Research Article

Evaluation of DNA damage in radiation workers by using Comet assay

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Received: 19 November 2018 Revised: 14 December 2018 Accepted: 2 January 2019

Abstract

Objective: The objective of the present work was to evaluate the DNA damage in radiation workers by using single cell gel electrophoresis (comet assay). **Material and Methods:** A total of 30 blood samples of radiations workers from different hospitals of Dharwad and Bagalkot districts of Karnataka were collected. The blood samples of alcoholic and smoking individuals were avoided so as to emphasize only on the effect of radiation. Collected blood samples were subjected for comet assay by following standard procedure. After the comet assay, cells were stained with fluorescent dye and observed in fluorescent microscope. The DNA damage was estimated by measuring the length of the comet tail by using visual scoring method. Microscopic examination of individual leukocytes samples embedded in agarose was subjected to electrophoresis and stained with a fluorescent DNA binding dye Acridine Orange Showed smear of DNA in damaged cells based on the exposure period. **Results:** The duration of exposure and the kind of level of DNA damage were directly proportional, an exposure of 18 months to the radiation environment showed a visual score of 0.1 in sample S3. Contrastingly as the duration of exposure increased the level of DNA damage as assessed by the visual score also increased in sample 20 with an exposure of 102 weeks. Out of 20 samples only few samples of X-ray technicians showed the considerable damage of DNA. And other samples did not show such severe damages. **Conclusion:** Study concludes that high exposure to radiation is main cause for DNA damage in selected population of X-ray technicians. In the course of our experiment we came across variability in the extent of DNA damage among 20 exposed individuals which can be attributed to their occupation. Our experiment has shown a significant increase in amount of DNA damage in workers with the increase in duration of exposure.

***Keywords**:* X-ray technicians; comet assay; Single cell gel electrophoresis; Blood lymphocyte and DNA damage

Introduction

Nucleic acids in the form of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) are present in all living organisms; they dictate the protein synthesis mechanism through transcription and translation. DNA is chemical basis of heredity and may be regarded as the reserve bank of genetic information. DNA is exclusively responsible for maintaining the identity of different species of organisms over millions of years. However this DNA can be damaged due to a number of factors such as radiations. DNA damage is an alteration in the chemical structure of DNA, such as a break in a strand of DNA, a base missing from the backbone of DNA, or a chemically changed base such as 8-OHdG. Damage to DNA that occurs naturally can result from metabolic or hydrolytic processes. Metabolism releases compounds that damage DNA including reactive oxygen species, reactive nitrogen species, reactive carbonyl species, lipid peroxidation products and alkylating agents, among others, while hydrolysis cleaves chemical bonds in DNA. Naturally occurring DNA damages arise about 10,000 to 100,000 times per day per mammalian cell. DNA damage and mutation have different biological consequences. While most DNA damages can undergo DNA repair, such repair is not 100% efficient. Un-repaired DNA damages accumulate in non-replicating cells, such as cells in the brains or muscles of adult mammals and can cause aging (Bernstein et al., 2008; Hoeijmakers. et al., 2009; Freitas and de Magalhães., 2011). In replicating cells, such as cells lining the colon,

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DOI: https://doi.org/10.31024/ajpp.2019.5.3.8

errors occur upon replication past damages in the template strand of DNA or during repair of DNA damages. These errors can give rise to mutations or epigenetic alterations (O'Hagan HM et al., 2008). Both of these types of alteration can be replicated and passed on to subsequent cell generations. These alterations can change gene function or regulation of gene expression and possibly contribute to progression to cancer.

Another indication that DNA damages are a major problem for life is that cells make large investments in DNA repair processes. As pointed out by Hoeijmakers et al. (2009), repairing just one double-strand break could require more than 10,000 ATP molecules, as used in signaling the presence of the damage, the generation of repair foci, and the formation in humans of the RAD51 nucleofilament an intermediate in homologous recombinational repair. Another type of DNA damage, the double strand break, was shown to cause cell death through apoptosis (Carnevale et al., 2008). This type of DNA damage would not accumulate with age, since once a cell was lost through apoptosis, its double strand damage would be lost with it. The Single Cell Gel Electrophoresis assay also known as comet assay is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. It was first developed by Östling & Johansson in 1984 and later modified by Singh et al. in 1988. The comet assay is an extremely sensitive DNA damage assay. This sensitivity needs to be handled carefully as it is also vulnerable to physical changes which can affect the reproducibility of results. Essentially, anything that can cause DNA damage or denaturation except the factor(s) being researched is to be avoided. The most common form of the assay is the alkaline version although there is as yet no definitive alkaline assay protocol. Due to its simple and inexpensive setup, it can be used in conditions where more complex assays are not available.

