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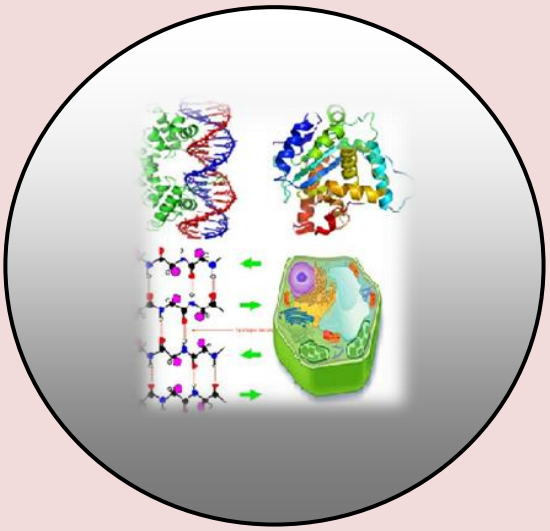

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RESEARCH PAPER

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Detection of DNA Crosslinking, A Direct Comparison Between Alkaline Comet Assay and DNA Diffusion Assay

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ABSTRACT

DNA crosslinking is a deleterious event that often leads to genomic instability and cell death. Crosslinkers are chemical mutagens that bind irreversibly to the DNA and interfere with the replication process resulting in gene loss. Therefore, early detection is critical to understand the inherent mechanism of DNA damage which involves crosslinking as a mutational phenomenon. Single cell gel electrophoresis or alkaline comet assay are long being used to detect crosslinking, however, the entire process is time-consuming and may result in artifacts due to prolonged alkaline exposure to the genetic material. In this study, we present DNA diffusion assay as an alternative of comet assay, which is rapid and devoid of alkaline exposure to the DNA. Formaldehyde and cisplatin are used as model crosslinkers, while methyl methanesulfonate (MMS) is used as standard DNA strand breaking agent. Human lymphocytes are taken as a cellular system to study the efficacy of DNA diffusion assay for the detection of crosslinking. The statistical analysis indicated DNA diffusion assay as a suitable alternative of comet assay, however, further research is warranted.
Keywords:DNA Crosslinking, Comet assay, DNA diffusion assay, Cisplatin and Formaldehyde.

INTRODUCTION

DNAcross-linking is a covalent linkage between the complementary strands of DNA duplex and/or protein or between the bases of a single strand of DNA. It is a kind of DNA damage, whichis extremely difficult to repair, often lead to genomic instabilityand cellular death[Murnane and Byfield, 1981; Lawley and Phillips, 1996].In case of DNA, it is formed in between the intra-strands and inter-strands of the molecules [Noll et al. 2006]. However, the presence of DNA–protein crosslink is also common [Macfieet al. 2009] and is reported to have many forms depending on the protein types available during crosslinking [Barker et al. 2005]. Detection and measurement of DNA crosslinking are therefore essential for proper understanding of the mechanism of DNA damage and subsequent repair.

Most commonly, formation and repair of DNA cross-links is measured with DNA filter elution (alkaline elution) [Kohn et al. 1981] and SDS-KCl precipitation methods [Oilve, 1988]. However, these techniques are time-consuming and provide no information on the extent of damage at the individual cell level. Single cell gel electrophoresis (SCGE) or comet assay (alkaline) is a sensitive method for the detection of DNA crosslinking at individual cells [Pfuhler et al. 1996; Merk and Speit, 1999]; generally considered to have several advantages in comparison with other available methods.

