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ORIGINAL RESEARCH

Micronucleus assessment as a biomarker and susceptibility to DNA damage in workers occupationally exposed to pesticides

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Abstract— Occupational exposures to hazardous chemicals are common in industries using solvent based materials as well as in indoor environments where people are exposed to volatile organic compounds from various sources. The health of the workers has several determinants, including risk factors at the workplace leading to cancer. The aim of the present investigation was to assess the potential cytogenetic damage associated with occupational exposure among pesticide workers by using micronuclei and other nuclear abnormalities as a biomarker. The micronucleus assay on exfoliated buccal cells is a useful and minimally invasive method for monitoring genetic damage in humans. To determine the genotoxic effects of pesticide workers, Micronucleus assay was carried out in exfoliated buccal cells of 50 pesticide workers and 50 controls. For each individual, 2,000 exfoliated buccal cells were analyzed. Micronucleus and other nuclear abnormalities frequencies in exposed were significantly higher than those in control groups ($P < 0.05$) and also significantly related to smoking, tobacco chewing and alcohol drinking habit ($P < 0.05$). Increased frequency of these nuclear abnormalities in buccal epithelial cells of exposed workers indicates adverse cellular reaction and/or a surveillance mechanism to eliminate cells with genetic damage. The present studied individuals may be at a higher risk of developing cancer and therefore monitored for any long term adverse effects of the exposure. Genotoxic studies are foremost for any occupational exposure studies. Evaluation based on genotoxic parameters is often useful in warranting environmental endowment and occupational health. Genotoxicity biomarkers have received a considerable interest as tools for detecting human genotoxic exposure and effects, especially in health surveillance programs dealing with chemical carcinogens.

Keywords—Occupational exposure, Pesticide workers, Micronucleus, Genotoxicity.

INTRODUCTION

To live in the 21st century means to live in a toxic world, where we are exposed daily to numerous environmental toxins and pollutants. Occupational health deals with all aspects of health and safety in the workplace and has a strong focus on primary prevention of hazards. The health of the workers has several determinants, including risk factors at the workplace leading to cancers, accidents, musculoskeletal diseases, respiratory diseases, hearing loss, circulatory diseases, stress related disorders and communicable diseases etc. Humans are diverse in their responses to exogenous exposures because of variability, the rate of metabolism, DNA repair processes and other factors (Anwar, 1997). Occupational exposures to hazardous chemicals are common in industries using solvent based materials as well as in indoor environments where people are exposed to volatile organic compounds from various sources (Barroa et al., 2009).

Health suffers the influence of inherited, nutritional, and environmental factors. Populations of industrial areas are intensely exposed to chemical substances that can cause mutations, cancer, and congenital defects (Hirvonen, 1995). Pesticides, as a heterogeneous category of biologically active compounds, are characterized by various degrees of toxicity also to non-target species, including human beings. Most pesticides are acutely toxic to humans. The widespread use

of pesticides in agriculture, public health and household environments results in continuous exposure of human populations. With green revolution and industrialization, they have become household items of the agriculturists. Unfortunately, because of their easy availability and accessibility, they have also been commonly abused for suicidal purpose in the developing countries. According to World Health Organization, three million cases of acute poisoning occur annually, of which about 2,20,000 die. Of these, 99% of the fatal poisonings occur in developing countries, particularly among farm workers (WHO, 1962). Exposure to low-level of pesticides is known to produce a variety of biochemical changes, some of which may be responsible for the adverse biological effects reported in human and experimental studies (Gupta et al., 1998; Banerjee et al., 1999). Conversely, some biochemical alterations may not necessarily lead to clinically recognizable symptoms, although all the biochemical responses can be used as markers of exposure or effect. The biochemical changes induced after exposure to pesticides or their active metabolites include target cell/receptor binding, protein and DNA adduct formation, and induction or inhibition of enzymes (Heinzow and McLean, 1994).