Materials and methods

The present study was undertaken to study the DNA damage in blood samples of radiation workers by single-cell gel electrophoresis or comet assay. As little as 20 microlitres of whole blood was sufficient for comet assay. Overall, this whole-blood technique for comet assay is expected to provide a simple, rapid, and cost-effective alternative for the existing comet assay using isolated lymphocytes in situations such as when time and cost are limiting factors.

Blood samples for comet assay

A total of 30 blood samples of radiations workers from different hospitals of Dharwad and Bagalkot districts of Karnataka were collected. The blood samples were drawn with the help of lab technician in sterilized conditions in 2ml quantity. The blood samples were processed and stored as per the ethical committee under CPCSEA guidelines of Karnatak University Dharwad. Basic information of the workers were collected which include their food habits, lifestyle, working pattern, daily hours of exposure to radiations and safety measures adopted during the operation. The collected blood was used immediately or it was stored in a cold storage at -20^{0}C when needed for further analysis. The blood samples of alcoholic and smoking individuals were avoided so as to emphasize only on the effect of radiation.

Chemicals and reagents

Chemicals necessary for performing Single cell gel electrophoresis comet assay likeAgarose-normal melting (molecular biology grade–MB), Agarose-low melting (MB), sodium chloride (analytical reagent-AR), disodium hydrogen phosphate (AR), Potassium dihydrogen phosphate (AR), disodium ethylene diamine tetra acetic acid (disodium EDTA), tris (AR), sodium hydroxide (AR), sodium dodecyl sulphate /sodium lauryl sarcocinate (AR), tritron X 100 (MB) trichloro acetic acid, zinc sulphite (AR), glycerol (AR) were purchased from Himedia chemcals.

Protocol for Single Cell Gel Electrophoresis (Comet Assay)

Preparation of slides: Frosted slides were used for Single Cell Gel Electrophoresis.

Preparation of agarose

0.5% LMPA (low melting point agarose) and 0.75% NMPA (normal melting point agarose) were prepared in phosphate buffered saline (PBS) and melted before use.

Pre -coating of agarose: 100µl of hot, 0.75% NMPA and smeared on the slide as a single layer, slides were labeled and allowed to dry at 37°C. Pre-coating of slides with agarose provides better anchorage for the subsequent agarose layers.

Layering of blood-LMPA gel mixture

On the NMPA solidified agarose layer add 100µl of LMPA was mixed with 30µl of blood were mixed thoroughly by pipetting and the mixture is layered over NMPA layer. Cover slip was placed carefully over the gel so that it forms an uniform layer over the NMPA coat, taking care to avoid air bubbles, Gels were allowed to solidify at 4°C in a refrigerator for 10-15min.

Layering of third layer of gel

Once the lymphocyte-LMPA cell layer was solidified, coverslip was removed carefully, avoiding avulsion of the underlying layer. 75µl of LMPA was added onto the agarose gel mixture layer and a fresh cover slip was replaced carefully over the gel mixture layer avoiding air bubbles. The gel was allowed to solidify at 4°C in a refrigerator for 10-15 min.

Lysis of lymphocyte

Once the third layer of agarose was solidified, the cover slip was carefully removed again and the slides are gently immersed into cold lysis solution and refrigerated for 1hr. The lysis treatment may extend up to a maximum 24hr for lysis of lymphocytes embedded in the gel.

Alkaline unwinding and electrophoresis of slides

After lysis, the slides were gently removed from the lysis solution and placed exactly perpendicular to both the electrodes with the agarose coated side facing upwards in gel electrophoresis system. The electrophoresis tank was filled with fresh cold electrophoresis buffer until the buffer completely covers the slides without formation of air bubbles over the agarose gel. The slides were allowed to stay in the alkaline buffer for 30 min in order to unwind DNA strands and expose the alkali labile sites (alkali unwinding). Power supply is turned on between electrodes and current is adjusted to 50-100v by raising or lowering the buffer level. Electrophoresis is carried out for 30min. Cold electrophoresis buffer is always used or the electrophoresis should be performed under refrigeration to avoid DNA damage due to heat generated during flow of current (Nandakumar et al., 2011).

