RESEARCH COMMUNICATION

Evaluation of DNA Damage Induction and Repair Inhibition in Welders Exposed to Hexavalent Chromium

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Abstract

The soluble hexavalent chromium Cr (VI) used in industrial welding is an environmental contaminant widely recognized to act as a carcinogen, mutagen and teratogen towards humans and animals. The carcinogenic potential of metals is a major issue in defining human health risk from exposure. In the present investigation, 93 welders and 60control subjects with similar mean ages, smoking prevalences and alcohol consumption were enrolled for DNA damage analysis in blood leucocytes by Micronucleus assay (MN) and the Comet assay. DNA repair inhibition was also analyzed by assessing XPD gene polymorphism. Welders showed a significant increase in micronucleated cells compared to controls with respect to their smoking habits and alcohol consumption, age and years of exposure (P<0.05). Results indicated that the welders had a larger mean comet tail length than that of the controls (P<0.05). The current study suggested that chronic occupational exposure to Cr (VI) during welding could lead to increased levels of DNA damage and repair inhibition.

Key Words: Hexavalent chromium - genotoxicity - micronucleus assay - comet assay - XPD gene polymorphism

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Introduction

Chromium is a widely used industrial metal to which millions of workers are exposed worldwide in industries, such as pigment production, chrome plating, leather tanning, stainless steel production and welding. The heaviest metal exposure occurs in the workplace among occupationally exposed groups (Danadevi et al., 2004). A person spends, on average, one-third of his life at his workplace and therefore the environment in which he works can be a major factor in determining health (Kawanishi, 1995).

Welders in India are inclined to possible occupational Cr exposure. Welders are estimated to receive some of the highest acute exposures to hexavalent chromium in welding fumes (Langard, 1990; Hartwig et al., 2002). Hexavalent chromium can easily pass the cell membranes, and it is reduced inside the cells to its trivalent form. Trivalent chromium, intermediates like Cr (V) and Cr (IV) and radicals are suspected to react with DNA and cause DNA damage (De Flora et al., 1989; Karahalil et al., 1999; Shi et al., 1999). Many metals have the potential to cause genetic alterations in the target tissues of exposed humans. Such alterations, if they occur in tumor suppressor genes, may lead to the development of cancer in the target organs (Kassie et al., 2000).

Cr and Cr compounds have been tested for genotoxicity in a variety of short-term tests using different end-points (De Flora et al., 1990; Simone et al., 2002; O'Brien et al., 2003; Kusal Das et al., 2009). Moreover, there are reports on positive genotoxic effects in populations exposed to Cr (Stich et al., 1982; Choi et al., 1987; Tice et al., 1995; Benova et al., 2002; Quievryn et al., 2003). Workers occupationally exposed to Cr are considered to be at an elevated risk for developing cancer (Pool et al., 1994; Keshava et al., 1999; De Flora et al., 2000; Gibb et al., 2000). However, the bulk of the literature data indicated that only Cr (VI) may pose a carcinogenic risk, and only when inhaled at very high doses in the three occupational settings indicated by the International Agency for Research on Cancer (1990).

Some of the biomonitoring studies carried out have shown negative results (Gao et al., 1994; Zhitkovich et al., 1996; Huvinen et al., 2002). The inconsistent genotoxicity data could be due to differences in levels of exposure, in the protective measures employed or in the end-points utilized. Therefore, results from one investigation in a specific occupational setting cannot be used to judge the genotoxic potential in another occupational setting. Hence, there is a need to evaluate different populations and to analyze different genotoxic parameters (Danadevi et al., 2004).

The present study aimed to investigate the genotoxic effects associated with occupational exposure to Cr (VI) on South Indian welders using the Comet assay and the Micronucleus test (MN test). The comet assay has been found to be a very sensitive method for measuring DNA damage (Schmid, 1975; Snow et al., 1989). It is a quick,

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reliable and fairly inexpensive way of measuring DNA damage. It has a further advantage that the observations are made at the single cell level. Moreover, it is an invaluable tool for investigating DNA damage in human populations (Collins et al., 1997). To substantiate our results and to provide a cytogenetic parameter, the MN test was also carried out. This test allows the detection of both clastogenic and aneugenic agents (Salama et al., 1999). The influence of confounding factors like age, smoking, alcohol drinking, duration of exposure on the differences in DNA damage was also analyzed.

