphocytes (MNL) evaluation were followed from Fenech et al (2003). To determine the frequency of BNMN and MNL, a total of 1000 binucleated cells with well preserved cytoplasm were scored per subject on the coded slides (Figure 1B, C). In addition, nuclear division index (NDI) was evaluated by scoring the number of nuclei in 400 cells and calculated using the following formula according to Eastmond and Tucker (1989):

Figure 1. Cell types observed in CBMN assay from MIC-affected population **(A)** mononucleate cell **(B)** ideal binucleate cell **(C)** binucleate lymphocyte with one MN **(D)** trinucleate cell **(E)** trinucleate lymphocyte with two MN **(F)** quadrinuclear cell

$$NDI = [M1 + (2 \times M2) + (3 \times M3) + (4 \times M4)]/N$$

where M1-M4 represents the number of cells with one to four nuclei, respectively, and N is the total number of viable cells scored (Figure 1 A, B, D, F).

Statistical methods

The data were analyzed using GraphPad PRISM 4 (GraphPad Software, Inc., San Diego California, USA) and SPSS 20.0 (SPSS, Chicago, IL, USA) for windows statistical package. Differences in the distributions of socio-demographic characteristics, lifestyle habits and dietary status amongst the groups (MIC-affected and controls) were evaluated accordingly. BNMN, MNL, and NDI frequencies were expressed as means ± standard deviation (SD). Significant difference in the frequencies between the groups was tested by unpaired t-test. Similarly, gender-age-group matched respective sub-groups (EF *vs* UEF, EM *vs* UEM, FOE *vs* FOUE, and MOE *vs* MOUE) were compared by unpaired t-test. Mann-Whitney U-test was applied to evaluate the influence of smoking, alcohol consumption, and tobacco-chewing on the differences in the frequencies between the groups (MIC-affected *vs* Controls) and between the sub-groups within the same group (smokers *vs* non-smokers, alcoholics *vs* non-alcoholics, tobacco chewers *vs* non-chewers). The minimum level of significance was set at P ≤ 0.05.
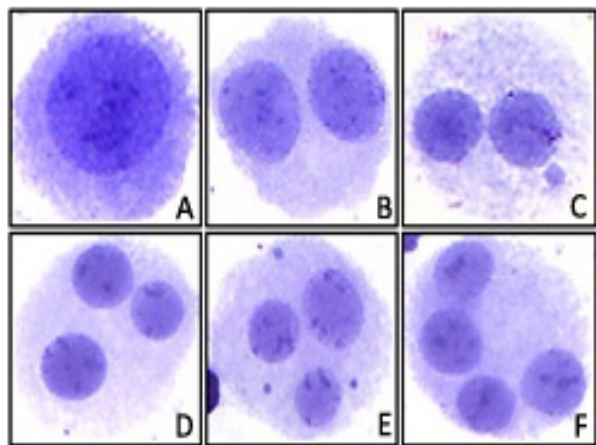
Table 1. Descriptive Statistics for Socio-demographic Characteristics within the Study Population

Characteristics	MIC-affected		Control		Total (%)
	n (%)	Mean±SD	n (%)	Mean±SD	
Number of subjects (n)	46(50)	-	46(50)	-	92(100)
Age[a]	46(50)	30.5±11.8	46(50)	30.0±8.91	
Gender					
Female	23(25)	-	23(25)	-	46 (50)
Male	23(25)	-	23(25)	-	46 (50)
Ethnic region					
Bhopal (By birth)	46(50)	-	16(17.39)	-	62 (67.39)
Non-Bhopal (Migrated after 1984)	-		30(32.60)	-	30 (32.60)
Marital status					
Married	23(25)	-	23(25)		46 (50)
Unmarried	23(25)	-	23(25)		46 (50)
Family History of cancer	3(3.26)		1(1.08)		4 (4.34)
Physical activity (Exercise, Yoga, Walking)					
Sedentary	8(8.69)	-	13(11.9)	-	19(20.65)
Occasionally	9(9.78)		1(1.08)	-	10(10.9)
Daily	29(31.52)		34(36.95)	-	63(68.47)
Smoking habit					
Smokers	3(3.26)	-	7(7.60)	-	10 (10.9)
Non-smokers	43(46.73)		39(42.39)	-	82(89.13)
Alcohol consumption					
Alcoholics	4(4.34)	-	11(11.9)	-	15(16.30)
Non-alcoholics	42(45.65)	-	35(38.04)	-	77(83.69)
Tobacco chewing					
Chewers	10(10.9)	-	8(8.69)	-	18(19.56)
Non-chewers	36(39.13)	-	38(41.30)	-	74(80.43)
Dietary status					
Vegetarian	10(10.9)	-	13(14.1)	-	23(25)
Non-vegetarian	36(39.13)	-	33(35.86)	-	69(75)