One way to study the effects on an exposed population is to conduct monitoring studies, using pertinent biological parameters with a short term manifestation, such as cytogenetic analysis, by which damages to the DNA or to the chromosomes resulting from exposure can be identified. Micronucleus (MN) assay for exfoliated cells have been used to evaluate the genotoxic effects produced by low doses of carcinogenic substances or carcinogenic mixtures, to which human populations are exposed (Maluf and Erdtmann, 2000). Micronuclei originated from aberrant mitosis and consist of acentric chromosomes, chromatid fragments or whole chromosomes that have failed to be incorporated in to the daughter nuclei during mitosis. The micronuclei test is the most frequent technique used to detect chromosome breakage or mitotic interference events thought to be associated with increased risk for cancer (Fairbain et al., 1995).

Knowledge of human health risks related to environmental exposure to hazardous chemical agents is a current concern (Franco et al., 2008). To add further knowledge to the genetic risk on an exposed population, we applied the MN and other nuclear abnormalities (NA) as a biomarker. Our objective was to evaluate the cytogenetic damage in exfoliated buccal cells obtained from pesticide workers and non-exposed control subjects, further to establish the relationship of the MN frequency with occupational and non-occupational factors, such as the smoking, tobacco chewing and drinking habits.

MATERIALS – METHODS

Study Subjects

The study population composed of 50 exposed pesticide workers and 50 unexposed controls. The exposed group included 14 smokers and 11 non-smokers, 12 smokers with alcohol drinking and 13 smokers with tobacco chewing habit, from 8 pesticide industries located in the rural area of Coimbatore City, South India. The respective control groups were matched for age and sex (11 smokers and 10 non-smokers, 16 smokers with alcohol drinking and 13 smokers with tobacco chewing habit) and had no occupational exposition to toxic agents. At the time of sample collection the subjects signed a term of informed consent. All subjects were selected based on questionnaire which included items about age, occupational exposure, smoking habit, use of drugs, such as alcohol, virus illnesses, recent vaccinations, and radiological exams. All the individuals who agreed to participate in the study were healthy, and they answered a detailed questionnaire according to the protocol published by the International Commission for Protection against Environmental Mutagens and Carcinogens (Carrano, 1988). For the exposed group, a further questionnaire was completed to evaluate the use of protective measure. These workers were engaged in work for more than 8 h per day with a minimum 7 yrs of exposure duration. None of these study groups showed significant differences with regard to lifestyle and personal factors. The study procedures used in the present study were approved by the Institutional ethical committee.

Cell sampling

Before sampling, each subject rinsed the mouth thoroughly with tap water. Buccal cells (BCs) were collected from consented volunteers at the end of the work shift according to the criteria established by Tolbert and his co-workers (Tolbert et al., 1991). Prior to BC collection the mouth was rinsed thoroughly with water to remove any unwanted debris. Buccal cell samples were obtained by rubbing the inside of both cheeks using a wooden spatula. The cells were collected in tubes containing 3 mL sterile saline.

Micronucleus analysis

Ten micro liters of buccal mucosal cell suspension was smeared on a microscopic slide, dried in air and fixed with cold methanol: acetic acid (3:1) solution in 0.1M phosphate buffer (pH 7.5) for 20 min. Then the slides were stained by Feulgen reaction essentially by the modified procedure of Belien and co-workers (Belien et al., 1995). In briefly, hydrolysis in 5N HCl for 10 min at room temperature, washing in distilled water for 5 min followed by staining with fresh Schiff's reagent (Sigma Chem, USA) for 90 min, and washing in tap water for 15 min. The cells were counter stained in Coplin jars containing 1% Fast Green reagent for 2-5 min and rinsed with distilled water. Slides were analyzed under light microscope (Leitz, Germany) with 1000 x magnification. A total of minimum 2000 cells per individual were scored for analysis of micronuclei. The slides were randomized and scored by a single observer. Micronuclei were scored in normal cells. In addition, the frequencies of nucle-