Although it is well established that tobacco smoking causes lung cancer, cancer at other sites and several other adverse health effects; less information is available concerning the outcome of combined exposure to cigarette smoke (CS), and other agents (Hedenstedt et al., 1977; Environmental Health Criteria 211, 1999).

An increase in MN and chromosomal abnormalities has been observed in alcoholics (Castelli et al., 1999; Kirsch et al., 2002). The present study analysed the synergistic genotoxic effect of smoking and alcohol consumption among welders.

Micronuclei induction reflects clastogenic and/or aneugenic events. The major pathway eliminating DNA base damage and helix distortion is the excision repair pathway, subdivided into nucleotide excision repair (NER) and base excision repair (BER). If the mutagenic potentials of metal compounds are rather weak, in contrast, they exert pronounced comutagenic effects, which may be explained by the inhibition of different DNA repair systems (Hartwig 1998). Among the repair proteins, the XPD protein is interesting because it is a major player in the nucleotide excision repair pathway and is also involved in transcription initiation and in the control of the cell cycle and apoptosis.

Mutations in the XPD gene can diminish the activity of transcription complexes giving rise to repair defects, transcription defects, abnormal responses to apoptosis and probably, hormonal dysfunctions. The XPD protein is a 5' 3' helicase with a molecular weight of 86.9 kDa and comprising 761 amino acids. (Sarto et al., 1990). The genomic DNA of the XPD gene comprising 23 exons spans ~54.3 kb at 19q13.3 and almost 100 mutations have been mapped in this gene (Hengstler et al., 2002). Most of them are point mutations, indicating that the presence of a full-length XPD protein is necessary for life (Coin et al., 1998).

The present study aimed also to analyze the incidence of polymorphisms in XPD DNA repair gene and thereby DNA damage evaluation by complementary genotoxic endpoints (MN assay and Comet assay) among South Indian welders.

Materials and Methods

Selection of subjects and collection of specimens

A total of 153 individuals (93 experimentals exposed to welding fumes and 60 controls) were analyzed in this study. All of them were employed in welding plants located at Coimbatore, South India. The welders had varying durations of exposure (5-15 years) and they were in the age group 25-46 years. All the welders were engaged in shielded manual metal arc welding. Welders were working with consumable stainless steel electrodes usually containing 20% chromium. The experimental group was further branched as smokers (53), non-smokers (40). The control group was selected from the general population with no history of occupational exposure to welding fumes or any known physical or chemical agent in the workplace, but belonged to the same age group and socio-economic status as the welders. The selection criteria for the subjects were based on a questionnaire. The questionnaire covered standard demographic questions (age, genetic disorders, number of X-ray diagnoses, vaccinations, medication, smoking, alcohol, etc.) and occupational questions (years of exposure). We ensured that the welders and the controls did not markedly differ from each other except for occupational exposure. We also ensured that all the subjects had not been taking any medicines nor had they been exposed to any kind of radiation for 12 months before sampling. The subjects who smoked >5 cigarettes/day at least for 1 year were considered as smokers and those who consumed >120gm of alcohol/day were considered as alcoholics in both groups. All subjects were informed of the objective of the study and gave their consent. The institutional ethical committee approved the research procedures used in this study.

Venous blood (3ml) was collected from all subjects using heparinized syringes. The samples were transported on ice to the laboratory and were processed. Micronucleus assay and Comet assay were performed using the collected blood samples and the DNA isolated from the sample was used for gene polymorphism analysis.

Genes and laboratory analysis

Micronucleus Assay: Leukocyte cultures were set up by adding 0.5 ml whole blood to 4.5 ml RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1% antibiotics (penicillin and streptomycin) and L-glutamine (all obtained from Gibco). Leukocytes were stimulated by 1% phytohaemagglutinin (PHA; Gibco) and incubated for 72 h at 37°C. A final concentration of 6 µg/ml cytochalasin B (Sigma) was added to the cultures 44 h later to arrest cytokinesis (Singh et al., 1988). At 72 h of incubation, the cultures were harvested by centrifugation at 1000 rpm for 8 min and treated with a hypotonic solution (2–3 min in 0.075 M KCl at 4°C). Cells were centrifuged thereafter and a 3:1 (v/v) methanol: acetic acid solution was gently added. This fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative solution and dropped onto clean slides. Finally the slides were stained with Acridine orange (Merck) analyzed under a fluorescent microscope and scored.