[a]Unpaired t-test, No significant difference were observed (P ≥ 0.05)

Results

As shown in Table 1, the socio-demographic characteristics of the population indicate no difference in the age of two groups (P > 0.05). Age in MIC-affected group (n=46) (50%) ranged from 9 to 55years (30.5±11.8) whereas the age in control group (n=46) (50%) was in the range of 12 to 50 years (30.0±8.91). Both the groups had 23(25%) females and 23(25%) male subjects each. Regarding marital status, 23(25%) were married and 23(25%) were unmarried in both the groups. Three (3.26%) of the MIC-affected and one (1.08%) from the control group had family history of cancer. That means, only 4 (4.34%) of the study population had family history of cancer. Physical exercise, yoga and walking were undertaken daily by 63(68.47%) of the study population and 10(10.9%) were occasionally active from these standpoints. Only 19(20.65%) had sedentary lifestyle. The frequency of such a lifestyle in the control group (11.9%) and the MIC-affected (8.69%) was statistically insignificant. As regards smoking habit, 3 (6.52%) were smokers and 43(93.47%) were non-smokers among the MIC-affected. Of the controls, 7(15.21%) were smokers and 39 (84.78%) were non-smokers. The study, as a whole, included 10 (10.9%) smokers and 82 (89.13%) non-smokers.

Among the MIC-affected, 4 (8.69%) were alcoholics and 42 (91.30%) non-alcoholics. In the control group, 11 (23.91%) were alcoholics and 35 (76.08%) non-alcoholics. The entire study thus had 15 (16.30%) alcoholics and 77 (83.69%) non-alcoholics. Interestingly, smoking habit and alcohol consumption was observed higher in the controls as compared to the MIC-affected.

With respect to tobacco-chewing, 10 (21.73%) of the MIC-affected were chewers and 36 (78.26%) non-chewers. Among the controls, 8 (17.39%) were tobacco chewers and 38 (82.60%) non-chewers. In total, 18 (19.56%) of the studied subjects were tobacco chewers and 74 (80.43%) non-chewers. In contrast to smoking habit and alcohol consumption, tobacco-chewing subject was higher in the MIC-affected as compared to controls. Amongst the affected and controls, we observed a higher frequency of tobacco-chewing habit (19.56%) as compared to other habits including smoking (10.9%) and alcohol consumption (16.30%).

BNMN and MNL frequencies were relatively higher (P<0.01) in the MIC-affected as compared with parallel control group. No difference was observed in the NDI between the MIC-affected and controls (Table 2). When stratified further in to gender-age-group matched sub-groups, BNMN and MNL were significantly higher (P≤0.01) in the EF as compared to UEF. A significant reduction in the NDI was also observed in the EF (P≤0.05) as compared to UEF. There was no difference in BNMN,

Table 2. Frequencies of BNMN, MNL and NDI in the MIC-affected and Control Subjects

Group	n	BNMN[a]	MNL[b]	NDI[c]
MIC-affected	46	4.30±3.34**	4.36±3.55**	1.60±0.12
Control	46	2.56±1.98	2.63±2.06	1.62±0.11
"t "value		3.032	2.867	0.8467
Total	92			

Table 4. Effect of Smoking Habit, Alcohol Consumption, and Tobacco-chewing on Cytogenetic Variables in the Population Studied

