

Original Research Article

The Use of Micronucleus Assay as A Measure of DNA Damage in Occupationally and Non-Occupationally Chromium Exposed Population

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ABSTRACT

Introduction and Aims: Trivalent chromium used in tanning industry has potential adverse effects in term of DNA damage on occupationally and non-occupationally exposed population. The present study aimed to emphasize the use of micronucleus assay as an ideal and rapid parameter for the estimation of DNA damage in chromium exposed population and to establish the association between micronucleus frequency and non-occupational factors such as smoking and alcohol consumption habits.

Materials and Methods: The study involved a total of 300 male subjects, including 100 male tanners (Occupationally exposed group), 100 male populations living near tanning sites (Non-occupationally exposed group) and 100 control males. Baseline characteristics were recorded for all the subjects. Blood Cr level was estimated by atomic absorption spectrophotometry. DNA damage was measured by micronucleus assay in term of micronucleus frequency. Effects of non-occupational factors on micronucleus frequency were estimated using univariate analysis.

Results: As a result, chromium level was significantly ($p < 0.0001$) higher in both the exposed groups, than in controls. Similarly, both exposed groups revealed a significant ($p < 0.001$) increase in micronucleus frequency, when compared with controls. Among both the exposed group, micronucleus frequency was significantly ($p < 0.05$) higher in occupationally exposed subjects as compared to non-occupationally exposed subjects. No significant effect ($p < 0.05$) of non-occupational factors were observed on micronucleus frequency.

Conclusion: The present study concludes the importance of micronucleus assay for the evaluation of DNA damage in occupationally and non-occupationally chromium exposed population. Further, our study also concludes that occupational exposure of chromium was associated with higher risk of DNA damage as compared to non-occupational exposure.

Keywords: Chromium, Occupational exposure, Non-occupational exposure, DNA damage, Micronucleus assay.

INTRODUCTION

Environmental toxic metals cause a great deal of ill health in the populations of most industrial areas and many of these a result of particularly tanning industries, which utilize trivalent chromium (Cr) as a major tanning agent. [1] Tannery workers as well as surrounding population near tanning industrial site are occupationally and non-occupationally exposed with a variable

concentration of trivalent Cr that causes moderate as well as severe adverse health effects in term of dermatitis, ulcers, stomach disorder, lung cancer, nasal cancer, respiratory illnesses, nasal sinuses, asthma as well as allergic bronchitis. [2,3] According to Hilali et al. (2008), long term professional exposure of trivalent Cr have been reported to induces several type of

cancer in human body organs including liver, lungs and kidney. [4]

DNA damage is one of the potential serious adverse health effects related with Cr, which consequently led to the development of various type of cancer. The relevance of an increased frequency of DNA damage as cancer risk is supported by the study of Bonassi et al. (2005), suggesting that a high frequency of DNA damage is predictive of an increased risk of cancer. [5]

Trivalent Cr has been found to interact with cell components such as DNA, causing DNA damage and conformational change, which further associated with severe health hazards. [6] Ambreen et al. (2012) reported deleterious effects of trivalent Cr on cells, in term of DNA strand break. [1] Balachandar et al. (2010), has also addressed the issue of chromosomal aberrations in tannery workers. [7] Many recent studies [8,9] also indicate positive genotoxic effects of Cr on exposed tannery workers.

In India, tannery workers and surrounding population near the tanning industry are at increased risk of adverse health effects of trivalent Cr. Early recognition of toxicity induced by Cr is very important to arrest its severe side effects, prevent cancer and ultimately, may be helpful to enhance environmental pollution prevention programs. The majority of the health problem encountered by Cr exposure is related with DNA damaging effect. [10]

In recent years, as increasing awareness of public, for removing of hazardous chemicals from the environment, there is a need of rapid and reliable method that may detect DNA damage in a short time. Therefore, much interest has been exhibited in the use of micronucleus assay, a useful and rapid biomarker for the estimation of genotoxic effects in populations exposed to genotoxicants. Micronucleus, a small microscopically visible, round or oval nucleus, which is originated from acentric chromosome fragments or whole lagging chromosomes that are not included in the main daughter

nuclei during metaphase or anaphase phase of cell division (Figure.1). A rise in the number of micronucleus in exposed cells with toxicants indicates increase frequency of DNA damage. [11]