Table 1. Demographic characteristics of the groups studied

	Study group	n	Age (yrs) mean ±SD	Average number of cigarettes/ day mean ±SD	Average alcohol intake in last 1 yr (g alcohol drinking/day) mean ±SD	Duration of employment (yrs) mean ±SD
Controls (n=50)	Smokers	11	34.26 ± 2.04	16	-	-
	Non-smokers	10	38.15 ± 1.96	-	-	-
	Smoking with Drinking	16	35.08 ± 1.69	15	74.61 ± 22.07	-
	Smoking with tobacco chewing	13	37.92 ± 1.47	13	-	-
Exposed (n=50)	Smokers	14	35.13 ± 1.04	20	-	19.52 ± 2.03
	Non-smokers	11	36.05 ± 2.31	-	-	16.19 ± 1.39
	Smoking with Drinking	12	35.01 ± 1.20	19	98.12 ± 41.56	15.82 ± 0.61
	Smoking with tobacco chewing	13	34.61 ± 3.95	18	-	13.03 ± 2.84

ar anomalies, namely binucleates, broken eggs, karyolysis were recorded. MN and other nuclear abnormalities were classified according to Tolbert and co-workers. MN must satisfy the following conditions: a) consist of nuclear material; b) be completely separated from the parent nucleus; c) be less than 1/3 of the diameter of associated nuclei; d) be smooth, oval- or roundshaped; e) be on the same plane of focus and f) be of the same color, texture and refraction as the main nucleus. Cells with two nuclei were considered to be binucleate. Besides MN, other NA, such as Binucleates (BN), broken eggs' (nuclei that appeared cinched), karyolysis (dissolution of nucleus) was recorded separately.

Statistical analysis

The samples were coded at the time of preparation and scoring. They were decoded before statistical analysis for comparison. The significance of the differences between control and exposed group means were analyzed using Student's t-test, whereas Pearson's rank correlation analyses were performed to assess the association between end-points and the independent variables. The MNC, BNC, BEC and KLC distributions of individuals, grouped by each of two-class factors, were compared with the Mann-Whitney test. All the calculations were performed using SPSS 11.01 statistical software (SPSS Inc., Chicago, IL).

RESULTS

The characteristics of the subjects used in the study are shown in **Table 1**. The individuals were classified according to their age, length of occupation, smoking, tobacco chewing, and alcohol drinking habits.

Results on micronuclei frequency and nuclear abnormalities are given in **Table 2**. Assessment of MN frequencies in exfoliated buccal cells revealed a significant difference ($P < 0.05$) between exposed workers with smoking (9.03±1.45) and controls with smoking habit (4.58±1.05). Smoking also had a marked effect on MN frequency among unexposed control group (4.58 vs. 1.26 between smokers and non-smokers, respectively). The average MN frequency in the exposed non-smokers was 5.02±1.56, and in the control non-smokers was 1.26±0.54 ($P < 0.05$). The average micronucleus frequencies were 9.98 and 5.81±1.07 between exposed smoking with drinking habit and unexposed, respectively (**Figure 1**).

Like MN, A significant difference ($P < 0.05$) in other NA was more prevalent in pesticide workers compared with that of controls (**Figure 2** and **Figure 3**). Among the three NA, karyolysis was predominant in smokers with chewing habit followed by smokers with drinking habit (**Figure 4**). Burgaz et al. (1995) reported a significant increase of micronucleated cells ($P < 0.001$) in smokers, as compared to non-smokers. Similarly our study showed synergistic effect between habitual usage and occupational exposure. We observed a significant increase in the frequency of micronuclei formation and nuclear changes in buccal epithelial cells. Individuals of the exposed as well as control groups with smoking habit and chewing habit showed an enhanced frequency of micronuclei in comparison to controls.