Studied variables	MIC-affected (n= 46)		Control (n= 46)	
	n (%)	Mean±SD	n (%)	Mean±SD
BNMN				
Smokers	3 (6.5)	3.66±1.52	7 (15.2)	4.85±1.06
Non-smokers	43 (93.5)	4.34±3.44**	39 (84.8)	2.15±1.82
Alcoholics	4 (8.7)	4.75±3.09	11 (23.9)	3.36±1.43
Non-alcoholics	42 (91.3)	4.26±3.40**	35 (76.1)	2.22±1.98
Tobacco chewers	10 (21.7)	7.80±3.67	8 (17.4)	4.50±1.69
Non-chewers	36 (78.3)	3.33±2.54	38 (82.6)	2.15±1.80
MNL				
Smokers	3 (6.5)	3.66±1.52	7 (15.2)	5.00±1.29
Non-smokers	43 (93.5)	4.41±3.66**	39 (84.8)	2.20±1.89
Alcoholics	4 (8.7)	4.75±3.09	11 (23.9)	3.72±1.73
Non-alcoholics	42 (91.3)	4.33±3.62**	35 (76.1)	2.28±2.06
Tobacco chewers	10 (21.7)	8.10±4.28	8 (17.4)	4.75±1.66
Non-chewers	36 (78.3)	3.33±2.54	38 (82.6)	2.18±1.87
NDI				
Smokers	3 (6.5)	1.56±0.07	7 (15.2)	1.53±0.06
Non-smokers	43 (93.5)	1.60±0.12	39 (84.8)	1.64±0.10
Alcoholics	4 (8.7)	1.54±0.07	11 (23.9)	1.56±0.10
Non-alcoholics	42 (91.3)	1.60±0.12	35 (76.1)	1.64±0.10
Tobacco chewers	10 (21.7)	1.61±0.09	8 (17.4)	1.57±0.09
Non-chewers	36 (78.3)	1.60±0.12	38 (82.6)	1.63±0.11

Mann-Whitney U-test, Significance level at * P<0.05 and ** P<0.01; (MIC-affected *vs* Control)

Table 3. Comparison of BNMN, MNL and NDI between the Respective Sub-groups According to Age Group and Gender in the Population Studied

Age/Gender		n	BNMN[a]	"t "	MNL[b]	"t "	NDI[c]	"t "
(>28-55yr)		(n=52)						
Females	EF	13	6.76±3.60**		7±4.14**		1.51±0.15*	
	UEF	13	3.07±1.80	3.301	3.07±1.80	3.131	1.63±0.11	2.068
Males	EM	13	4.76±2.27		4.76±2.27		1.62±0.10	
	UEM	13	3.23±1.64	1.975	3.38±1.75	1.735	1.59±0.12	0.727
(<28yr)		(n=40)						
Female offspring	FOE	10	3±3.26*		3±3.26*		1.63±0.07	
	FOUE	10	0.7±0.82	2.159	0.7±0.82	2.159	1.64±0.11	0.234
Male offspring	MOE	10	1.8±1.75		1.8±1.75		1.65±0.08	
	MOUE	10	2.9±2.47	1.149	3±2.62	1.203	1.63±0.08	0.461
Total		(n=92)						

a, b, c Unpaired t-test, Significance level at * P <0.05 and ** P <0.01

Table 5. Effect of Smoking Habit, Alcohol Consumption, and Tobacco-chewing on Cytogenetic Variables between the Subgroups within the Same Group

