Evaluation of DNA Damage in Lymphocytes of Radiology Personnel

by Comet Assay.

Muhammad Khisroon\textsuperscript{1,\dagger,*}, Ajmal Khan\textsuperscript{1,\dagger}, Maryam Naseem\textsuperscript{1,\dagger}, Naheed Ali\textsuperscript{1}, Sardar Khan\textsuperscript{2}

and Syed Basit Rasheed\textsuperscript{1}

\textsuperscript{1} Department of Zoology, University of Peshawar, Peshawar. Pakistan

\textsuperscript{2} Department of Environmental Sciences, University of Peshawar, Peshawar. Pakistan

\dagger These authors contributed equally to this work.

* Corresponding author (E. mail, m_khisroon@upesh.edu.pk; Tel.: +92-91-9216754)

Running title. DNA damaging effects of radiation by comet assay.
Abstract

Objectives: The importance of X-rays as a diagnostic medical tool cannot be denied. However, continuous exposure to X-rays can cause DNA damage. This study aimed to use the comet assay technique to investigate the level of DNA damage in lymphocytes due to X-rays in occupationally exposed personnel.

Methods: Blood samples were collected from 74 exposed and 70 control subjects for analysis. A total of 100 randomly captured cells from each slide were examined using an epifluorescent microscope. The comets were analyzed by a visual scoring method according to comet tail length.

Results: The results indicated a significant increase ($p < 0.05$) in DNA damage in X-rays technicians ($129.8 \pm 17.2$) as compared with the control group ($53.0 \pm 25.0$). A significant increase ($p < 0.02$) in DNA damage was also observed with an increase in exposure duration of technicians because of their service length.

Conclusions: The present study suggests that the exposed radiology personnel should carefully comply with radiation protection procedures such as wearing of lead apron during diagnostic procedures and minimize radiation exposure where possible to avoid potential genotoxic effects due to X-rays.

Key words: X-rays, DNA damage, Comet assay, Blood lymphocytes.

Abbreviations: CA, comet assay; DMSO, Dimethyl sulfoxide; EDTA, Ethylenediaminetetraacetic acid; IARC, International Agency for Research on Cancer; LMPA, low melting point agarose; NMA,
normal melting agarose; PBS, phosphate buffered saline; SCGE, Single cell gel electrophoresis; TCS, total comet score.

41 **Introduction**

42 The X-ray is one of the most important components of medical technology. In the past few decades, X-radiation has been put into use for many purposes in medical science such as for therapy and diagnosis of human ailments\(^1\). Diagnostic radiology, a field of physical medicine uses X-rays to obtain functional and anatomical information about an individual’s body\(^2\). X-rays have been classified as carcinogenic by the International Agency for Research on Cancer (IARC) and Government of the United States\(^3\). Medical X-rays account for 0.6 to 3% of all types of cancers worldwide and at least 20% of cancer in developed countries\(^4, 5\). Radiation in heavy doses has been proved to be lethal for cells, while in mild doses have been proved to damage the DNA of the exposed cells\(^6\). The DNA molecule may be damaged directly by interaction with ionizing radiation or indirectly by interaction with reactive products of the degradation of water by ionizing radiation\(^7\). Damage to DNA is regarded as the most important initiating step in the development of cancer and genetic diseases after exposure\(^8\).

Previously, numerous studies have focused on radiation-induced genetic effects using animal models\(^9\). Radiation has been proved to be carcinogenic in all types of experimental animals (mice, rats and monkeys for X-radiation and mice, rats, rabbits and dogs for gamma radiation) and has been shown to induce tumors in at least 17 different tissue sites, that is, sites at which tumors have been observed in humans (i.e., leukemia, thyroid gland, breast and lung)\(^10\).
Radiation technicians are qualified and authorized personnel performing different radiation-related examinations in hospitals and private clinics. They are occupationally exposed to radiation emitted from X-ray devices for a long time during handling of patients for diagnostic and therapeutic purposes\textsuperscript{11}). However, the level of exposure depends on the working area, exposure time, job rotation and protective measures adopted by the individual workers\textsuperscript{12)}. Trends in radiation exposure for both patients and staff are affected not only by advances in radiation protection but also by the level of doses used in the practice of medicine\textsuperscript{13)}.