Although sensitive, alkaline comet assay involves certain artifacts such as prolonged exposure in an alkaline environment may raise a chance to induce additional damage to the cellular DNA [Spanswick et al. 2002;

Kalaude et al. 1996]leading to false positive or negative results. Loss of small fragments (less than 50 kb) during electrophoresis is also possible in this method [Olive, 1999], as well. However, all such possibilities always open an avenue to envisage the efficiency of other easily available convenient methods (s), in parallel with the comet assay. In this study, we investigated"DNA diffusion" assay [Gichner et al. 2005; Singh 2005; Prabhavathy et al., 2006] to evaluate its effectiveness for the detection of DNA cross-linkingin comparison with alkaline comet assay on human peripheral blood lymphocyte cells. In this assay, low molecular-weight DNA fragments are allowed to diffuse in the agarose gel in all directions,followed byprecipitation with a mixture of spermine and ethanol and finally visualized in a fluorescence microscope by staining with a DNA-binding fluorescent dye (ethidium bromide). Cross-linking agents connect DNA with DNA and/or proteins; they would be expected to cause decreased diffusion. Cells are treated with a combination of cross-linker and an agent, which can induce DNA strand breaks. Hence, the extent of DNA cross-linking by a particular agent can be indirectly measured by analyzing the relative reduction of DNA diffusion, induced by a strand-breaking agent. We used methyl methanesulphonate (MMS, 70 μM) for strand-break induction andtested known crosslinkers were formaldehyde and cisplatin (250, 500 and 1000 μM).

MATERIALS AND METHODS

Chemicals

Normal melting point agarose (NMPA), low melting point agarose (LMPA), Tris-HCl, ethidium bromide, dimethyl sulphoxide and methyl methanesulphonate (MMS) were purchased from Sigma Aldrich. Formaldehyde and cisplatin were purchased from Qualigen Ltd. and Sigma Aldrich respectively. The rest of the chemicals, such as phosphate buffer saline (PBS), sodium chloride, and sodium hydroxide were purchased locally and were of analytical grade.

Test system

Human peripheral blood was collected from healthy male donors aged 20-25 years (non-smoker, nonalcoholic and not undergoing any medication) after obtaining the written informed consent. The blood was collected by venipuncture into Na-heparin coated vacutainers. Lymphocytes were isolated from fresh blood according to[Boyum 1976], with slight modifications. Fresh blood (1 ml) was diluted with an equal volume of PBS and was layered over 3 ml of Histopaque and centrifuged at 800g for 20 min. The buffy coat was aspirated into 3–5 ml of PBS and was centrifuged at 250g for another 10 min. The supernatant was discarded and the pellet was suspended in RPMI-1640 media. The viability of the cells were evaluated by trypan blue dye exclusion method[Tennant 1964], and the range of viable cells was above 90%. All experiments were conducted in accordance with the Institutional ethical guidelines.

Treatment

The isolated cells were treated with MMS (Final concentration: 70 μM) and with the cross-linking agents and then incubated for 2 hours at 37^{o}C. For the detection of cross-linking potency, three different methods have been followed- (I) A combined treatment of MMS (70μM) and the crosslinker agents (viz. formaldehyde andcisplatin) in increasing concentrations (250, 500 and 1000 μM) was carried out and incubated along with the controls (Both negative and positive) for 2 hours and 37^{o}C. MMS (70μM) treated cells were used as positive control; (II) In the second method, cells were treated for 1 hour with MMS (70μM), followed by centrifugation in 2000 rpm for 5 min and cells were resuspended in fresh media for further 1 hour incubation with crosslinkers (viz. formaldehyde, cisplatin) of different concentrations (250, 500 and 1000 μM); (III) A third protocol was performed with treatments with crosslinkers first of different concentrations (250, 500 and 1000 μM) for 1 hour, followed by centrifugation in 2000 rpm for 5 min and cells were resuspended in fresh media for further treatment of MMS (70μM) in crosslinker for 1 hour. After final incubation, the lymphocytes were centrifuged at 2000 rpm for 5 min and the cells were suspended in PBS. Two aliquots were prepared: one was immediately used for comet assay and the other was used for viability tests.

Single cell gel electrophoresis (alkaline comet assay)

Comet assay was carried out according to the method of [Singh et al. 1988]with minor modifications [Ghosh et al. 2010] Slides were prepared by mixing the cell suspension with 1% low melting point agarose; layered on slides base coated with 1% normal melting point agarose. Slides were prepared in triplicates per concentration. Slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mMTrizma base , 1% Triton X–100, 10% DMSO; pH 10) and kept at 4 °C for at least 1h. Followed by lysis the DNA was allowed to unwind in the electrophoresis buffer (300 mMNaOH, 1 mM Na_2EDTA; pH 13.5) for 20 min.