Comet assay: An aliquot of 40 µl of whole blood was used to quantitate basal DNA damage. Cell viability determined by the trypan blue exclusion technique (Maxild et al., 1978) ranged from 90 to 95% (data not shown). Slides were prepared in duplicate per person according to Singh et al (1988).

The cell suspension was centrifuged, the pellet obtained was mixed with 0.7% low melting agarose (LMA) and placed on fully frosted roughened slides

previously coated with 1% normal melting point agarose. To the solidified agarose, a third layer of 0.1% LMA was applied and were immersed in freshly prepared ice cold lysis solution for 1 hour. The slides were then electrophoresed, neutralized, dried and stained with ethidium bromide.

A total of 100 randomly captured comets from each slide were examined at 400X magnification using an epifluorescence microscope (Zeiss) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd, UK). A computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components and then evaluates the range of derived parameters. To quantify the DNA damage tail length (TL) and tail moment (TM) were evaluated. Tail length (length of DNA migration) is related directly to the DNA fragment size and presented in micrometers. It was calculated from the centre of the cell. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail.

An undamaged cell resembles an intact nucleus without a tail and a damaged cell has the appearance of a comet. The length of the DNA migrated in the comet tail, which is an estimate of DNA damage, was measured.

Restriction Fragment Length Polymorphism of XPD Gene: Polymorphism of XPD gene was analyzed by a PCR-RFLP procedure with the following oligonucleotide primer: FP: 5'-GCCCGCTCTGGATTATACG-3' RP: 5'-CTATCATCTCCTGGCCCCC-3' which gave a 436 bp product. The PCR conditions were standardized as initial denaturation at 94˚C for 3 min, followed by 38 cycles of denaturation at 94˚C for 45 sec, annealing at 57˚C for 45 sec, extension at 72˚C for 60 sec and a final extension of 72˚C for 7 min. A negative control without template DNA was used in each run. The amplified products were then resolved in 2% agarose gel stained with ethidium bromide.

The products were then digested with restriction endonuclease, PstI (Invitrogen Life Technologies), as recommended by the manufacturer. The fragments obtained were analyzed in a 1.2% agarose gel stained with ethidium bromide and photographed under UV light. The bands obtained were confirmed in triplicate along with undigested sample and DNA marker.

Statistical analysis

The samples were coded at the time of preparation and scoring. They were decoded before statistical analysis for comparison. Mean and standard deviation (SD) were calculated for each biomarker. The significance of the differences between control and welder end-point means were analyzed using Student's t-test. Mean values and standard deviations were computed for the scores and the statistical significance ($P<0.05$) of effects (exposure, smoking and age) was determined.

Results

The demographic characteristics of the study subjects are presented in Table 1. The age, alcohol consumption and smoking status distributions were similar among exposed workers and controls. Among the smokers, the years of smoking and daily cigarette consumption were similar in the two groups.

The frequency of micronuclei (MN) was studied in 93 welders and in 60 controls. Welders revealed a significant induction of MN when compared with controls (9.09 versus 4.05; $P < 0.05$). Individuals of the exposed as well as control groups with smoking habit and alcohol consumption showed an enhanced frequency of micronuclei (8.94 and 9.24) vs. (6.36 and 6.78) when compared to non smokers and non alcoholics. (6.73 and 6.92) vs. (4.29 and 3.93). Welders who are smokers and alcoholics showed a highly significant increase ($p<0.05$) in MN frequency when compared to all other groups and subgroups (Table 1).

An age-dependent increase in MN frequency was observed in welders (9.14 vs. 6.08; 8.67 vs. 5.61, $P<0.05$). Welders also showed an increased MN frequency with an increase in duration of work ($P<0.05$). A significant correlation was observed between MN induction and duration of exposure in welders (Table 2).