Several research methods including sister chromatid exchange, chromosomal aberration and the micronucleus assay are normally used for investigating genetic damage. However, these methods are economically costly, time-consuming and require proliferating cells. Therefore, the use of single cell gel electrophoresis (SCGE) or/and the comet assay for genotoxicity studies have greatly increased during the past few decades\textsuperscript{14-16)}.

The comet assay (CA) was first introduced by Ostling and Johanson (1984)\textsuperscript{17)} as a technique to determine DNA single-strand breaks that caused relaxation of DNA supercoils. This technique was further modified by Singh et al. (1988)\textsuperscript{18)}. The CA or SCGE is a precise, simple and fast test that has been extensively used to calculate both in-vitro DNA damage and repair following exposure to a variety of genotoxic agents and even for human biomonitoring\textsuperscript{19-20)}. Different possible modifications of the comet assay have helped in the detection of single strand breaks (SSBs), double-strand breaks (DSBs), alkali-labile sites, incomplete excision repair sites, interstrand cross linkages and cell death or apoptosis\textsuperscript{21)}. 
Considering the hazardous effects of X-rays and lack of awareness among radiation personnel, this study was conducted to assess the level of DNA damages in local radiology technicians using the CA technique.

**Materials and Methods**

*Study population*

A total of 74 radiology personnel employed in various government and private hospitals of Peshawar, Pakistan, were included in this study (Table 1). Their average age ranged from 20 to 52, with mean age of $35.2 \pm 8.5$ years and a median age of 37.5 years. Among the exposed group, 11 subjects were smokers and 63 subjects were nonsmokers. The control population comprised 70 healthy individuals selected from students and staff of the University of Peshawar with ages ranging from 18 to 62, a mean age of $36.2 \pm 13.2$ years and a median age of 32.5 years (Table 1). The control group had no previous history of occupational exposure to X-rays or known genotoxic chemicals and none reported medicine intake, presence of chronic diseases or any known inherited genetic disorders. Prior to the start of the study informed consent was obtained from all participants.

*Questionnaire*

A questionnaire regarding personal data, type and duration of occupational exposure, working activities and information on exposure to possible confounding factors (smoking habits, medicine intake, viral diseases, presence of known inherited genetic disorders, recent vaccinations, chronic disease, family history of cancer and radiodiagnostic examinations) was completed by each individual before collection of blood samples.
Blood sample collection and lymphocyte separation

Peripheral blood was collected by venipuncture into EDTA tubes. Blood samples from both exposed and control subjects were handled in the same manner. After collection, all blood samples were coded, refrigerated at 4°C, transported to the laboratory and processed within 2 to 3 hours. Lymphocytes were separated from the blood by Histopaque 1077 density gradient centrifugation and washed in phosphate buffered saline (PBS). The viability of the cells was tested by trypan blue exclusion. The number of dye-excluding cells was kept greater than 90%.

Alkaline comet assay (CA)

The alkaline CA was performed according to the protocol described by Singh et al. (1988)\textsuperscript{18} with some modifications. Cleaned conventional microscopic slides were dipped into 0.7% normal melting agarose (NMA) solution. Then the slides were gently removed, their undersides were wiped to remove extra agarose, and they were then stored at room temperature (25°C) until needed. First layer slides were generally prepared one day before use. The cell suspension (15 μL) was mixed with 70 μL of low melting point agarose (LMPA) (0.7%) and spread on top of first layer slide. The slides were kept at 0°C for 5 minutes and then a second layer of 85 μL LMPA was added to fill any residual holes; the slides were kept at 0°C for 5 minutes to solidify. After solidification, slides were gently immersed in freshly prepared cold-lysing solution [(2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, pH 10) with 1% Triton X-100 and 10% DMSO added just before use] overnight at 4 °C.

Electrophoresis and neutralization
The slides were then removed from lysing solution, immersed in freshly made electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13) and left for 20 minutes to allow the unwinding of DNA and the expression of alkali-labile sites. The slides were then subjected to electrophoresis for 30 min at 300 mA and 25 V. All the steps were conducted in the dark at 4°C. After electrophoresis, the slides were neutralized by washing three times with neutralization buffer (400 mM Tris, pH 7.5) for 5 minutes each.