Electrophoresis was conducted at a constant voltage of 26 V and current of 300 ampat 4ºC. Slides were neutralized in 0.4 M Tris (pH 7.5) for 5 min and rinsed in distilled water.
The slides were stained with 50-75µl of ethidium bromide (EtBr; 20µg/ml) for five minutes and then rinsed in chilled water to wash off the excess stain. Slides were scored using an image analysis system (Kinetic imaging; Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters (N2.1). The microscope was connected to a computer through a charge-coupled device (CCD) camera to transport images to software (Komet 5.5) for analysis. The final magnification was 40X. The parameters studied were tail extent moment (µm) which gave us a clear indication of the extent of DNA damage induced by the test chemicals. Images of 150 (50 x 3) cells per concentration were analyzed.

DNA diffusion assay
DNA diffusion assay was conducted as per the protocol of Singh2005 with minor modifications [Gichner et al., 2005].Slides were immersed in cold lysis solution at pH 10. The lysing solution consisted of 2.5 M NaCl (Sigma-Aldrich, USA), 100 mM Na_2EDTA (Sigma-Aldrich, USA), 10 mMTrizma base (Sigma-Aldrich, USA), 1% Triton X–100 (Merck, India), 10% DMSO (Sigma-Aldrich, USA) and kept at 4°C for 60 min. After lysis, the DNA was allowed to unwind in the electrophoresis buffer (300 mMNaOH (SRL Chemicals, Mumbai, India): 1 mM Na_2EDTA (pH 13.5) for 20 min. Slides were then immersed in freshly prepared 0.4M Tris (pH 7.5) in 50% ethanol with 1mg/ml of spermine for 30 minutes. This step was repeated once more. After washing in cold double distilled water, the slides were stained with 75µl of ethidium bromide (EtBr; 20µg/ml) for five minutes and then rinsed in chilled water to wash off excess stain and analyzed. Slides were scored using an image analysis system (Kinetic imaging; Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters (N2.1).

Statistical analysis
For statistical analysis median values of each concentration with respect to the comet parameters were calculated and one way analysis of variance (ANOVA) test was done by using Sigma Stats.3 software (SPSS Inc., Chicago, Illinois, USA). 2 sample t-test was performed for comparing the results between comet and DNA diffusion assays. For all statistical analysis, the level of significance was established at $P \leq 0.05$.

RESULTS
The sensitivity and reproducibility of the comet assay for quantifying DNA cross-linking in human lymphocytes were initially determined. The lymphocytes were treated with 70µM methyl methanesulphonate (MMS) to induce an obvious increase in DNA migration in comet assay and also to increase mean nuclear area of the cells in DNA diffusion assay. Both formaldehyde and cisplatin clearly reduced the DNA migration incomet assay, caused by MMS treatment in a dose-dependent manner. Length of Tail Extent Moment (TEM) represents the extent of damage in cellular DNA.
Table 1 shows a consistent decrease of TEM with the increasing concentration of crosslinkers (*viz*., cisplatin, formaldehyde) along with MMS, using three different methods, as mentioned in materials and method. However methodII and IIIwere found to be less efficient than the co-treatment method (**I**),less efficiency in the reduction of % tail extent moment (%TEM:(Average {3 replicates} tail extent of the test substance - Average tail extent of negative control)/average tail extent of MMS] x 100) for both the compounds was observed as well (Fig.1). Owing to this reason, the methodI was only exercised for DNA diffusion assay.
Table:2 reveals a definite dose-dependent decrease in the mean nuclear area (μm^2) in DNA diffusion assay.
Figure2 represents a comparative account of comet assayand DNA diffusion assay in terms of%crosslinking[100-%TEM] for formaldehyde and cisplatin respectively. A concentration of 1000 µM in the method I was found to cause maximum %crosslinking (more than 90% and 85% in comet assay and DNA diffusion assay respectively).
Figure 3 and 4 ensure direct visualization of representative images of crosslinked nuclear DNA at different microscopic magnification (100X &400X respectively) for comet assay and DNA diffusion assay respectively.These images are also supplemented with pseudocolored images and graphs for proper understanding.A clear dose-dependent decrease in the tail moment and mean nuclear area can be observed in comet assay and DNA diffusion assay respectively.