Neutralization

The slides were gently lifted from the electrophoresis buffer and placed on staining tray. The slides are carefully flooded with neutralizing tris buffer (pH7.4) for 5 min and the buffer was drained; the process was repeated twice followed by several washes with distilled water.

Fluorescent staining

The 50µL of acridine orange stain was dropped onto each slide and covered with a clean cover slip. Before viewing the slides, excess stain from the back and edges of the slides was blotted away. For visualization of acridine orange stained slides, fluorescent microscope with a magnification of 40x-100x was used. The following are the specifications of Fluorescent Microscope, (Make – Carl Zeiss; Model – Imager. M2; Camera – Janoptik – ProgRes® C5 Software – ProgRes Capture Capture Pro 2.8 Version).

Evaluation of DNA damage

The DNA damage was estimated by measuring the length of the comet tail by using visual scoring method. In this method scores are given to the selected cells as o to 0.2 to 0.4……to 2. Based on this DNA damage was detected, even the change in the shape of cells, and their diameter was used as a parameter to evaluate DNA damage.

Results and discussion

Search for simple reliable techniques to asses DNA damage has led to the discovery of single cell gel electrophoresis technique otherwise also called as the Comet assay and has been used for years to assess DNA damage. In this assay, only a small number of cells are required for analysis, and data are collected at the individual cell level, providing information on the intracellular distribution of DNA damage (Collins et al., 1997). The one-dimensional comet assay is highly reproducible, easy to perform, and capable of identifying DNA damages. Sensitivity of the comet assay has been realized during the outcome of our experiments which also matches with the findings of Blasiak and Trzeciak (1998). Comet assays applications in various fields of biological science are well documented (Jackman et al., 2002). Our present findings are in correlation with the earlier workders (Tice et al., 2000; Wojewodzka et al., 2002; Donggeam et al., 2003). An effort was made to understand the plight of radiation workers, working in different diagnostic centers and hospitals in the region.

Table 1. Details of non-alcoholic radiation workers, exposure period and scoring S1 to S20 are individual blood samples

Sl. No.	Sample	Age	Duration of exposure (in months)	Score
1	S1	24	6	0
2	S2	25	12	0.05
3	S3	25	18	0.1
4	S4	26	24	0.1
5	S5	25	30	0.15
6	S6	24	36	0.15
7	S7	25	42	0.2
8	S8	24	48	0.25
9	S9	25	54	0.3
10	S10	27	60	0.4
11	S11	26	66	0.5
12	S12	27	72	0.5
13	S13	26	78	0.5
14	S14	25	84	0.6
15	S15	26	90	0.6
16	S16	25	96	0.65
17	S17	27	96	0.65
18	S18	26	90	0.65
19	S19	26	96	0.7
20	S20	30	102	1

The comet assay was successfully performed using the blood samples of radiation workers (X-ray technicians) from different diagnostic centres of Dharwad and Bagalkot districts. Microscopic examination of individual leukocytes samples embedded in agarose was subjected to electrophoresis and stained with a fluorescent DNA binding dye acridine orange Showed smear of DNA in damaged cells based on the exposure period. When cells are embedded in agarose, lysed and subjected to an electric field, broken DNA is able to migrate towards the anode due to shearing or damage of DNA. Each cell which is completely damaged has the appearance of a "comet" with a brightly fluorescent head and a tail with an intensity that is related to the amount of damage sustained by the cell (Figure 3b).

In the present study, a total of 30 blood samples of radiation workers were collected including the control of same age group. The samples were differentiated into alcoholics, smokers and non-alcoholic, non-smokers. As many as 20 samples were non-alcoholic, non-smokers and 5 samples were non-alcoholic controls. Only 25 samples were considered for the comet assay, the details of subjects chosen for the study are presented in table 1. Subsequently the control batches were also tested to compare the normal cell with the damaged cell and the details of normal samples used for testing is presented in table 2. It was found that, medical workers who were occupationally exposed to ionizing radiation for different period of time showed significant increase in levels of DNA damage compared to control (normal). The influence of duration of exposure to the level of visual score of comet assay reveals that the higher the duration of exposure the more is the damage caused to DNA as indicated in figure 1.