To the best of our knowledge, in the literature, there is no previous study which differentiate occupationally exposed and non-occupationally exposed population, with respect to micronucleus formation induced by Cr exposure, using micronucleus assay. Therefore, the present study aimed to estimate the frequencies of micronuclei using micronucleus assay, as an index of genotoxic effects of trivalent Cr on occupationally exposed tannery workers as well as non-occupationally exposed surrounding population near the tanning industry and further, to establish the association between micronucleus frequency and non-occupational factors such as smoking and alcohol consumption habits.

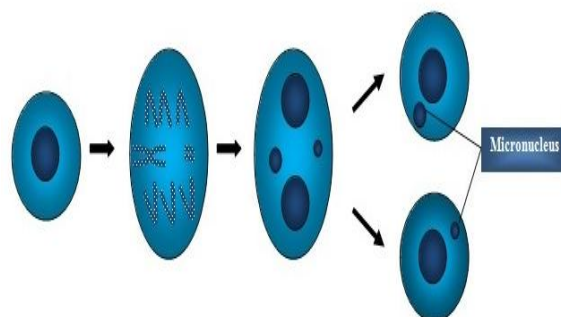


Figure: 1. Micronucleus formation through lagging chromosome or acentric chromosome fragment at anaphase stage of cell division.

MATERIALS AND METHODS

Sample collection and experimental design

The study involved a total of 300 male subjects, which were further divided into three subgroups including 100 male tannery workers (Occupationally exposed group), 100 male population living near tanning sites (Non-occupationally exposed group) and 100 control males (Unexposed group). The age range of our study subjects were 15–65 years. 2 ml of blood sample was collected in heparinized tubes from each subject in accordance with the Guidelines of Ethical and Bio-safety Committee of

University of Lucknow. Exposed subjects were recruited visiting Jajmau, Kanpur, India. In occupationally exposed group, all the tannery workers were directly involved in Cr tanning process. In non-occupationally exposed group, male population living near tanning industry were indirectly exposed with trivalent Cr. The general population with no history of Cr exposure and no history of radiation treatment were considered as controls. The exposed subjects, who had a history of exposure to any kind of radiation treatment, were excluded in this study.

Before the collection of blood sample, informed consents were taken from the whole participant and they were informed of the objective of our study. Also, individual questionnaire was filled with respect to baseline characteristics such as age, marital status, smoking, alcohol consumption habits and duration of exposure.

Blood Cr estimation

The level of blood Cr was measured according to the method of Granadillo et al. (1994) with slight modifications. [12] 1 ml of whole blood was digested with 5 ml, 3 ml and 2 ml of mixture of HNO₃: HClO₄ (in ratio of 5:1) respectively on hot plate in STI-FUME Hood. The digested sample was dissolved in 5 ml of Distilled water and filtered with Whatman paper to obtain clear solution. Finally Cr concentration was determined using atomic absorption spectrophotometry (AAS) with a graphite furnace and Zeeman Effect background correction (Perkin-Elmer Model 5100PC). Standard curves were included in each run, obtained by adding known amounts of Cr standard solution to a control biological sample. No matrix modifier was used. Cr concentration was expressed as µg/l.

Micronucleus estimation

Micronucleus estimation was done using micronucleus assay by the method of Rajeswari et al. (2000) with slight modifications. [13] 5 µl drop of whole blood was used to perform micronucleus assay. Thin smear of blood was prepared on pre-

cleaned slide with the help of glass slide. These slides were then air-dried overnight in a dust and moisture free environment at room temperature and fixed by dipping it in absolute methanol for 5-10 min. Again, slides were air dried for at least 1 hour. These slides were further allowed to stain in 6-10% Giemsa stain in phosphate buffer for 30 min. After staining, these slides were washed with Double Distilled water and tried to remove all the Giemsa particles carefully by washing. Slides were again air dried for overnight. Slides were made permanent with DPX-mountant and drying it over hot plate at 60 °C for overnight. Observed the slide under microscope using 40/100 X objective lenses and scored the micro nucleated cells. For each subject 500 cells were calculated for determining the frequency of micronucleated cells.