DISCUSSION

Single cell gel electrophoresis (alkaline comet assay) has been widely used in the detection of cross-linking [Pfuhler et al. 1996; Merk and Speit 1999]because of a small number of cells, sensitivity, and reproducibility. In the present study, initially, we aimed to examine the effect of interaction between the respective crosslinker (cisplatin and formaldehyde) and DNA damaging/strand breaking agent (MMS). Three approaches namely co-treatment as well as sequential (incubation by crosslinking agents for initial 1 hr, followed by incubation with MMS in a crosslinking free media and vice-versa) were designed to study the interaction between compounds. Results obtained by using these three methods, all have shown fairly dose-dependent decrease of DNA migration. However, co-treatment protocol came up with a better result in terms of reduction in % tail extent moment (% TEM) for both the compounds when compared with other methods. This result suggested that probably formaldehyde/cisplatin –MMS interaction is somewhat critical to exhibit maximum cross-linking potential. Hence, we opted for the co-treatment procedure and the data gained were compared with the DNA diffusion assay results. Interestingly, formaldehyde showed more crosslinking capability than cisplatin.

Table 1.Comet assay: Effect of the combined treatment of MMS and different concentrations of formaldehyde/cisplatin on tail extent moment (TEM) of human lymphocyte cells; FA: Formaldehyde * statistically significant difference from control (p≤0.05).

Concentration (µM)						
0	250		500		1000	
	FA	Cisplatin	FA	Cisplatin	FA	Cisplatin
5797.43 ± 471.70	3387.70 ± 348.00*	4089.42 ± 286.18*	2925.00 ± 335.00*	3664.60 ± 399.44*	2846.34 ± 48.30*	3262.64 ± 195.29*

* statistically significant difference from control (p≤0.05)

Table 2. DNA diffusion assay:Effect of the combined treatment of MMS and different concentrations of formaldehyde/cisplatin on the mean nuclear area (µm2) of human lymphocyte cells, using the co-treatment method; FA: Formaldehyde.

Method	Concentration (µM)						
	0	250		500		1000	
		FA	Cisplatin	FA	Cisplatin	FA	Cisplatin
1	9.27 ± 0.75	0.61 ± 0.05*	1.67 ± 0.05*	0.18 ± 0.05*	1.56 ± 0.12*	0.06 ± 0.02*	0.19 ± 0.02*
2	5.03 ± 0.27	3.45 ± 0.27*	2.57 ± 0.51*	0.49 ± 0.07*	1.53 ± 0.25*	0.1 ± 0.02*	1.37 ± 0.06*
3	6.00 ± 1.84	2.48 ± 0.09*	3.18 ± 0.15*	0.81 ± 0.01*	1.67 ± 0.51*	0.38 ± 0.08*	0.84 ± 0.17*

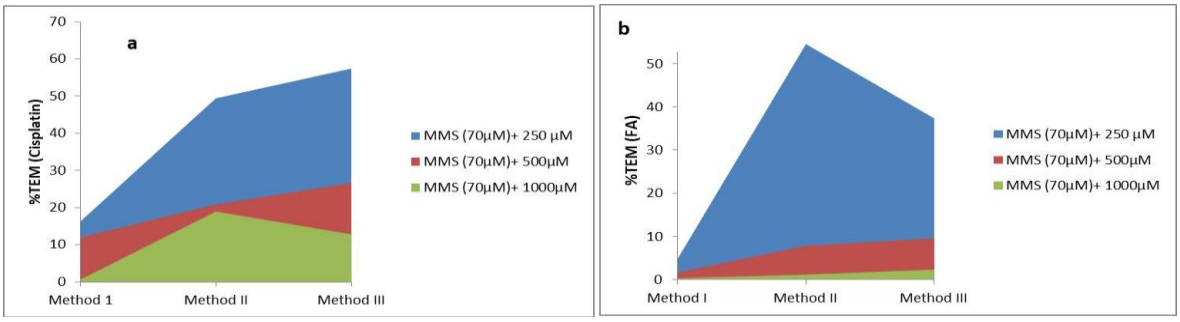

Figure 1.The values of DNA migration are given in percent of the tail extent moment (%TEM) induced by MMS, calculated by the formula: [(average tail extent of test substance-average tail extent of negative control)/average tail extent of MMS] x 100. Results are represented as mean of three independent experiments ± SEM.