Table 2. Micronuclei and other NA frequencies in exfoliated buccal epithelial cells of control and exposed pesticide workers

	Study group	n	MN (mean ±SD)	BNC (mean ±SD)	BEC (mean ±SD)	KLC (mean ±SD)
Controls (n=50)	Smokers	11	4.58 ± 1.05	5.24 ± 1.68	5.94 ± 2.90	3.91 ± 0.74
	Non-smokers	10	1.26 ± 0.54	2.06 ± 1.05	3.38 ± 1.03	1.05 ± 1.02
	Smoking with Drinking	16	5.81 ± 1.07	7.93 ± 1.98	6.72 ± 2.27	3.98 ± 1.64
	Smoking with tobacco chewing	13	6.84 ± 1.96	8.03 ± 1.06	7.93 ± 2.84	4.38 ± 1.87
Exposed (n=50)	Smokers	14	9.03 ± 1.45*	10.19 ± 1.63*	14.26 ± 3.87*	8.32 ± 2.07*
	Non-smokers	11	5.02 ± 1.56*	4.91 ± 1.28*	10.07 ± 1.91*	4.19 ± 1.21*
	Smoking with Drinking	12	9.98 ± 1.64*	11.42 ± 1.10*	16.29 ± 3.83*	8.79 ± 1.92*
	Smoking with tobacco chewing	13	10.94 ± 1.47*	13.98 ± 1.82*	17.07 ± 3.56*	10.06 ± 1.15*

*MN=cells with micronuclei; BNC=binucleated cells; BEC= broken egg cells; KLC= karyolytic cells; *Significantly different with their respective controls, P < 0.05.*

Discussion

Exposure to environmental agents and byproducts of cellular metabolism results in DNA damage, which if left unrepaired, could lead to the process of carcinogenesis. The molecular epidemiologic studies have shown that cancer is mediated by both genetic and environmental factors that play a role in the individual's susceptibility to carcinogenesis. The development of cancer is not only due to endogenous or exogenous carcinogens, but also to their interactions with genes that are also involved in the detoxification of these carcinogens.

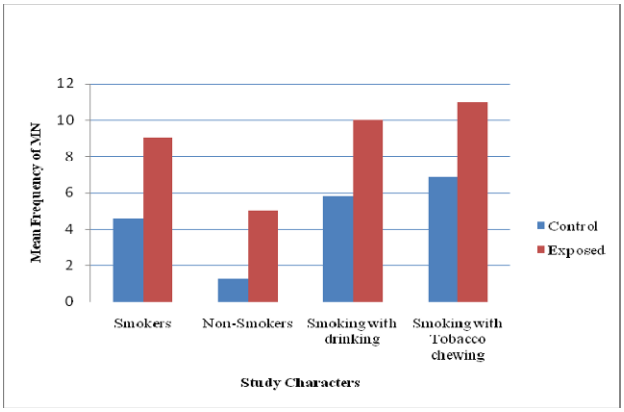

Figure 1. Mean frequency of MN among control and exposed pesticide workers

The molecular epidemiologic studies have shown that cancer is mediated by both genetic and environmental factors that play a role in the individual's susceptibility to carcinogenesis. Genetic variation in the ability to repair DNA lesions induced by smoking tobacco or environmental carcinogens may contribute to the inter-individual variation in cancer. Humans are exposed to a large number of physical or chemical agents, which can cause a variety of health hazards. Majority of human cancers are known to arise as a direct consequence of environmental exposure to mutagenic and carcinogenic agents, mainly through diet, habit, and occupation.