The duration of exposure and the kind of level of DNA damage were directly proportional, an exposure of 18 months to the radiation environment showed an visual score of 0.1 in sample S3 (Figure 4b). Contrastingly as the duration of exposure increased the level of DNA damage as assessed by the visual score also increased in sample 20 with an exposure of 102 weeks, the visual score was 1as shown in (Figure 3b)

Table 2. Details of control samples, age and scoring.C1 to C5 control samples

Samples	Age	Score
C1	25	0.0
C2	27	0.0
C3	26	0.0
C4	28	0.03
C5	30	0.05

There is no significant damage in the DNA of these samples, however sample C4 and C5 showed some damage which may be due to number of factors such as aging.

Figure 1. Graph of non-alcoholic radiation workers showing increase in the DNA damage with the increase in duration of exposure. The highest score is 1 i.e, sample S20 for 102 months.

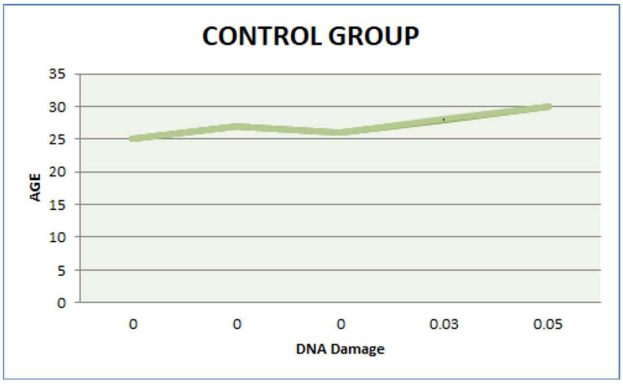

Figure 2. Graph of Control group showing negligible DNA damage in the aged cells from sample C1 to C5 respectively

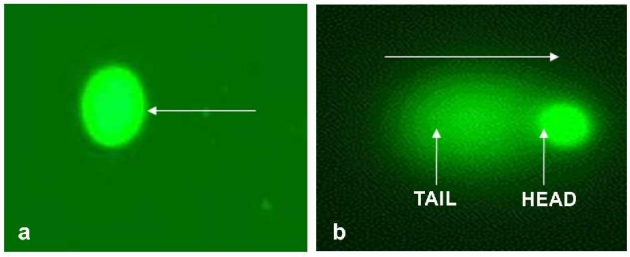

Figure 3. Photograph of (a). Normal Healthy Blood Cell (b). Damaged Cell showing Comet Shape

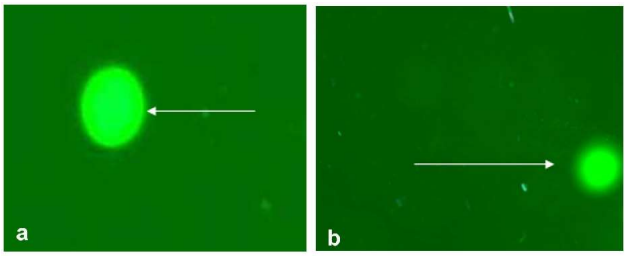

Figure 4. Photograph of (a). Normal cell without any damage (b). Cell with round smear

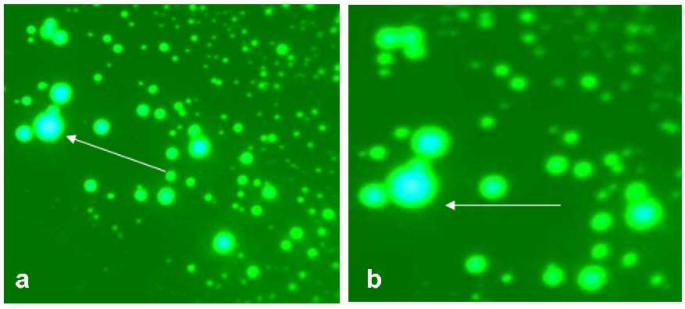

Figure 5. Photomicrograph for (a). Cell showing high flouroscence (b). Large circular shape cell with high flouroscence