The following criteria were used for the selection of micronucleus: [14] i. The diameter of the micronucleus should be less than one-third of the main nucleus. ii. Micronucleus should be clearly separated from the main nucleus. iii. Micronucleus must have a smooth oval or round shape. iv. It should have similar staining as the main nucleus.

Statistical analysis: The entire categorical variables were expressed in term of percentage. Mean and standard deviation (SD) were calculated for each continuous variable. ANNOVA test was used to compare continuous variables. Similarly, categorical variables were compared by using chi-square (χ^2) test. The unpaired t test was used to compare two mean values. The univariate analysis of variance was used to find out the effect of non-occupational factors including smoking and alcohol consumption habits on micronucleus formation. For all statistical analysis, the value of $p < 0.05$ was used to determine significance level. Joint effect of smoking and alcohol consumption habit on micronucleus frequency was also analysed using multivariate linear regression.

RESULTS

Demographic characteristics

The distribution of subjects with respect to age, marital status, alcohol consumption habit, smoking habit and duration of exposure are listed in Table I. Controls and both the exposed groups

showed a significant ($p < 0.05$) difference only with respect to smoking habit. We also observed a significant ($p = 0.001$) difference with respect to duration of exposure, when we compared between occupationally and non-occupationally exposed groups.

Table 1: The baseline characteristics of Subjects (Controls and Exposed groups)

Characteristics	Controls (n=100)	Occupationally exposed (n=100)	Non-Occupationally exposed (n=100)	p-value
Age	37.12±12.28	38.44±6.83	35.09±12.79	0.09 ¹
Marital Status				
Married (%)	61 (61.0)	67 (67.0)	63 (63.0)	
Single (%)	39 (39.0)	33 (33.0)	37 (37.0)	0.66 ²
Smoker				
Yes (%)	37 (37.7)	59 (59.0)	51 (51.0)	
No (%)	63 (63.0)	41 (41.0)	49 (49.0)	0.007 ^{2*}
Alcohol				
Yes (%)	19	23	26	
No (%)	81	77	74	0.49 ²
Duration of Exposure	-	20.56±5.65	25.42±12.66	0.001 ^{3*}

¹ANOVA, ²Chi-square, ³Unpaired t-test, * for $p < 0.05$.

Occupationally exposed group- Tanners; Non-occupationally exposed group- Population living near tanning industry.

Cr concentration in whole blood of controls and exposed subjects

Both the exposed groups including occupationally as well as non-occupationally, showed significant ($p < 0.0001$) higher Cr concentration (occupationally exposed group- $167.58 \pm 23.44 \mu\text{g/ml}$; non-occupationally exposed group- $143.99 \pm 25.61 \mu\text{g/ml}$), as compared to controls ($22.09 \pm 3.78 \mu\text{g/ml}$). Further, In our study, when we compared among both the exposed groups with respect to Cr concentration, we observed that the increment of Cr concentration was significantly ($p < 0.05$) higher in occupationally exposed group as compared to non-occupationally exposed group. Results are illustrated in Figure 2.

Micronucleus frequency

All the exposed groups revealed a significant increase ($p < 0.001$) in micronucleus frequency, (occupationally exposed group - 6.73 ± 2.02 ; non-occupationally exposed group - 5.02 ± 2.60), when compared with controls (1.94 ± 1.75). As shown in Figure 3. Among both the exposed groups, significantly ($p < 0.05$) higher frequency of micronucleus was observed in occupationally exposed group as compared to non-occupationally exposed group.

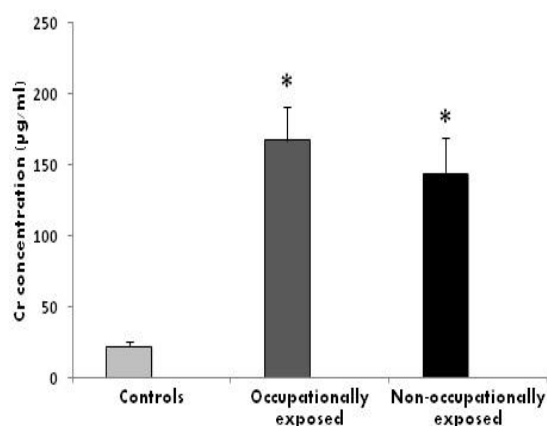


Figure 2. Cr concentration in whole blood of controls (unexposed group- 100), occupationally exposed group (tanners- 100), non-occupationally exposed group (population living near tanning industry- 100). Each experiment was done in duplicate. Data were represented as means ± SD. * for $p < 0.05$.