Basal DNA damage (BDD) was studied in a total of 153 subjects using the Comet assay. The results of DNA damage are given in Table 1. In welders a significant increase ($P < 0.05$) in DNA mean tail length indicating BDD was observed when compared with controls (15.08 vs. 11.40 μm) (Table 1). In welders, a significant difference was observed between smokers and non-smokers and

Table 1. Micronucleus Frequency and DNA Mean Tail Length (μm) with Respect to Smoking Habit and Alcohol Consumption in Controls and Welders

Characteristics	Sample Size (n=153)	Age (Mean ± SD)	Exposure Duration (Mean ± SD)	Cigarettes/day (Mean ± SD)	Alcohol (gm/day)	MN (Mean ± SD)	Comet tail length (Mean ± SD)
Exposed	Total 93	35.5 ± 6.99	8.63 ± 3.28	-	-	9.09 ± 4.36*	15.1 ± 4.12*
Smoking	Yes 51 (55%)	35.1 ± 6.94	8.33 ± 3.28	19.7 ± 7.78	-	8.94 ± 4.33-*	15.0 ± 4.11*
	No 42 (45%)	35.7 ± 7.13	8.88 ± 3.20	-	-	6.73 ± 3.29*	14.8 ± 4.01*
Alcohol	Yes 54 (58%)	35.5 ± 6.59	8.33 ± 3.19	-	223.4± 67.2	9.24 ± 4.40*	14.1 ± 3.75*
consumption	No 39 (42%)	36.0 ± 7.57	9.36 ± 3.48	-	-	6.92 ± 2.98*	12.9 ± 3.92*
Controls	Total 60	35.3 ± 7.16	-	-	-	4.05 ± 1.84	11.4 ± 3.59
Smoking	Yes 36 (60%)	35.3 ± 7.30	-	21.4 ± 8.24	-	6.36 ± 1.10	12.3 ± 3.70
	No 24 (40%)	35.3 ± 6.91	-	-	-	4.29 ± 0.80	10.1 ± 3.05
Alcohol	Yes 32 (53%)	34.7 ± 6.86	-	-	220.9 ± 65.7	6.78 ± 1.07	11.5 ± 4.28
consumption	No 28 (47%)	35.6 ± 7.29	-	-	-	3.93 ± 0.97	10.6 ± 2.33

*P<0.05 Values are mean ± SD data

Table 2. Micronucleus Frequency and DNA Mean Tail Length (μm) with Respect to Work Duration and Age in Controls and Welders

Characteristics		Controls (n=60)			Welders (n=93)		
		Sample (n)	MN (mean ± SD)	Mean tail length (μm)	Sample (n)	MN (mean ± SD)	Mean tail length (μm)
Years of exposure	<10	-	-	-	44	8.47 ± 1.04	12.29 ± 1.50
	≥10	-	-	-	49	9.94 ± 1.03*	14.83 ± 1.89*
Age (years)	<35	23	5.61 ± 1.73	10.30 ± 2.22	42	8.67 ± 1.20	12.71 ± 2.31
	≥35	37	6.08 ± 1.48	12.62 ± 3.52	51	9.14 ± 1.09*	14.25 ± 2.98*

*P<0.05; Values are mean ± SD data

between alcohol drinkers and never drinkers in relation to DNA migration (15.04 vs. 14.83 and 14.06 vs. 12.85). A significant difference between smokers and non-smokers among the control group was observed (12.25 vs. 10.13). Similarly, there was a marginally significant (11.5 vs. 10.57) effect of alcohol in the control group. DNA damage was significantly higher in subjects with a longer duration of work. An age dependent increase in DNA damage was also observed ($P < 0.05$) (Table 2).

DNA was amplified by polymerase chain reaction for XPD gene exon 23 using specific primer. Individuals were genotyped to identify the presence of polymorphism. The XPD gene exon 23 has an amplification product at 436bp. 100bp molecular DNA marker was used for the identification and differentiation of gene. Bands at 436bp region were observed for the presence of XPD gene.

The XPD gene polymorphism was analyzed by PCR-RFLP. Bands at 227, 146 and 63bp products were observed for the presence of normal XPD gene. Samples with an altered gene failed to give bands at these sites.