(a) Effect of the combined treatment of MMS and different concentrations of cisplatin on % tail extent moment of human lymphocyte cells using three different protocols, as found in the comet assay.
(b) Effect of the combined treatment of MMS and different concentrations of formaldehyde (FA) on % tail extent moment of human lymphocyte cells using three different protocols, as found in the comet assay.
all values shown are statistically significant than control ($P \leq 0.05$)

MMS treated cells in the DNA diffusion assay (positive control) revealed completely diffused nuclear DNA with the large nuclear area and similarly in the comet assay significantly long tail was observed. These findings indicated that the observation in DNA diffusion assay is consistent and comparable with that of comet assay. While cells were treated with 250 to 1,000 µM of crosslinkers (*viz*. formaldehyde or cisplatin) combined with MMS, the comet assay images showed a gradual decrease in comet tail with an increase in brightness of comet head in consistent with the earlier literature [McKenna et al. 2003]. On the other hand, theresults of diffusion assay showed the increase in brightness and the decrease in diameter of the nuclear due to the formation of DNA-crosslinks.

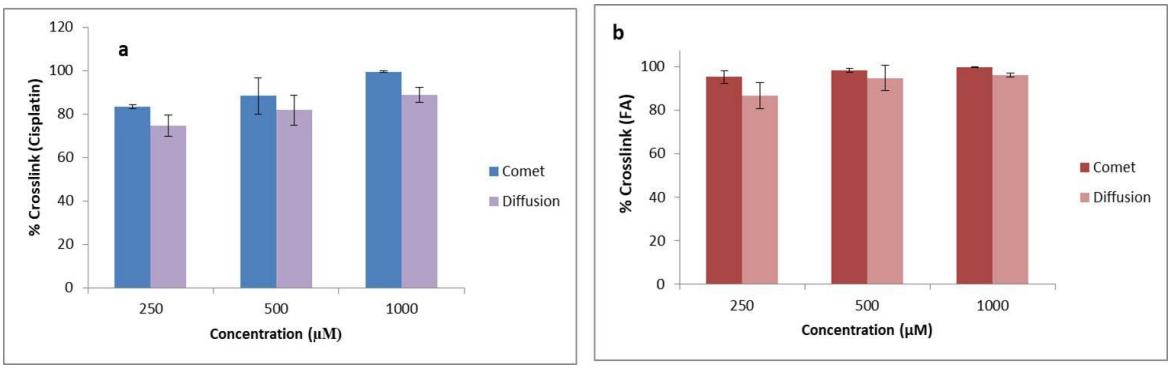

Figure 2. Comparative representation of % crosslinking, obtained from comet assay and DNA diffusion assay, calculated by the formula: [100-%TEM]. Results are represented as a mean of triplicate data ± SEM.
(a) Co-incubation of MMS and different concentrations of formaldehyde in human lymphocyte cells.
(b) Co-incubation of MMS and different concentrations of cisplatin in human lymphocyte cells.