Studied variables	MIC-affected (n= 46)		Control (n= 46)	
	n (%)	Mean±SD	n (%)	Mean±SD
BNMN				
Smokers	3(6.5)	3.66±1.52	7(15.2)	4.85±1.06**
Non-smokers	43(93.5)	4.34±3.44	39(84.8)	2.15±1.82
Alcoholics	4(8.7)	4.75±3.09	11(23.9)	3.36±1.43
Non-alcoholics	42(91.3)	4.26±3.40	35(76.1)	2.22±1.98
Tobacco chewers	10(21.7)	7.80±3.67***	8(17.4)	4.50±1.69**
Non-chewers	36(78.3)	3.33±2.54	38(82.6)	2.15±1.80
MNL				
Smokers	3(6.5)	3.66±1.52	7(15.2)	5.00±1.29**
Non-smokers	43(93.5)	4.41±3.66	39(84.8)	2.20±1.89
Alcoholics	4(8.7)	4.75±3.09	11(23.9)	3.72±1.73*
Non-alcoholics	42(91.3)	4.33±3.62	35(76.1)	2.28±2.06
Tobacco chewers	10(21.7)	8.10±4.28***	8(17.4)	4.75±1.66**
Non-chewers	36(78.3)	3.33±2.54	38(82.6)	2.18±1.87
NDI				
Smokers	3(6.5)	1.56±0.07	7(15.2)	1.53±0.06**
Non-smokers	43(93.5)	1.60±0.12	39(84.8)	1.64±0.10
Alcoholics	4(8.7)	1.54±0.07	11(23.9)	1.56±0.10*
Non-alcoholics	42(91.3)	1.60±0.12	35(76.1)	1.64±0.10
Tobacco chewers	10(21.7)	1.61±0.09	8(17.4)	1.57±0.09
Non-chewers	36(78.3)	1.60±0.12	38(82.6)	1.63±0.11

Mann-Whitney U-test, Significance level at * P <0.05, ** P <0.01 and *** P <0.001

MNL and NDI among EM and UEM. BNMN and MNL were significantly higher (P≤0.05) in the FOE as compared to FOUE. However, there was no difference in the NDI (P>0.05) among these sub-groups. Similar to EM and UEM, no differences were observed in the values of BNMN, MNL and NDI between the MOE and the MOUE (P>0.05) (Table 3).

As depicted in Table 4, the effect of lifestyle influences, such as smoking habit, alcohol consumption, and tobacco-chewing on BNMN, MNL and NDI frequencies between the groups (MIC-affected *vs* controls) was negligible. No difference existed in the BNMN and MNL between smokers and alcoholics of both the groups (P>0.05). On the other hand, BNMN and MNL were significantly higher (P≤0.01) in the MIC-affected non-smokers and non-alcoholics than in the parallel control group. Difference for BNMN and MNL between the tobacco chewers of both the groups were not statistically significant (P>0.05). No difference existed in the BNMN and MNL between the non-chewers of the two groups (P>0.05). With reference to smoking habit, alcohol consumption and tobacco-chewing, no difference was observed with respect to NDI between the groups (P>0.05).

As represented in Table 5, there was no difference with respect to BNMN and MNL among the smokers *vs* non-smokers and alcoholics *vs* non-alcoholics in the MIC-affected group. However, BNMN and MNL were significantly higher (P<0.001) in the tobacco chewers as compared to the non-chewers in the affected population. With respect to smoking habit, alcohol consumption and tobacco-chewing, no difference was observable for NDI in the MIC-affected (P>0.05). In the controls, however, BNMN was significantly higher (P<0.01) in the subjects with smoking habit and tobacco-chewing. Surprisingly, no difference could be observed amongst the alcohol consuming subjects with respect to BNMN (P>0.05). MNL was significantly higher in the control subjects with smoking habit (P<0.01), alcohol consumption (P<0.05) and tobacco-chewing (P<0.01). A significant reduction in NDI was observed in the individuals with smoking (P<0.01) and alcoholic habits (P<0.05). Tobacco-chewing, however, showed no difference with respect to NDI (P>0.05).

Discussion

Cytogenetic damage in relation to cancer has long been established (Hagmar et al., 1994; 1998; 2001; 2004; Bonassi et al., 2000; 2007; 2008; 2011; Rossner et al., 2005; Norppa et al., 2006; Boffetta et al., 2007). We have attempted to understand if there is a relationship between cancer risk and cytogenetic damage in the MIC-affected population. Assessment of the cytogenetic damage was made following CBMN assay.

