DNA Damage in Leukocytes from Fanconi Anemia (FA) Patients and Heterozygotes Induced by Mitomycin C and Ionizing Radiation as Assessed by the Comet and Comet-FISH Assay

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ABSTRACT

Background: Lymphocytes of Fanconi anemia (FA) show an increased sensitivity to the alkylating agents such as mitomycin C (MMC), but their responses to gamma-irradiation is controversial. The extent of DNA damage in leukocytes of FA patients following irradiation and MMC treatment was studied at cellular and single chromosome level. **Methods:** DNA damage induced by gamma-rays and MMC was measured in leukocytes of FA patients and carriers at whole genome level using the comet assay. Also, at the DNA level of specific chromosome involved in this disease using a modified comet-FISH protocol with whole chromosome painting probes (chromosomes 16 and 13), DNA damage in leukocytes of FA patients and heterozygotes were compared to healthy individuals. **Results:** Baseline DNA damage in leukocytes of patients and heterozygotes was higher than in controls. Net induced DNA damage by gamma-rays in leukocytes of FA cases was not significantly different from that of healthy donors and heterozygotes. Net induced DNA damage by MMC was statistically higher and significantly different ($P<0.05$) in patients than other groups. Hybridization of chromosome 16 reveals more signals in the tail but the number of spots in the tail was not significantly higher than the hybridization spots for chromosome 13 in both gamma-irradiated and MMC treated samples. **Conclusion:** Results indicate that DNA damage induced by MMC could be a better index for diagnosis of FA patients compared to gamma-rays. Results of comet-FISH showed no difference between the sensitivity of chromosome 16 and 13 to MMC and radiation. It may indicate that, although the FA-A gene is located on chromosome 16, this chromosome might have a similar sensitivity as other chromosomes. *Iran. Biomed. J. 13 (1): 1-8, 2009*

*Keyword*s: DNA damage, Fanconi anemia patients, Mitomycin C, Gamma-rays, Comet assay

INTRODUCTION

To maintain genomic integrity, cells have developed various repair mechanisms specific for each type of lesion. Genetic diseases related to a defect in a DNA repair pathway generally show hypersensitivity to one or several DNA damaging agents. Fanconi anemia (FA) is an autosomal recessive disease described as an instability syndrome with cellular hypersensitivity to DNA cross-linking agents [1]. The hallmark of the FA cellular phenotype is a high level of spontaneous chromosomal breakage [2] that is enhanced by exposure of the cells to DNA cross-linking agents such as mitomycin C [3] and diepoxybutane [4]. In addition, FA cells have a cell cycle disturbance [5] and have increased susceptibility to oxygen-induced damage [6]. A less-known and still controversial feature of FA cells is their radiosensitivity [7-9]. It is recognized that individual with risk of cancer may be related to genetically determined differences in the ability of a cell to identify and repair DNA damage. Also, it has been suggested that individuals who are genetically susceptible to cancer manifest this by exhibiting increased DNA radiosensitivity [10]. By using linkage analysis, it was possible to map the FA-A gene to chromosome $16q^{24.3}$. This group consists approximately 65-70% of total FA patients [11]. However, up to now most of the investigations on the sensitivity of FA cells were

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focused on chromosomal aberrations studies. Chromosome aberrations are cytogenetic end points after un-repaired or mis-repaired DNA damage mainly double strand breaks (DSB). Therefore, these are considered as processed DNA damage. Initial DNA damage induced by chemicals or ionizing radiation at whole genome level or specific genomic region might serve as a diagnostic end point and valuable tool for studying sensitivity of cells from FA patients to DNA damaging agents. Research on DNA damage induced by agents such as ionizing radiation, environmental toxins, DNA binding substances or therapeutics has been performed during the last years with the comet-assay. This technique, first described by Ostling and Johanson [12] in 1984 and in a modified version by Singh *et al.* [13] in 1988, allows easy and fast visualization and measurement of overall DNA damage on the level of the whole genome of single cells. Through the variation of lysis, electrophoresis buffers and electrophoresis conditions (variation of pH and ionic strength) different DNA damages such as single strand break or DSB can be monitored [14-17]. The comparison of the intensities of tail DNA and head DNA under a conventional fluorescence microscope allows quantification of the DNA damage, which can today be performed with automated software systems. With the combination of the comet-assay and fluorescence *in situ* hybridization [18-20], termed comet-FISH [21], specific genomic regions can be examined in the comet-assay which allows measuring sequence specific DNA fragmentation on an intermediate scale (10-800 kb). This assay is a combination of the comet assay with fluorescence *in situ* hybridization. Up to now, several hybridization procedures including those of DNA whole chromosome painting probes [22, 23], gene-specific probes and centromere probes [19, 24] have been described. Thus, specific sequences can be located in the head or the tail of the comet. By counting the ratio of cells with signals in the head and those with signals in the tail, the sensitivity of the specific genomic regions towards damaging agents can be calculated. In this way, this technique allows rapid and easy screening of DNA breakage sensitivity after exposure to various kinds of damaging agents on the level of individual cells, and is a good tool to perform studies on region/ locus specific DNA damage distribution in the human genome [22]. In the present study, MMC and gamma-ray induced DNA damages in leukocytes of FA patients and their obligate heterozygotes were measured by alkaline comet assay. Moreover, comet-FISH was used with two different whole chromosome painting probes (for chromosomes 16 and 13) to study whether gamma-ray and MMC-induced DNA damages in these chromosomes are distributed randomly all over the genome or occur at preferable sites in these study groups. Chromosome 16 was selected because the FA-A gene is located on it, and chromosome 13 as a control chromosome.