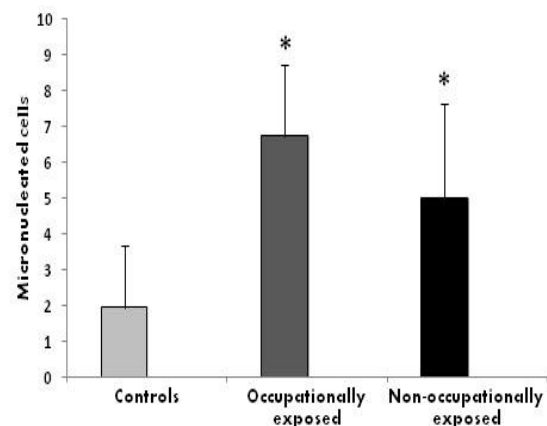


Figure 3. Micronucleus frequency in controls (unexposed group- 100), occupationally exposed group (tanners- 100), and non-occupationally exposed group (population living near tanning industry- 100). Each experiment was done in duplicate. Data were represented as means ± SD. * for $p < 0.05$.

Association between micronucleus frequency and non-occupational factors (smoking and alcohol consumption habits) We observed no significant ($p > 0.05$) effect of smoking and alcohol consumption habits on the frequency of micronucleus in

controls as well as both the exposed groups (Table 2). In our study, we also analysed joint effects of smoking and alcohol consumption habits on the frequency of micronucleus. However the result was statistically insignificant (Table 3).

Table 2: Effects of smoking and alcohol consumption habits on micronucleus frequency in controls and exposed groups, using univariate analysis of variance.

Parameters	Controls (n=100)	Occupationally exposed (n=100)	Non-occupationally exposed (n=100)	p-value
Micronucleated cells				
Smoker	2.10±2.01	6.89±1.85	4.80±2.52	0.79
Non-smoker	1.84±1.59	6.48±2.25	5.24±2.68	
Alcoholic	1.89±1.41	6.43±2.12	5.26±2.58	0.88
Non-alcoholic	1.95±1.83	6.81±2.00	4.93±2.62	

Table 3: Joint effect of smoking and alcohol consumption habit on micronucleus frequency in both exposed groups using multivariate linear regression analysis.

Parameters	Occupationally exposed (n=100)		Non-occupationally exposed (n=100)	
	Beta coefficient, p-value		Beta coefficient, p-value	
	Smoker	Alcoholic	Smoker	Alcoholic
Micronucleated cells	0.45, 0.27	-0.44, 0.36	-0.46, 0.37	0.37, 0.52

DISCUSSION

Exposure to hazardous metals near the industrial areas plays an imperative role in the progression of severe toxic health effects on human population. Among the hazardous metals, trivalent Cr is widely used in tanning industry throughout the world, and has the potential to cause serious health hazards on tannery workers as well as surrounding population near the tanning industry. [15]

A number of studies have been designed to evaluate the potential toxic effect of Cr in term of genotoxicity and in most of the studies, comet assay was proposed as a biomarker for the measurement of Cr induced genetic effects. However, this assay is considered as a time consuming method. [8] Therefore, during few years, a need for method that are less time consuming has arisen, in order to obtain early diagnosis of DNA damage as well as to minimize the toxic health hazards of Cr.

Consequently, micronucleus assay has been receiving much attention as a simple and rapid method for the estimation of genotoxicity. [16] Our study particularly highlights the importance of micronucleus assay for the evaluation of DNA damage in occupationally and non-occupationally Cr

exposed population. The finding of our study may be used as an early warning for those populations, who are at high risk of Cr exposure.