Results of this study indicate a significant association between MIC-exposure and cytogenetic damage (Table 2) which is in agreement to our previous work (Malla et al., 2011). It is, however, important to note that earlier, we observed an increased incidence of chromosome-type aberrations in the EM as compared to EF. But, the present study shows greater incidence of BNMN and MNL in the EF than in the EM and all other sub-groups of the affected and control subjects. Genetic differences, age effect, gender and individual susceptibility, exposure level, subject selection may be responsible for these contradictory findings, however, at present it is difficult for us to explain the underlying mechanisms involved in the observed gender differences without future larger cohort analysis. This result is in agreement with the findings of Fenech (1998) who reported higher MNL frequency in the females than males. Similarly, Thierens et al. (2000) and Maffei et al. (2002a) also reported increased MNL in the females exposed to the ionizing radiation. Besides, age and gender also seems to influence BNMN and MNL frequencies (Fenech and Morley, 1985b; 1986; Fenech et al., 1994; Fenech and Rinaldi, 1994; Fenech and Bonassi, 2011). The previous cross-sectional study (Senthilkumar et al., 2011), also reported higher cancer incidence in the EF as compared to the EM among the long-term survivors. We observed 34.6% and 12.5% of cancer incidence in EF of the age group ranging from 39-50 years and 27-38 years (children's at the time of MIC-exposure), respectively. This finding correlates well to our results that cytogenetic damage is greater in the EF than in the EM, amongst the survivors. The observations of Ghosh et al (1990) in course of cytogenetic studies during the three years post-disaster of Bhopal gas tragedy that there is a higher incidence of CAs in the MIC-exposed females than in the males lend further support to our data. It is interesting to note that *in vivo* animal cytogenetic studies made by Tice et al (1987) observed significantly higher incidence of MN-PCEs by 6 ppm of MIC in the male mice than in the female. Similarly, Shelby et al. (1987) also observed a significant increase of micronucleated-PCEs in the PB of male mice exposed to 0, 1, 3, or 6 ppm MIC. The differences in observations on the animal and human may be due to many reasons

including physiological and genetic diversities. Closely agreeing to this, Pala et al (2008) and Viegas et al (2010) have presented contradictory reports on the cytogenetic effects in the *in vivo* animal and human studies.

We observed a significant reduction in NDI in the EF as compared to UEF (Table 3). Reduction in NDI might be due to prior MIC-exposure of this sub-group. This observation suggests that exposure to MIC may influence lymphocyte proliferation and cell cycle which is in agreement with an earlier report (Deo et al. 1987). Another study conducted by Tucker et al. (1987) in animals reported a moderate suppression of mitogen-induced lymphoproliferative responses to MIC, especially in the female B6C3F1 mice that suggests an obvious difference between the animal and the human models.

Our studies on the influence of lifestyle factors suggest that non-smokers and non-alcoholics are more sensitive to MIC with respect to incidence of BNMN and MNL that show higher frequencies in the MIC-affected non-smokers and non-alcoholics as compared to the parallel control subjects (Table 4). This genotoxic damage could be attributed to MIC- exposure.

We did not find any association in terms of BNMN and MNL in the MIC-affected smokers and this finding was supported by other studies, found a negative association between smoking and MN frequency (Anwar and Gabal, 1991; Mäki-Paakkanen et al., 1991; Bolognesi et al., 1993; 1997; Tates et al., 1996; Scarpato et al., 1996). Contrary to the results of MIC-affected smokers, an elevated BNMN and MNL were found in the control group smokers than non-smokers (Table 5). There are only a few studies reported the association between smoking and MN (Hogstedt et al, 1983; Hogstedt, 1984; Sorsa et al., 1988; da Cruz et al., 1994; Di Giorgio et al., 1994; De Boeck et al., 2000). With regard to alcohol consumption, we observed higher MNL only in the control group alcoholics. There are strong evidences that heavy smoking with occupational exposure to genotoxic agents (Bonassi et al., 2003; Fenech and Bonassi, 2011) and excessive alcohol consumption (Maffei et al., 2000; 2002b; Ishikawa et al., 2003; 2006; 2007; Kim et al., 2005) may influence higher MN frequency. In addition, smoking and alcohol consumption also seems to affect lymphocyte proliferation in the controls (Table 5) and this finding is in agreement with other studies observed a significant reduction in NDI associated with smoking (da Cruz et al., 1994; Maffei et al., 2002a) and alcohol consumption (Figgs et al., 2000). In contrast, Savage et al. (1980) observed a significantly increased lymphocyte response to PHA in smokers.