MATERIALS AND METHODS

Study subjects, blood cells and treatments. Five FA patients, aged between 4.5 and 15 years (mean age = 8.5 ± 4.13) were recruited at Ali Asghar Children Hospital in Tehran. None of them had been treated with chemotherapy or radiotherapy. On the basis of chromosome breakage after mitomycin C treatment, the clinical diagnosis of FA was confirmed in all ten patients. Five FA obligate carriers, aged between 33 and 39 years (mean age = 35.60 ± 2.19) were also examined. Five phenotypically normal non-smoker healthy individuals aged between 28 and 41 years (mean age = 35 ± 5.09), who hadn't received any antibiotic or drug for at least 2 months were randomly selected regardless of sex, race or age to serve as the control group. The study was approved by the Ethical Committee of the School of Medical Sciences of the Tarbiat Modares University (Tehran). Patients gave their informed written consent. All donors completed a written questionnaire to obtain information related to their life style, such as dietary habits, medical history and exposure to chemical and physical agents.

Heparinized venous blood (7.5 μl) was added to the 1.5-ml micro centrifuge tube containing 1 ml RPMI 1640 medium (Gibco, BRL) supplemented with 10% fetal calf serum (Gibco, BRL). Diluted blood obtained from controls, FA patients and hetrozygotes were irradiated on ice with gamma-rays at a dose of 4 Gy with a dose rate of 2.75 Gy/min generated from a ^{60}Co source (Theratron 780E, Canada). This radiation dose was selected using the dose response curve constructed for a healthy individual showing intermediate levels of damage in cells in our previous study [25]. For MMC treatment, all blood samples were treated for 2 h with MMC (Sigma, Aldrich) at 37°C. The MMC concentration was 5 μg/ml and was chosen according to the literature with minimal cytotoxic effects on control cells [26, 27].

Slide preparation and alkaline comet assay. After irradiation, the samples were centrifuged at 0°C. The supernatant was poured off and the pelleted cells were mixed with 100 µl of 37°C LMP, low melting point agarose (Merck, Germany, 0.75% agarose in PBS, Ca^{2+}, Mg^{2+} free). The cell mixture was added to the windows made on frosted slides with clear windows, precoated with 1% normal agarose (Merck, Germany) and was immediately covered with a coverslip. The slides were placed on a tray kept on ice for 10 min to solidify. After solidification, the coverslip was removed.