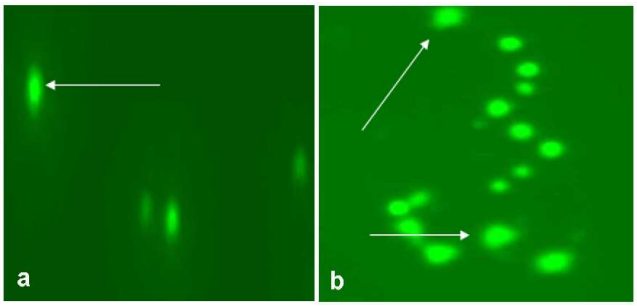

Figure 7. Photomicrograph for (a). Cell damage with smear around the cell (b). Cell damaged with round smear

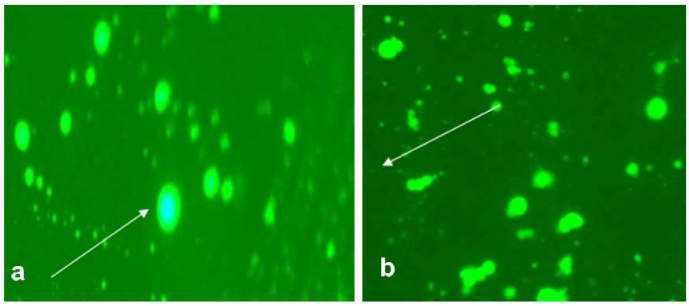

Figure 6. Photomicrograph for (a). Cell with high flouroscence (b). Scattered cells with different shapes

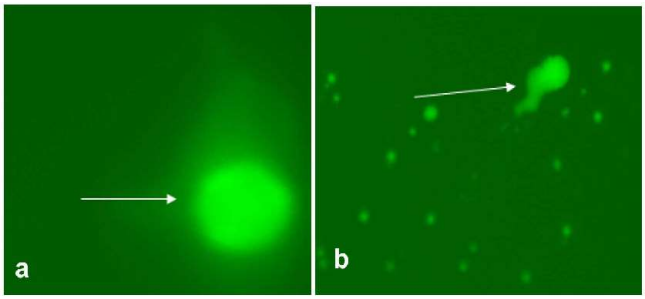

Figure 8. Photomicrograph for (a). Cell damage with smear around the cell (b). Cell with irregular shape

Formation of typical comet like structure due to the migration of damaged DNA is seen prominently during single cell electrophoresis, during our study we also noticed that in addition to the comet shape, there were conditions like smearing of DNA, irregular cell migration etc, were observed as shown in figure (3, 4, and 5). But the control groups showed a negligible DNA damage as shown in figure 2 which can be attributed to aging. It is evident that the duration of exposure is directly related to DNA damage and in turn represented by visual scores as seen in the figures. There was a considerable DNA damage in the samples (non alcoholic and non smoking) of medical workers occupationally exposed to ionizing radiations as shown in figure 1.

All the above images are captured by the help of a camera attached to fluorescent microscope after single gel electrophoresis at 100v for 30 minutes.

Conclusion

Comet assay technique was used for the evaluation of DNA damage in radiation workers. Totally 30 blood samples were collected in 2ml quantity, out of which, 5 controls samples of healthy individuals of same age group were considered. 20 blood samples of radiation workers were considered for assay. Collected blood samples were analyzed using single cell gel electrophoresis (comet assay) and each sample was made into 3 slides (triplicates), from 3 slides atleast 100 cells were analyzed by using fluorescent staining method under fluorescent microscope with 20x or 40x magnification. The images are captured with the help of camera attached to the fluorescent microscope. The damage was assessed by visual scoring method where scoring to at least 50 cells was given. The scores were given from 0 to 1. The normal cells were scored as 0 and based on the damage of cells score is increased to the highest damaged cell as 1. The mean of all the cells was taken to establish the results.

Out of 20 samples only few samples of X-ray technicians showed the considerable damage of DNA. And other samples did not show such severe damages. This concludes that high exposure to radiation is main cause for DNA damage in selected population of X-ray technicians. In the course of our experiment we came across a variability in the extent of DNA damage among 20 exposed individuals which can be attributed to their occupation. Our experiment has shown a significant increase in amount of DNA damage in workers with the increase in duration of exposure.

Acknowledgement

The Authors are thankful to Department of Biotechnology and Microbiology, Karnatak University, Dharwad for providing necessary facilities for conduct of this experiment. The authors are also thankful to **University Science Instrumentation Centre (USIC)** for providing fluorescent microscope for study.

Conflict of interest

We wish to confirm that there are no known conflicts of

interest associated with this publication.

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