### Staining, scoring and visualization of slides

For slide staining, 70 μl acridine orange (20 μg/ml) was applied to each slide for 5 min and then covered with a cover slip before observation under a fluorescent microscope. Images of 100 randomly selected cells were taken at 400x-magnification using a fluorescent microscope (Nikon Eclipse 80 i) equipped with a 450-490 nm excitation filter. Comet tail lengths (consisting of the nuclear region and tail) were scored visually as suggested by Collins\(^{20}\) into 5 comet classes (Figure 1): a) comet class 0 (no damage, hence no tail), b) comet class 1 (tail up to 1.5 times the diameter of the comet nucleus), c) comet class 2 (tail 1.5–2.0 times the diameter of the comet nucleus), d) comet class 3 (tail 2.0–2.5 times the diameter of the comet nucleus) and e) comet class 4 (maximally damaged with total DNA in its tail). A final overall total comet score for all 100 cells was obtained by summing up the number of cells in each class times the class number, giving a rating between 0 (completely undamaged) and 400 (maximum damaged) Collins\(^{20}\) i.e. total comet score \(TCS = 0(n) + 1(n) + 2(n) + 3(n) + 4(n)\), where \((n)\) indicates the number of cells in each class.

### Statistical analysis
Mean and standard deviations of TCS and differences among the means of X-rays exposed and control subjects were calculated using the Student's t-test with the SPSS (V.16.0) for Windows software. Correlation was calculated for the duration of occupational exposure and TCS using online software (Wessa, 2011) \(^2\). The \(P\) value was kept at 0.05 for statistical significance.

**Results**

Table 1 summarizes the main characteristics of the studied population including age, tobacco consumption, duration and nature of occupation (Table 1).

Undamaged cells had an intact nucleus without a tail and damaged cells had the appearance of a comet (Figure 1). Significantly greater DNA damage was observed in the radiology personnel (TCS = 129.8 ± 17.2) compared with that observed in the control group (TCS = 53.0 ± 25.0, \(P < 0.001\)) (Table 2). In addition, comet class 3 (13.9 ± 3.0 cells) and class 4 (10.2 ± 3 cells) were observed more frequently in radiology personnel than in the control group (comet class 3 = 4.0 ± 3.9 and comet class 4 = 3.7 ± 3.2 cells) (Table 2). The opposite results were observed with undamaged cells; comet class 0 was observed more frequently in the control group (72.4 ± 11.4) as compared with the radiology personnel (41.9 ± 5.8 cells) (Table 2).

A strong positive correlation \((r = 0.62, \ P < 0.001)\) was observed between the duration of occupational exposure and TCS values. Among the technicians, the lowest TCS value (119.6 ± 14.1) was observed in those working for less than one year, while the highest TCS value (136.6 ± 18.3) was observed in those working for more than 11 years (Table 3). The results indicate that the TCS values increased with the increase in duration of exposure of technicians to X-rays. In the exposed group, a very weak and nonsignificant \((r = 0.31, \ P > 0.05)\) positive correlation was observed between age and TCS. Furthermore, TCS values were also closely connected with use of a lead apron by the technicians. The TCS values of the radiology technicians (132.0 ± 16.7)
who had not used a lead apron for protection were significantly higher \((P < 0.01)\) than those who had used a lead apron \((111.0 \pm 5.6)\) while performing their duties (Table 4). DNA damage in the X-ray technicians who were smokers (Table 5) was slightly lower \((128.8 \pm 18.8)\) than in X-ray technicians who were nonsmoker \((129.9 \pm 17.0)\) but this difference was not significant \((P > 0.05)\).

**Discussion**

This study showed using the CA technique that DNA damage occurred in personnel (radiology technicians) occupationally exposed to X-rays. The DNA damage in the radiation technicians was significantly higher \((P = 0.001)\) compared with that in controls. This increase in DNA damage may be associated with the professional conditions to which radiology personnel were exposed. The radiation workers were exposed to more X-rays; therefore, they are at higher risk of developing detrimental effects than the general public\(^{22}\).