On the other hand, we observed significantly higher BNMN and MNL in tobacco-chewing individuals of both the groups. Tobacco-chewing had a preponderant effect than smoking habit and alcohol consumption. However, no effect found for NDI. These observations are in agreement with Das and Das (1992) who reported tobacco-chewing has profound effect on the occurrence of MNL in men and women. Similarly, Patel et al. (2009) also showed that tobacco-chewing may influence higher MNL in males.

Interestingly, we observed an increased BNMN and MNL in the FOE as compared to FOUE (Table 3). Both of these sub-groups had subjects that never smoked, never

consumed alcohol and never chewed tobacco at all. This outcome signifies genotoxic effect in FOE reflects their parental exposure to MIC might be getting manifested as increase in the BNMN and MNL. Without doubt, the observed genotoxic damage is the parental effect perceived by FOE. Although we included relatively low numbers of subjects in this sub-group, this is the first study suggesting the cytogenetic damage as a transgenerational effect in FOE subjects. In support, there are recent evidences related to transgenerational effects on the offspring of MIC-exposed survivors (Kapoor, 1991; Vijayan, 2010; Sarangi et al., 2010). Future investigations are needed to explain the correlation between MIC-exposure and genotoxic effects, especially in FOE population. These finding are needed to explain the correlation between MIC-exposure and genotoxic effects, especially in FOE population. These finding strengthens the evidence that EF and FOE are susceptible to cancers as compared to EM and MOE.

Our findings corroborate with previous reports (Saxena et al., 1988; Goswami et al., 1986; 1990; Ghosh et al., 1990; Malla et al., 2011) that the observed cytogenetic damage as prior MIC-exposure-related long-term effect in the MIC-affected population. We also conclude that genotoxic effect is consistent in the MIC-exposed survivors and transgenerational to their future generations. Therefore, it seems reasonable that the MIC-affected population is at greater risk for developing cancers as a result of prior MIC-exposure and in particular, lifestyle habits mainly tobacco-chewing also tends to enhance the cancer risk.

In conclusion, CBMN assay actually emerged as noteworthy cytogenetic end-point that indicates link between MIC-exposure and possible cancer risk. A significant increase in BNMN and MNL rates indicate the cytogenetic damage and suggest greater cancer risk in the MIC-affected population. As prophylaxis, the affected population can be counseled through interventional programs for healthy lifestyle and dietary habits to minimize their cancer risk. This study highlights the genotoxic effect of MIC-exposure and suggests the urgent need for genetic surveillance and health risk management in Bhopal MIC-affected population.

A major strength of the present study is the inclusion of offspring of the MIC-exposed survivors that allowed us to predict their impending risk and transgenerational effects. However, there are several limitations to our study. A major limitation was small sample size in all the sub-groups. The smaller sample size is because that many of the MIC-exposed survivors are non-responders, expressed that they were not interested to participate in this study. This was one among the difficulties we faced during this biomonitoring study. This may be due to the lack of health awareness in MIC-exposed population.

Another limitation is the selection criterion followed in the study did not classify the subjects according to exposure status such as severely-and moderately-exposed to MIC. Almost 27 years elapsed post-catastrophe, it is difficult and not feasible to categorize this population on the basis of exposure criteria. Investigation on the genetic differences may be followed to elucidate why some individuals survived the MIC-exposure better than others. Genes involved in thiol-redox homeostasis in the airways and resistance to oxidative stress may be important keys

to answer this question.

However, the current work is the first cytogenetic study to report cancer risk in Bhopal MIC-exposed long-term survivors and their offspring. To support our findings, population-based (larger cohort) biomonitoring studies with other cytogenetic end-points are needed to further substantiate transgenerational effects in the offspring.

Acknowledgements

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100.0

6.3

75.0

56.3

50.0

25.0

31.3

0

Newly diagnosed without treatment

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