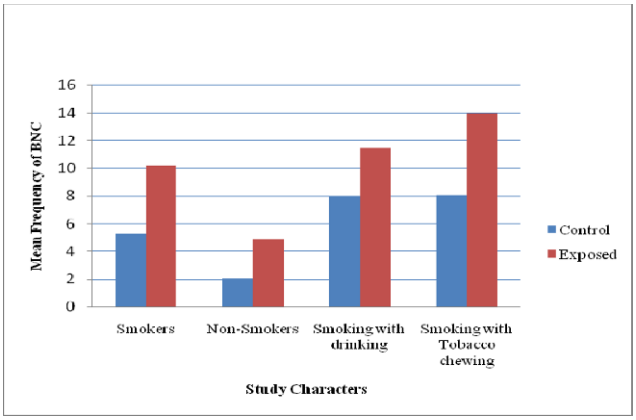

Figure 2. Mean frequency of BNC among control and exposed pesticide workers

Excesses in risk have been found in most (Aronson et al., 1996; Fleming et al., 1999; Alavanja et al., 2003; Mills and Yang, 2003; Settimi et al., 2003; Ritchie et al., 2003), but not all (Van Der Gulden and Vogelzang, 1996) studies investigat-

ing the relation between exposure to pesticides and prostate cancer. Some other studies have reported excess risk among farmers or herbicide applicators. Yet, farmers perform a wide variety of tasks, and are therefore exposed to numerous potential carcinogenic substances like solvents, fuels and oils, pesticides, and more.

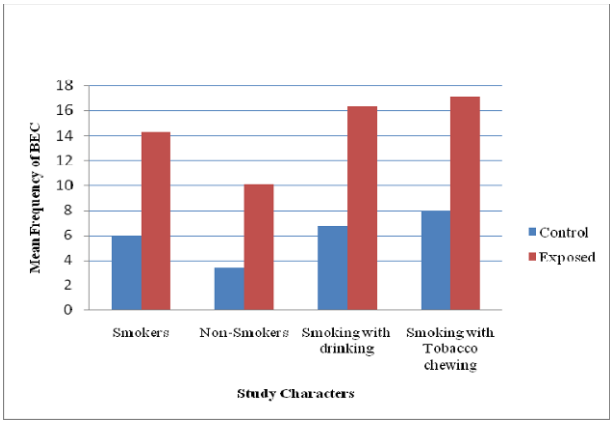

Figure 3. Mean frequency of BEC among control and exposed pesticide workers

Exposure to pesticides has been associated with an increase in the incidence of non-Hodgkin's lymphoma (Hardell and Eriksson, 1999; Zheng et al., 2001), multiple myeloma (Khuder and Mutgi, 1997), soft tissue sarcoma (Kogevinas et al., 1995), lung sarcoma (Blair et al., 1993), pancreatic, stomach, liver, bladder and gall bladder cancer (Ji et al., 2001; Shukla et al., 2001), Parkison's disease (Gauthier et al., 2001) and reproductive outcomes (Arbuckle et al., 2001), among others. Regarding pesticide exposure, many reports dealing with chromosomal aberrations (Au et al., 1999; Zeljezic et al., 2001), sister chromatid exchange (Shaham et al., 2001; Zeljezic et al., 2002), micronuclei (Falck et al., 1999; Pastor et al., 2003) and Comet cells (Grover et al., 2003) found significant increases in these biomarkers, providing suggestive evidence of genotoxic effects induced by pesticides. Dittberner et al. (1997) reported alcohol use can increase the number of micronuclei. Piyathilake et al. (1995) and Sarto et al. (1997) have reported smoking also increase the MN frequency in buccal cells.