Characteristically, X-rays have been thought to be responsible for various cytogenetic defects. An increase in chromosomal aberrations has been observed among the X-rays workers, and even at low levels of X-rays the same consequences along with dicentrics chromosomes have been observed among the hospital workers\(^{23-24}\). High frequencies of centromere-positive and centromere-negative micronuclei have been reported in the peripheral lymphocytes of hospital workers occupationally exposed to X-rays\(^{25}\). Our research findings are in agreement with these studies and showed a significant increase in DNA damage in X-ray technicians as compared with the control group (Table 2).

X-radiation has been proved to be carcinogenic in different animals. This radiation has been found to induce genetic defects and tumors in various tissues\(^{26}\). The first cases of leukemia were reported in 1911 in radiation workers\(^{27}\). It has been reported that skin cancer and leukemia in
Radiology technicians and their occurrence rates increased with increasing service duration\textsuperscript{28}. The present study also showed a significant increase in DNA damage with increasing service duration of radiology personnel, which may lead to the most important initiating step in the development of cancers and genetic diseases.

Exposure to X-rays may cause deterministic and stochastic effects in living organisms and a lead apron can be used to protect personnel from this radiation\textsuperscript{29}. The findings of this study indicated a significant decrease in DNA damage resulting from use of protective lead aprons by the radiology personnel. However, a lead apron could not ensure full protection against DNA damage. This may be due to improper use and handling of the aprons by the technicians. It has been reported that the quality of aprons and improper storage and use can decrease their protection efficiency/level and make them more radioparent than the defined limit. This may be the reason why significant DNA damage was still observed in the radiation technicians who used lead apron as compared with the control group.

A previous study related to DNA damage in workers occupationally exposed to X-rays showed a significant correlation between age and DNA damage index in the comet assay\textsuperscript{31}. In the present study, no correlation was observed between age and the DNA damage index in the exposed group, which is in agreement with other studies showing no correlation between age and the DNA damage index\textsuperscript{34, 35}. However, in X-ray-exposed personnel, the DNA damage index was significantly correlated with time of exposure or experience as reported by various studies\textsuperscript{29-31}.

In the past, several studies on monitoring DNA damage caused by occupational exposure have demonstrated the damage caused by smoking\textsuperscript{30, 31}. Tobacco smoke contains a high number of mutagenic and carcinogenic substances; hence, smoking is one of the important variables and therefore should be considered in biomonitoring studies. Cigarette smoke extract has been shown
to have carcinogenic and mutagenic activities in rodents and human cells during \textit{in vitro} studies\textsuperscript{32}. Smoking has been associated with increased DNA damage in a study conducted both on young and old individuals\textsuperscript{33}. Furthermore, an increase in DNA damage has also been reported in the workers of a cigarette factory\textsuperscript{34}. The level of DNA damage is closely related to the intensity of smoking; therefore, the level of DNA damage in individuals who quit smoking has been shown to decrease over the course of 1 year\textsuperscript{35}. In contrast, some studies have reported no difference in terms of DNA damage between smokers and nonsmokers\textsuperscript{36, 37}. In the present study, no correlation was established between the smoking and nonsmoking radiation technicians. Certain studies have reported that cigarette smoking is not a significant confounding factor for the comet score\textsuperscript{42-45} and this may be the possible reason for nonsignificant correlation of the DNA damage index observed between smokers and nonsmokers.

The results of the present study confirmed the harmful effects of X-rays on human DNA. These radiations-imposed genotoxic effects can cause significant DNA damage in radiology personnel during their occupational exposure. The increase in DNA damage may be attributed to handling of X-ray examinations without the use of any protective measures; therefore, the DNA damage in the workers using a lead apron was lower than in those who did not use a lead apron. Thus radiation personnel need to be aware about the hazards associated with excessive exposure to radiation.