Slides were then submersed in an alkaline lysis solution (2.5 M sodium chloride, 100 mM EDTA, 10 mM Tris base, 10% dimethylsulfoxide, 1% sodium N-lauroyl sarco-sinate, and 1% Triton X-100, pH 10 (Merck, Germany) at 4ºC for 1 h. Lysis was followed by unwinding step by immersing the slides in a freshly prepared alkaline solution, (0.3 M NaOH and 1 mM EDTA, pH>13Merck, Germany) in a horizontal-gel electrophoresis tank (SEU-7305, Paya Pajouhesh, Iran) at 4 ºC for 40 min. Electrophoresis was done at 0.75 V/cm at 4ºC for 30 min. The slides were washed three times in neutralization buffer (400 mM Tris buffer, pH 7.5) and rinsed in ethanol for 5 min and air dried. Cells were stained with 20 µl ethidium bromide (2 µg/mL, Merck, Germany) under a coverslip. Observations were made at a magnification of ×200 using a Nikon E 800 epifluorescence microscope equipped with 546-516 wave length band and a 590 nm barrier filter attached to a CCD camera. The comets were analyzed by visual classification [28] and for each sample 1000 cells were scored. Visual analysis of the induced comets under the microscope revealed distinct differences in shape and length between the applied mutagens. Gamma-rays induced longer and thinner comet tails than did MMC (which induced rather short and more voluminous tails). Damage was assigned to five classes (0-4) based on the visual aspect of the comets (Fig. 1), considering the extent of DNA migration according to the established criteria [29-31]. Comets with a bright head and no tail were classified as class 0 (cells with no DNA migration) and comets with a small head and a long diffuse tail were classified as class 4 (severely damaged cells). Comets with intermediate appearance were classified into classes 1, 2 and 3. Damage scores were calculated based on the following equation adopted from Jaloszynski *et al.* [28] that ranged from 0 to 400 arbitrary units, corresponding to situations ranging from no damaged comets to all comets extremely damaged:

$$DD\ (au) = (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4) / (\Sigma n /100)$$

Where DD (au): Arbitrary unit DNA damage score, n_0-n_4: number of Class 0-4 comets, Σn: total number of scored comets.

Coefficients 0-4 are weighting factors for each class of comet. One may suspect that the visual classification may be inferior to computerized analyses, such as tail moment analysis of images captured by CCD camera. However, it has been clearly shown that there is no statistical difference between visual quantification and image analysis by computer for tail moment quantification [25, 29]. However, to verify the validity of results obtained by visual analysis, pictures of 100 cells for each MMC treatment, captured by CCD camera were analyzed automatically using COMET IV software. COMET is a tool to image analysis in comet assay and has been developed to work with either color, or gray-scale images of fluorescence-stained comets.

Comet-FISH. To do comet-FISH, the slide preparation was similar to the comet assay. Before hybridization, the gels were stored in absolute ethanol for dehydration at 4°C for at least 3 days. The gels were rehydrated in H_2O for 15 min and subsequently the DNA was denatured by incubation in 0.5M NaOH for 25 min. The denatured DNA was immediately dehydrated in an ethanol series (75, 80 and 95%, 5 min each) and the gels were carefully air-dried until all ethanol had evaporated. Whole chromosome probes (Cytocell, UK) were used according to the instructions of the manufacturer. Since thermal co-denaturation was not possible with agarose gels, 10 µl of the hybridization probe was pre-warmed to 60 ºC then applied to the slide on an area of ~20 × 20 mm. The gels were sealed with a plastic coverslip and the slides were placed in a humidified chamber at 37ºC overnight. The next
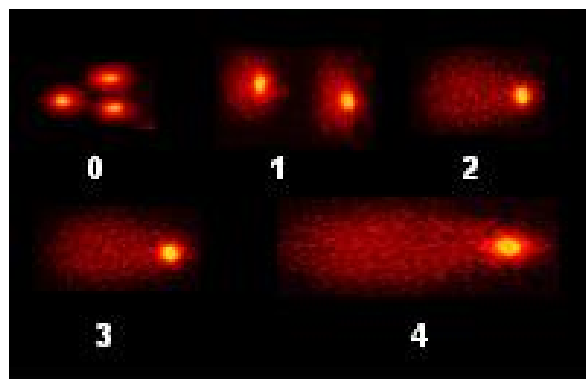

Fig. 1. Photomicrographs of comets showing classifications of comets based on DNA migration (magnification ×200).

day, the slides were placed at room temperature for 30 min. The coverslips were carefully removed and the slides were washed in SSC solution (1-2×) at 60ºC for 5 min. Finally, the slides were immersed in ice-cold phosphate buffered detergent. For counterstaining, the slides were embedded in 1:2000 diluted DAPI (Cytocell, UK) including 50% antifade. The number of signals and the localization of the signals (comet head or comet tail) were additionally recorded for each cell. A double band pass filter set (Chroma HiQ, Japan), tuned for DAPI (excitation 370 nm, emission 450 nm) was utilized to observe comets with probe signals under E800 fluorescent microscope.

For all used probes, hybridization on metaphase spreads performed before the probes was used in comet-FISH to check their quality, stringency and the optimal hybridization conditions. The hybridization on metaphase chromosomes was performed according to the standard cytogenetic protocols on methanol acetic acid fixed chromosomes and nuclei [32].