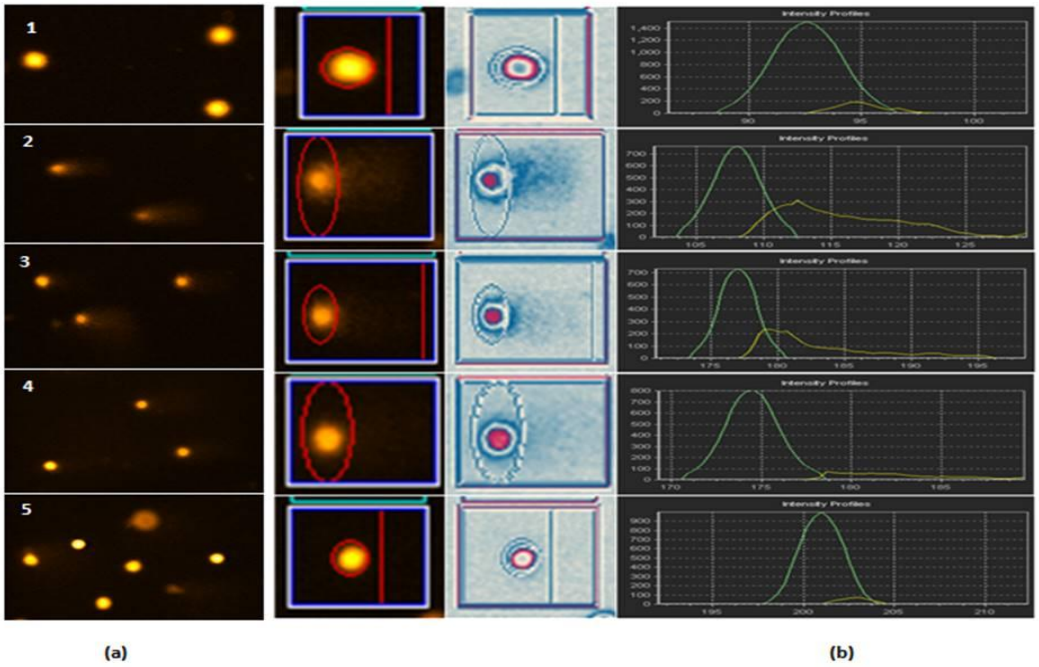

Figure 3. Single cell gel electrophoresis (modified alkaline comet assay) of lymphocyte cell DNA.

[1: negative control, **2**: 70µM MMS, **3**: 70µM MMS + 250 µM FA, **4**: 70 µM MMS + 500 µM FA and **5**: 70µM MMS + 250 µM FA**]**

(a) Representative images of individual lymphocyte cell DNA after electrophoresis (Column 1: 100X and Column 2: 400X)

(b) Individual comets are oriented with the head to the left. The image on the right of each (Column 3) shows a pseudocolor of the image with a graphic representation (Column 4) of staining intensity. This intensity is proportional to the amount of DNA at that location in the gel.

The parameters of the comet assay (TEM and %TEM) corresponded well with the DNA diffusion assay analysis. While the crosslinker concentration was increased to 1000 µM, the comet tail almost completely disappeared. In the comet assay, tail moment indicates the breakage of DNA strands [Pfuhler and Uwe 1996; Hartley 1999]. The disappearance of the comet tail, therefore, suggested that the DNA fragments were greatly reduced, hence fully crosslinked in this case[Pfuhler and Uwe 1996; Hartley 1999]. Similarly, in the diffusion assay protocol, the nuclear area is drastically reduced in 1000 µM of crosslinker-MMS (70 µM) combination when compared with the control, however slightly in a less efficient way that its comet counterpart (although statistically non-significant).