Discussion

The wide use of welding rods containing Cr in industrial settings has elicited concern over the safety of workers and surrounding populations. There is no study available on the biomonitoring of Indian welders. The current investigation reports genotoxicity in welders from South India. The health concerns raised by welders were welder's ash, sore eyes, headache, nose bleeds and discharges from the nose. Most welders expressed concern regarding excessive smoke levels in the workplace and inadequate ventilation. None of the welders wore protective gear and hence chances of exposure are more (Danadevi et al., 2004).

The molecular mechanisms of Cr (VI) carcinogenicity are not well understood. It is not possible to measure the concentration of hexavalent chromium in biological material because its oxidizing properties mean that it readily reacts with a number of substances present in the human body. In this situation the observation that only hexavalent chromium is able to pass cell membranes is of great value. In this manner chromate ions also enter erythrocytes. They are reduced there and bound to constituents of the cell. In contrast, trivalent chromium ions do not succeed in passing cell membranes (Gray et al., 1950). Based on this property of hexavalent chromium, the present study investigated welders for genotoxic effects using the Comet assay and the MN test.

The MN test has been increasingly accepted as a reliable biomarker of genotoxicity in occupationally exposed groups (International Agency for Research on Cancer, 1990). Benova et al., (2002) found double the frequency of buccal MN in Cr platers when compared with control persons. On the other hand, electroplaters showed no significant increase in MN in buccal or nasal mucosa (Rosenman et al., 1996).

The present investigation suggests that welders under their particular conditions of exposure (cigarette smoke and alcohol) reveal clear evidence of genotoxicity in lymphocytes when evaluated by MN test. Previous investigations reporting genotoxic effects in welders using the MN test are scanty. Our study revealed a significant induction of MN in welders when compared to controls with respect to their age and years of exposure.

The Comet assay is increasingly being used to monitor genotoxic effects in occupationally exposed humans (Itin et al., 2001). In the present study, a significant increase in BDD was observed in welders when compared with controls by the Comet assay. These results indicate that the level of exposure in the workplace is sufficiently high and also highlights the sensitivity of the assay used. Studies of welders utilizing the Comet assay are lacking. However, elevated levels of DNA protein cross-links were observed in a study of welders (Costa et al., 1993). A statistically significant enhancement in the frequency of protein cross-linking and DNA strand breaks was noticed in the blood lymphocytes of welders (Nordberg et al., 2001). Similarly, welders showed a significantly higher level of DNA single-strand breaks with the alkaline filter elution method (Stich et al., 1982). Our results are inline with these observations.

In the present study; smoking and alcohol consumption showed no significant effect on DNA damage as reported by Danadevi et al., (2004) and Fracasso et al., (2002). Heavy metals may not only be genotoxic, but also inhibit DNA repair systems including NER and BER at low, non-cytotoxic concentrations (Hartwig, 1998). DNA damage in the exposed individuals might also be a secondary event due to DNA repair inhibition, since a decreased repair capacity will enhance susceptibility to direct DNA strand breaks as previously suggested (Hedenstedt et al., 1977). Several studies have indicated the clastogenic and aneugenic ability of Chromium salts (Seoane et al., 2001; Wise et al., 2004). Chromosome breakage (clastogenic events) is caused by DSB induced either directly or by the conversion of a SSB into a DSB after cell replication. A significant proportion of induced micronuclei may be the result of aneugenic effects of metals. Micronuclei result either from lesions/adducts at the level of DNA or

chromosomes, or at the level of proteins directly or indirectly involved in chromosome segregation (Kirsch-Volders et al., 2002)

This study takes into consideration the involvement of genetic polymorphisms in DNA repair genes in genotoxic effects evaluated by the comet and MN assays. In this study, we focused on XPD gene, which play an important part in the BER pathway of DNA. XPD gene when analyzed for polymorphism showed a positive result which could lead to deficient DNA repair and thereby an increase in MN and comet tail length. This result suggests a further investigation of gene such as hOGG1 involved in DNA repair and their possible interactions with smoking, alcohol, age and other risk factors.

Workers in many occupational settings are exposed to certain genotoxic agents. 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 heavy metals about the potential hazard of occupational exposure and the importance of using protective measures.

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