In order to quantify different distribution of probes, we have introduced a classification of the hybridization patterns according to Figure 2. In addition to the standard comet parameters, measured as described above, the number and location of hybridization spots within each comet were also recorded. In a typical experiment, approximately 50-100 cells are evaluated per microscope slide. The number of cells counted depended on the quality of
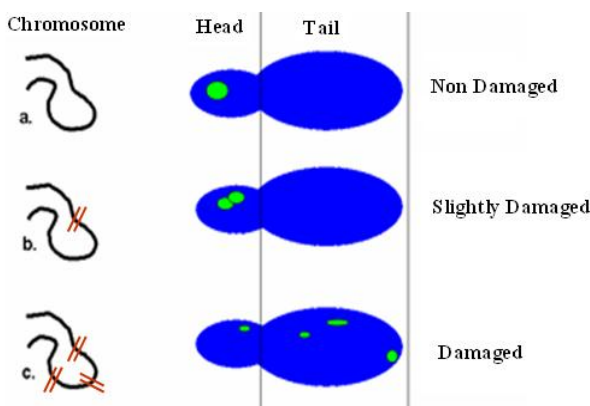

Fig. 2. Schematic overview of possible breakage patterns and the resulting hybridization signal distribution after comet-FISH. (**a**) Represents a completely undamaged chromosome, which results in only one signal in the head. This case would be correctly judged as 'undamaged'; (**b**) shows a low level fragmentation, which results in large unmovable fragments that form a fragmented cluster in the head. This case will be judged as undamaged, whereas it is slightly damaged; (**c**) shows a damaged chromosome with signals clustered in the tail. This pattern would be correctly interpreted as 'damaged'.

the gel matrix and the location of the comets. The number of comets counted was not dependent on the probe, but different from slide to slide. To achieve statistical security, a minimum number of 150 cells were analyzed for each treatment. Typical photomicrographs of comet-FISH images are shown in Figure 3.
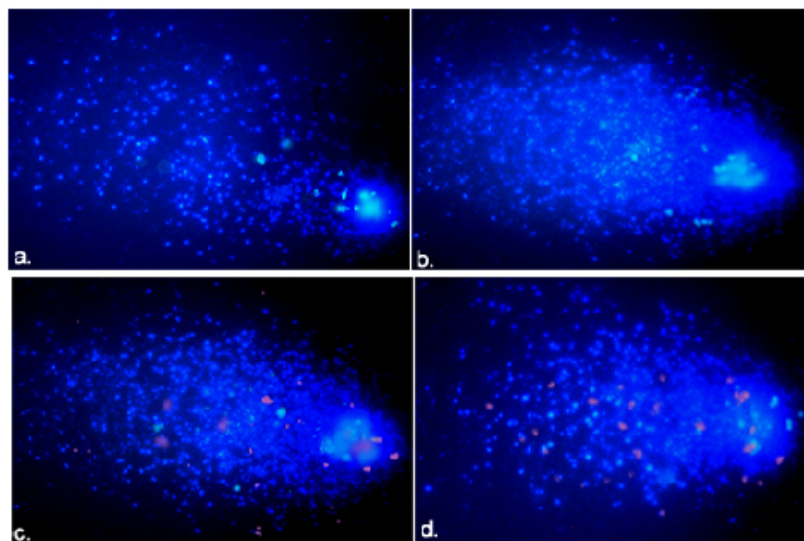

Fig. 3. Typical photomicrographs of comet-FISH images after hybridization with whole chromosome probes and treatment with gamma-rays and MMC. Panels a and b show typical damage in chromosome 13 following 4 Gy γ-radiation (**a**) and MMC treatment (**b**). Panels **c** and **d** show damage in chromosomes 13 (green spots) and 16 (red spots) following gamma irradiation (**c**) or MMC treatment (**d**).
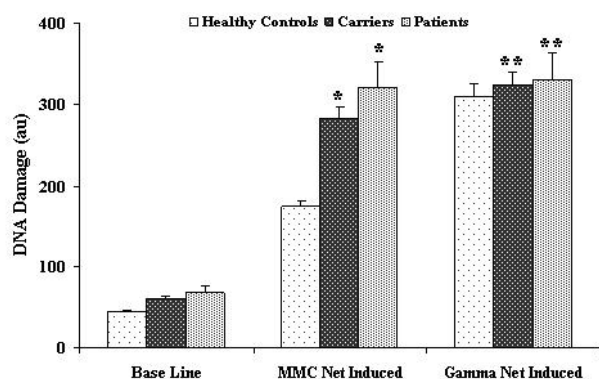
Fig. 4. Visual analysis of baseline (DD_0) and net induced DNA damage ($DD-DD_0$) in leukocytes of healthy controls, carriers and FA patients measured by the alkaline comet assay following treatment with either 5 µg/ml of MMC or irradiation with 4 Gy gamma-rays. Error bars show standard deviation from mean values. * Significantly different from control ($P<0.05$); ** not significantly different from control ($P>0.05$).