This study recommends reduction of the exposure duration in any radiation area because the radiation dose received by a person is closely related to the time spent in the radiation area. The current study emphasizes proper use and storage of good quality lead aprons to minimize X-ray penetration and it should be made compulsory for hospital administrations to take proper steps to reduce exposure to X-rays in the workplace. Individual biomonitoring should be conducted
regularly, and new radioprotection policies should be introduced on the basis of genetic studies like the present one.

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Contribution

Ajmal Khan and Maryam Naseem prepared samples for analysis and performed lymphocyte isolation and the comet assay. Muhammad Khisroon designed and prepared the initial draft of the study. Sardar khan collected the samples and interviewed the subjects. Syed Basit Rasheed arranged, statistically analyzed and interpreted the results of lab work. Naheed Ali was involved in extensive writing/editing of the paper and correcting draft versions of the manuscript.

Conflict of Interest Statement: We declare that there is no conflict of interest.

References


Figure 1. Acridine orange stained images (400x magnification) of lymphocytes of radiology personnel subjected to single cell gel electrophoresis. Numbers indicates classes, i.e., 0, 1, 2, 3 and 4, of comets according to the visual scoring method.
Table 1. Distribution of the main characteristics of the radiology Personnel and control group.

<table>
<thead>
<tr>
<th>Main Characteristics</th>
<th>Radiology Personnel</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>74</td>
<td>70</td>
</tr>
<tr>
<td>Mean Age (Years)</td>
<td>35.2 ± 8.5</td>
<td>36.2 ± 13.2</td>
</tr>
<tr>
<td>Mean Duration of Job (Years)</td>
<td>7.8 ± 5.3</td>
<td>............</td>
</tr>
<tr>
<td>Nature of Job</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-ray Technician</td>
<td>74 (100%)</td>
<td>............</td>
</tr>
<tr>
<td>Occupation of Control group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>University students</td>
<td>............</td>
<td>28 (40%)</td>
</tr>
<tr>
<td>University employees</td>
<td>............</td>
<td>22 (31.4%)</td>
</tr>
<tr>
<td>Unemployed</td>
<td>............</td>
<td>20 (28.6%)</td>
</tr>
<tr>
<td>Yes</td>
<td>11 (14.8%)</td>
<td>............</td>
</tr>
<tr>
<td>No</td>
<td>63 (85.1%)</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 2. Mean frequency of each comet class per 100 cells (± standard deviation) and overall mean of total comet score (± standard deviation) of the radiology personnel and control group.

<table>
<thead>
<tr>
<th>Comet Class</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>TCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiology Personnel (n=74)</td>
<td>41.9 ± 5.8</td>
<td>20.4 ± 3.5</td>
<td>13.6 ± 3</td>
<td>13.8 ± 3</td>
<td>10.2 ± 3</td>
<td>129.8 ± 17.2</td>
</tr>
<tr>
<td>Control (n= 70)</td>
<td>72.4 ± 11.4</td>
<td>13.6 ± 6</td>
<td>6.3 ± 4.3</td>
<td>4.0 ± 3.9</td>
<td>3.7 ± 3.2</td>
<td>53.0 ± 25.0</td>
</tr>
</tbody>
</table>

\[ P = 0.001. \text{TCS: total comet score} \]
Table 3: Comet score distributed according to duration of occupational exposure.

<table>
<thead>
<tr>
<th>Duration in Years</th>
<th>TCS Subject</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>2</td>
<td>2 to 5</td>
</tr>
<tr>
<td>3</td>
<td>6 to 10</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 11</td>
</tr>
</tbody>
</table>

$r = 0.96, P = 0.02$

Table 4: Protective measures and total comet score.

<table>
<thead>
<tr>
<th>Subject</th>
<th>TCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Lead Apron Users</td>
<td>111.0 ± 5.6</td>
</tr>
<tr>
<td>2 Nonusers of Lead Apron</td>
<td>132.0 ± 16.7</td>
</tr>
</tbody>
</table>

Table 5: Total comet scores of smokers and nonsmokers.

<table>
<thead>
<tr>
<th>Subject</th>
<th>TCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Smokers</td>
<td>128.82 ± 18.80</td>
</tr>
<tr>
<td>2 Nonsmokers</td>
<td>129.92 ± 17.03</td>
</tr>
</tbody>
</table>

$P > 0.05$