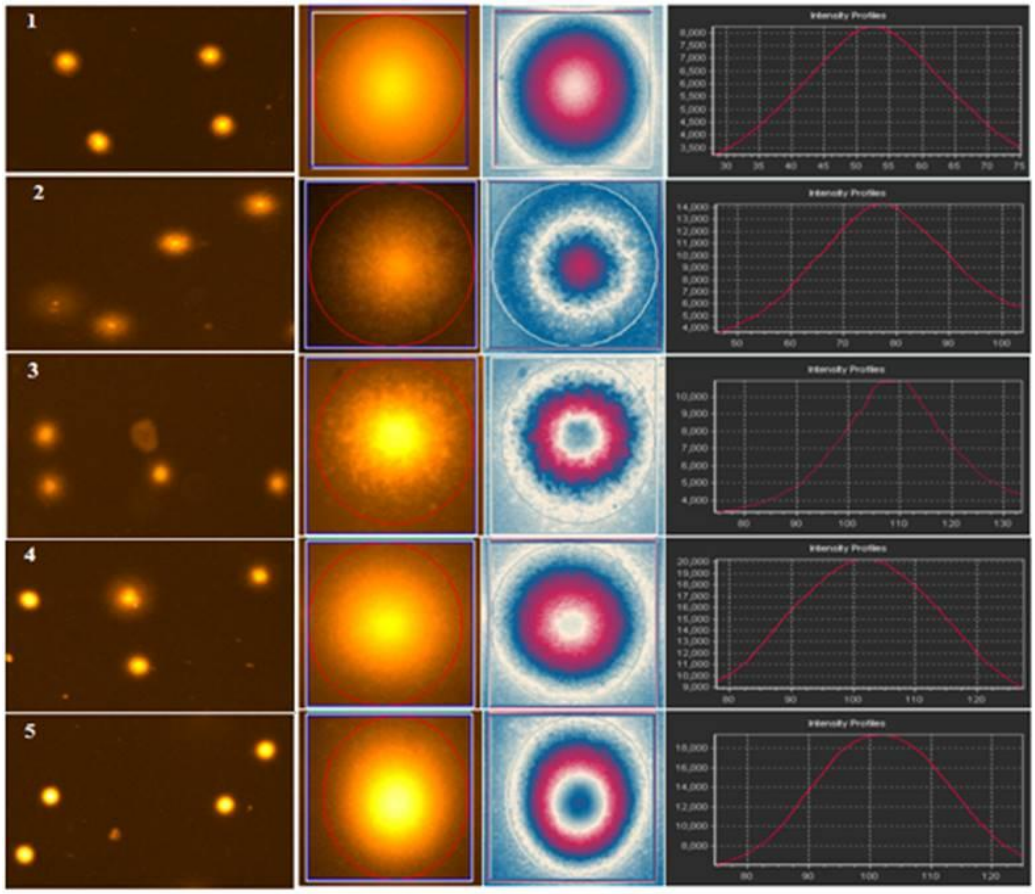

Figure 4.DNA diffusion assay of lymphocyte cell DNA.

[1: negative control, 2: 70 µM MMS, 3: 70µM MMS + 250 µM FA, 4: 70 µM MMS + 500 µM FA and 5: 70 µM MMS + 250 µM FA]

(a) Representative images of individual lymphocyte cell DNA after DNA diffusion assay (Column 1: 100X and Column 2: 400X)

(b) The image on the right of each shows a pseudocolor of the image (Column 3) with a graphic representation of staining intensity (Column 4). This intensity is proportional to the amount of DNA at that location in the gel.

The possible advantage of DNA diffusion assay can be less dependence on alkaline condition as well as complete exclusion of the electrophoresis step. This method completely eliminates alkaline electrophoresis condition. When DNA strand breaks take place, DNA fragments migrate out from the nucleus to form the comet tail. The migrated DNA includes short DNA fragments and long DNA strands or loop rings [Collins, 2004] as well as small crosslinked DNA fragments. However, small fragments of DNA are vulnerable to lose during electrophoresis[Olive 1999]. Bifunctional alkylating agents like cisplatin can form a number of DNA adducts, and most of them are monoadducts. Apurinic sites can result from these monoalkylations that can be converted to DNA strand breaks under alkaline conditions[Spanswick et al. 2002; Klaude et al. 1996]and could potentially interfere with the detection of DNA crosslinking by the comet assay. However, seems advantageous, the major constraint of this assay is its sole dependence on unforced diffusion, if affected result may be misleading.

In the current study, DNA diffusion assay showed comparable efficiency in the detection of % crosslinking than the alkaline comet assay for both the compounds (Fig 2). However, considering the need of further optimization, as of now this method can be used for initial screening for the detection of probable crosslinking agents followed by comet assay as the confirmatorytest.

CONCLUSION

The study established DNA diffusion assay as a suitable alternative for alkaline comet assay.However, validation will require investigation of protocol factors affecting detection (especially cell type, lysis, and exposure times) and modification of the conditions for precipitation (exposure, temperature, and duration) to assay substances requiring metabolic activation. Further investigations of the specificity and sensitivity of the assay and measurement of intra-laboratory and inter-laboratory variability are warranted.

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