Statistical analysis. All statistical analyses were carried out using SPSS software version 15. Differences among means of initial and induced DNA damage among groups were tested for significance with the two-sided, unpaired Student's *t*-test. To analyze the results of DNA damage among groups, the non-parametric Mann-Whitney U-test and one way analysis of variance (ANOVA) were also used. *P* value of less than 0.05 was considered as significant level.

RESULTS

The comparison of initial MMC and radio-sensitivity of FA patients and carriers with healthy volunteers. As shown in Figure 4, non-irradiated cells of FA patients exhibited noticeably higher baseline amounts of DNA fragmentation. The mean values of background DNA damage were statistically different in the FA patients and healthy individuals using alkaline comet assay ($P<0.05$). Induced DNA damage following 4 Gy gamma irradiation showed identical median and mean values for the controls and the patients ($P<0.05$). Net induced DNA damage was higher in FA patients than carriers and healthy controls, but this failed to reach statistical significance. However, as show in Figure 4, net induced DNA damage by MMC is significantly different for three studied groups, indicating different sensitivity of leukocytes from normal and carrier individuals and FA patients to MMC and gamma-rays.

Tail migration induced by MMC in leukocytes of FA patients and carriers with healthy controls. Results are shown in Figure 5. As seen, non-treated cells of FA patients exhibited noticeably higher baseline amounts of DNA fragmentation. The mean values of background DNA damage were statistically different in the FA patients and healthy individuals using alkaline comet assay ($P<0.05$). Induced DNA damage by MMC showed identical median and mean values for the controls, heterozygotes and the patients ($P<0.05$). DNA damage after MMC treatment was also higher in heterozygotes than in normal individuals ($P<0.05$).

Effect of irradiation and MMC on individual chromosomes. The results of mean signal numbers in the tails of the comets are summarized in Table 1. From the distribution of the whole chromosome painting, FISH signals in the comet tail or head, a correlation of damaged versus undamaged DNA in the respective chromosome can be evaluated. Comet-FISH can detect all DNA breaks (single- or double-strand) that exceed a given detection limit, defined by the minimum size of the fragments that can be separated from the un-fragmented DNA within the electric field. As this limit is similar to all DNA fragments from all chromosomes, the counted cases can be correlated to an induced number of breaks. As an example, the image of Comet-FISH for a chromosome 13 painting probe (green spots) and chromosome 16 (red spots) is shown in Figure 3. The labeled sites are nearly always located in the head, showing the typical shape of chromosome territories known from interphase FISH [33, 34] on standard microscope slides. Result of hybridization
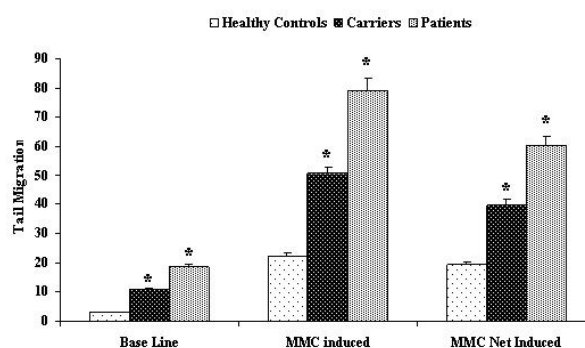

Fig. 5. Computerized analysis of baseline (DD_0), induced (DD) and net induced ($DD-DD_0$) Tail Migration in leukocytes of controls, carriers and FA patients measured by the alkaline comet assay following treatment with 5 µg/ml of MMC. Error bars show standard deviation from mean values. *Significantly different from control ($P<0.05$).

Table 1. Mean numbers of chromosome 13 and 16 signals in the tails of comets in different groups and different treatments.