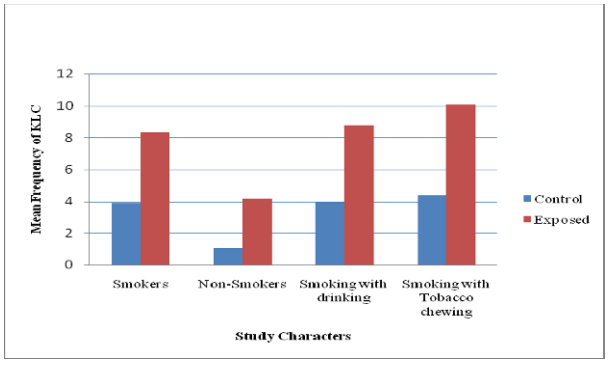

Figure 4. Mean frequency of KLC among control and exposed pesticide workers

These results show that individuals with smoking, drinking, tobacco chewing and pesticide exposure to have significantly higher frequencies of MN induction, indicative of cytogenetic damage in these individuals. Our earlier study provides an evidence of MN and other nuclear abnormalities in the buccal mucosa cells of automobile mechanics and spray painters exposed to PAHs (RafiqKhan and Sudha, 2012; Mohammed RafiqKhan and Sudha, 2013). The use of the micronuclei test (MNT) to detect and quantify the genotoxic action of carcinogenics is well established in several systems, either *in vitro* or *in vivo*, its sensitivity being compared to the analysis of chromatid breaks and exchanges (Kliesch and Adler, 1980). This test presents great advantages over other techniques, not requiring cell culture nor metaphase preparations, it is applicable on interphase cells, is a good indicator of chromosome mutations, is not invasive and has a low cost (Calvert et al., 1998; Titenko-Holland et al., 1998). When humans interact with chemicals in their workplaces or natural environments, it may result in both detoxification or activation processes, which ultimately can lead to an interaction with hemoglobin, proteins, DNA, or normal cells and the formation biological end products that may result in genotoxic effects if no biological repair is done. Several factors including the host, agent, and the environment influence how the disease interaction will progress. Our study showed that smoking status affected genetic damage in both groups studied, but a significant association emerged only among exposed workers. In our study, smoking, tobacco chewing, and alcohol drinking is associated with a significant induction of MN and other NA among exposed pesticide workers. This shows synergistic effect between habitual usage and occupational exposure.

Conclusion

Correlations among biomarkers, an important issue in biomarker research, provides enhanced insight and understanding of the complexity encountered on a molecular level during exposure to genotoxic agents. Explorations of correlations between biomarkers will contribute to the development of human monitoring to genotoxic exposures and will help to select optimal biomarkers for more efficient monitoring of various human exposures. MN and other NA, such as binucleates, karyorrhexis and karyolysis are also useful indices of chemical exposure and toxic response. Stages of biomonitoring exist such that within this progression from exposure to disease, systems can be developed to detect any adverse changes.

The present investigation suggests that pesticide workers under their particular conditions of exposure revealed clear evidence of genotoxicity in exfoliated buccal cell when evaluated by MN test. This shows synergistic effect between

habitual usage and occupational exposure. Besides elevated MN frequency, exposed individuals also exhibited raised prevalence of other NAs like BNC, BEC and KLC. These abnormalities occur at an elevated level in response to cellular injury. These workers may not be aware that they have been exposed to genotoxic agents nor do they know the type and amount of agent to which they have been exposed. Therefore, there is a need to educate those who work with chemicals, about the potential occupational hazards and the importance of using protective measures. The rate of occupational diseases and injuries are very high in India, and thousands of workers are routinely exposed to various chemicals, there is currently little or no survey data due to lack of proper reporting or monitoring procedures. Nonetheless, it is important to create better awareness of occupational hazards among workers to promote occupational safety. The obtained information can be used as an early warning about the potential risk of health problems' developing in the long run, also more comprehensive, larger-scale study is needed to further explore the effects of gene-environment interactions.

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Abbreviations

BCs: Buccal cells; BEC: Broken egg cells; BNC: Binucleate cells; KLC: Karyolytic cells; MN: Micronucleus; MNT: Micronucleus test; NA: Nuclear abnormalities; PAHs: Polycyclic aromatic hydrocarbons; WHO: World health organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors read and approved the final manuscript. MRK designed the study, GTK and RK contributed to the study design, literature review and analyzed the data. SNS, UDP and YRR participated in the designing of the study. MRK prepared the manuscript in cooperation with all the other authors.

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