In our study, we observed that Cr concentration was significantly higher in both the occupationally and non-occupationally exposed groups, as compared to controls. Similar to our study, Were et al. (2014) reported higher level of Cr in urine sample of exposed tannery workers, compared to those in the control group. [2] Ambreen et al. (2014) reported increased blood Cr concentration in exposed population than in unexposed individuals. [1] Goulart et al. (2005) also measured total plasma and urine Cr level in occupationally exposed tannery workers and found approximately twofold increase in plasma and urine Cr concentration in these exposed tannery workers in comparison with controls. [17] Significantly higher blood Cr concentration was observed in exposed individuals as compared to controls, by other previous studies. [3, 18] In our study, we also observed that among both the exposed groups, Cr concentration was significantly higher in occupationally exposed group as compared to non-occupationally exposed group.

In our study, formation of micronuclei was used to identify DNA damage in controls as well as both the exposed groups. Similarly, in other previous studies, [11,18,19] micronucleus assay was used as an important measure of DNA damage. According to Volders et al. (2014) the appearance of micronucleus using micronucleus assay is associated with increased risk of DNA damage. [20]

As a result of our experimental analysis, the percentage of micronuclei was found to be significantly higher in exposed individuals as compared to controls. We also observed that occupationally exposed group, which was directly involved in chrome tanning process, showed significantly enhanced frequency of micronucleus, when compared with non-occupationally exposed group (Indirectly associated with Cr exposure), Which clearly indicate that the direct exposure of Cr is associated with higher risk of DNA damage as compared to indirect exposure. However, in the literature, no previous study demonstrates the frequency of micronuclei between occupationally and non-occupationally exposed populations. Amaral et al. (2015) utilized micronucleus assay as a measure of DNA damage and found increased prevalence of micronucleus in exposed tannery population as compared to unexposed population. [21] Sellapa et al. (2011) also reported increased frequency of micronucleus in tannery workers than in controls. [9] Similarly, other previous studies, [4,7,16,22] also revealed increased incidence of micronucleus in exposed subjects.

In our study, we found no significant effect of smoking and alcohol consumption habits on the frequency of micronucleus. Amaral et al. (2015) also observed no significant difference between smoker and non-smoker or between alcohol taker and non alcohol taker with respect to micronucleus. [21] Similarly, Danadevi et al. (2004) reported no significant effect of smoking and alcohol consumption habits on DNA damage in Cr-exposed population. [18]

Smoking and drinking habits, did not represent significant factors in terms of increasing the production of micronuclei, when the controls and the exposed groups were compared. [23] However, Sellapa et al. (2011) showed positive relation between smoking and genotoxicity. [9] Similarly, Fenech et al. (2011) reported that exposed individual with high smoker had statistically greater micronuclei compared to non-smokers. [14] Significant effect of alcohol consumption habit on the incidence of micronucleus frequency was also observed in other previous studies. [24, 25]

CONCLUSION

Our results conclude that micronucleus frequency was significantly higher in occupationally and non-occupationally Cr exposed population as compared to unexposed population. Also, occupationally exposed population was at higher risk of DNA damage as compared to non-occupationally exposed population. Further, our results highlight the importance of micronucleus assay as an ideal and rapid parameter to measure DNA damage in Cr exposed population.

This is the first reported study, which differentiates occupationally and non-occupationally exposed population in term of micronucleus frequency using micronucleus assay as a reliable parameter for the estimation of Cr induced DNA damage. However, further study is also needed to establish reliable and rapid biomarker for the early identification of genotoxic effect of Cr, which may be used as an early warning about the potential risk of health problem in exposed population and ultimately may be imperative to reduce the health hazardous effect of Cr.

ACKNOWLEDGEMENT

The authors thank Dr. Rajeev Kacker, Chaudhary Ehsan Kareem Hospital, Jajmau, Kanpur, for providing blood sample of tannery workers as well as surrounding population near tanning industry and Dr Kr. P. Singh (Scientist), Indian Institute of Toxicology Research for total

Cr estimation by atomic absorption spectrophotometry.

This research was supported by University Grant Commission (UGC F.No. 37-435/2009-SR), New Delhi, India.

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How to cite this article: Ambreen K, Kumar S. The use of micronucleus assay as a measure of DNA damage in occupationally and non-occupationally chromium exposed population. *Int J Health Sci Res.* 2017; 7(4):388-395.