Samples	Chromosome 16 Signals	Chromosome 13 Signals
Control + MMC	19.48 ± 5.01	17.63 ± 2.21
Control + Gamma-ray	15.37 ± 3.32	14.29 ± 5.64
Patient + MMC	46.53 ± 2.90	43.11 ± 4.81
Patient + Gamma-ay	19.91 ± 6.27	16.26 ± 7.15

of comets with whole chromosome probes used in this study does not show a significantly higher number of spots for chromosome 16 than the hybridization spots for chromosome 13 in both gamma-radiated and MMC treated samples (Table 1).

DISCUSSION

Mitomycin C (MMC) is an alkylating agent that mainly induces DNA-DNA interstrand cross-links [5]. MMC is regarded as an important chemotherapeutic agent and is widely used in treatment of some cancers and diagnosis of FA patients [35]. Under our conditions, its specificity was sufficient to be employed as a primary diagnostic test in the differential diagnosis of bone marrow failure, because no false negative or positive results were seen in our research using comet assay. After using alkaline comet assay, the results show that the patients have higher baseline values than controls. Other studies have also reported higher levels of baseline DNA damage in blood and lymphocytes of cancer cases than in benign controls [25, 36, 37]. Persistent basal DNA damage may reflect higher exposure to carcinogens and deficient DNA repair [37]. It could be argued that pre-existing DNA damage, as indicated by increased spontaneous chromosome breakage rates in FA, may be responsible for the high initial DD values in patients' and carriers' cells. Moreover, such an interpretation is encouraged by the higher DD of both the FA patients and heterozygotes at a zero radiation dose. Although metaphase analysis after MMC treatment, cannot detect heterozygotes, comet assay showed a statistically higher DD in heterozygotes than normal individuals. It seems comet assay can be a trustable method for screening of carriers after MMC treatment (Figs. 4 and 5). After irradiation *in vitro*, there was a trend toward an increased induced DNA damage level (DD) in the cells from FA patients, but this failed to reach statistical significance (Fig. 4) which may be in part

due to the inter-individual variability. In addition to the comet data generated, we can also examine the number and position of hybridization spots within the comet to provide information about damage of the probed region (Fig. 3).

The modified protocol for comet-FISH [20, 21], described in this paper, is appropriate to perform hybridization with whole chromosome painting probes. This makes comet-FISH a fast and easy method to study stabilities of specific chromosomes towards DNA damaging agents. However, in interpreting comet–FISH results we must take into account the amount of DNA that can migrate into the tail in response to one strand break under the conditions of a given comet assay experiment and also we can estimate this as follows. Breaks induced by ionizing radiation in mammalian cells are, essentially random at the macro-sequence level [38]. A dose of 1 Gy gamma-rays induces 0.31 breaks per 10^9 Da DNA [39], therefore with an average base pair mass of 622 Da; 2Gy induces 0.39 breaks per Mb, or 1 break per 2.6 Mb. Furthermore, 2Gy drives on average 10% of the DNA into the comet tail. Hence, each break drives a region of approximately 260 kb into the tail, assuming both strands of the region affected go into the tail, or a region of 520 kb, if only the strands with free ends derived from the break migrates (it is still disputed in the comet community as to which of these assumptions is correct). These are estimates of the average amount of DNA moving into the tail.

We know that DNA from regions closely and extensively associated with the nuclear matrix, such as replicating DNA, does not move into the tail in standard alkaline comets even though it must be adjacent to free ends [40]. In our view, the different molecular mechanisms of action of the agents examined are reflected in the outcome of our experiments indicating that gamma irradiation and MMC induced comets of different shapes and sizes (Fig. 3.). These differences were also reflected in the distribution of signals in the heads and tails of the comets (Table 1). While after treatment of the cells with gamma irradiation sharp signals were found spread over the whole tail, a considerable number remains close to or within the head in the shorter comets after MMC treatment. These findings confirm previous data obtained by other authors with these standard mutagens.

While Anderson *et al.* [41] suggested that bleomycin which is a radiomimic agent can react through oxygen radical mechanisms, while MMC mainly induces DNA-DNA inter-strand cross-links

[42]. Pfuhler and Wolff [26] found an increase in DNA migration in the comet assay when MMC was added to human whole blood at a concentration of 100-800 µmol. However, McKenna *et al.* [43], using the comet-FISH technique in RT4 human bladder cancer cells, reported a decrease in signals in the tail with increasing MMC dose [43]. They suggested that the effect of reduced DNA, as well as hybridization signals in the tail, was due to increase cross-linking, which prevents DNA migration. This was supported by data on the role of inter strand cross-links in the reduction in free DNA fragments, mainly detected under alkaline conditions [16, 44].

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