

Comet assay in the assessment of the human genome damage induced by γ -radiation *in vitro*

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Background. The aim of the present study was to estimate a possible application of comet assay in the evaluation of DNA damage caused by different gamma radiation doses in peripheral human lymphocytes *in vitro*.

Materials and methods. Whole blood samples of young healthy, non-smoking donors were taken. The samples were divided in 4 specimens. The first specimen was used as the control. Other three specimens were irradiated using constant gamma irradiation source (⁶⁰Co) giving the dose rate of 0.907 cGy/s. Different specimens were irradiated for 51 s, 437 s and 1099 s, giving the doses of 0.5 Gy, 4 Gy and 10 Gy. In order to estimate dose-response curve on the control and all 3 irradiated whole blood samples, the comet assay under alkali conditions was performed.

Results and conclusions. The comet assay endpoints showed statistically significantly higher values for all irradiated blood samples compared to the control. For both, tail length and tail moment, dose-effect relationship was found to be linear in a dose range of 0.5Gy and 10 Gy. By this work we also pointed out possible usage of the comet assay in the detection of DNA lesions caused by extremely high radiation dose, which is not possible by using standard cytogenetic methods.

Key words: lymphocytes-radiation effects; DNA damage; comet assay

Introduction

In radiobiology there is always a need for the development of new rapid and more sensitive methods for DNA damage evaluation. So far,

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an analysis of structural chromosomal aberrations and micronucleus test have had great value in radiation biomonitoring.^{1,2} Recently, the comet assay, also called the single-cell gel electrophoresis (SCGE) assay appeared as new method of choice because it is a rapid and sensitive method for the detection of various DNA damages (strand breaks and alkali-labile sites) in individual cells, induced by a variety of genotoxic agents. Since radiation may cause SSB, DSB, DNA-DNA as well as DNA-protein crosslinkings and damage to bases, all detectable by comet assay, this method could provide information on the to-

tal DNA damage caused by ionizing radiation. This is not the case for standard cytogenetic methods that, due to heterogeneity in genome damage caused by ionizing radiation, provide only average DNA damage information.³

Comet assay was first introduced by Östling and Johanson⁴, and later modified independently by Singh *et al.*⁵ and Olive *et al.*⁶. The assay is based on the embedding of cells in agarose, their lysis in alkaline buffer and finally subjection to an electric current. The electric current pulls the charged DNA from the nucleus so that relaxed and broken DNA fragments migrate further from the nucleus than intact DNA. The resulting images, named for their appearance as comets, are measured to determine the extent of DNA lesion. Image analysis provides three important parameters for each comet: tail length, tail fluorescence intensity (percent of DNA in tail) and tail moment (roughly, the product of tail length and tail intensity).³

Although there were many papers considering dose-DNA damage relations for structural chromosome aberration analysis, until recently only few authors investigated those effects applying the comet assay^{2,6-9}, which is essential for better understanding and interpretation of the results in the field of radiation biomonitoring obtained by this method.

In the present paper, possible application of comet assay in the evaluation of DNA damage caused by different gamma radiation doses in peripheral human lymphocytes *in vitro* was studied. We also wanted to point out possible usage of the comet assay in the detection of DNA lesions caused by extremely high radiation dose, which is not possible by using standard cytogenetic methods. On the basis of these results using polynomial regression dose, a response curve for tail length and tail moment as comet assay endpoints was plotted.

Materials and methods

In vitro whole blood sample irradiation

Two whole blood samples of young healthy, non-smoking donors from the cubital vein by using heparinized syringes were taken. Twelve months before blood sampling donors were not exposed to any physical or chemical agent that might interfere with the results obtained by radiation. Immediately after the sampling, the blood from each donor was divided in 4 specimens. The first specimen was used as the control. Other three specimens were irradiated using Gammacel irradiator, Model 220, with constant gamma irradiation source (⁶⁰Co) giving the dose of 0.907 cGy/s.¹⁰ Different specimens were irradiated for 51 s, 437 s and 1099 s, giving the doses of 0.5 Gy, 4 Gy and 10 Gy.

Description of gamma irradiation source

The ⁶⁰Co source consists of 48 linear source elements equidistantly spaced in a stainless steel rack to form a cylindrical shell or annulus, with a diameter of 20,9 cm, measured between the centers of opposing elements. Each linear element consists of a welded stainless steel pencil filled with ⁶⁰Co in the form of metallic cobalt. Internal dimensions of each pencil are 1 cm in diameter and 20.3 cm in length.¹⁰

The drawer is centrally located in the radiation shield and is power driven vertically through the center of the source. The material to be irradiated is placed in the sample chamber, then lowered to the irradiation position, *i.e.* the sample chamber is then in the center of the source.¹⁰

Comet assay

After the irradiation the blood was put in ice and transferred to the laboratory. In order to estimate dose-response curve on the control and all 3 irradiated whole blood samples, the comet assay was immediately performed. The comet assay was conducted under alkali con-

ditions according to Singh *et al.*⁵ All chemicals used to perform the comet assay were obtained by Sigma. Two μ l of whole blood were suspended in 0.5% low melting agarose and sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. During the polymerization of each gel-layer, the slides were kept on ice. After the solidification of 0.6% agarose layer, the slides were immersed in lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%) at 4°C. After 1 hour, the slides were placed in electrophoresis buffer (0.3 M NaOH, 1 mM Na₂EDTA, pH 10) for 20 minutes at room temperature to allow for DNA unwinding. Electrophoresis was conducted in a horizontal electrophoresis platform in fresh, chilled electrophoresis buffer for 20 minutes at 300 mA and 19 V. The slides were neutralized with Tris-HCl buffer (pH 7.5) three times for 5 minutes and stained with 10% ethidium-bromide for 10 minutes. Each slide was analyzed by using Leitz Orthoplan epifluorescence microscope equipped with an excitation filter of 515 - 560 nm. For each irradiation dose, 100 cells were analyzed by automatic digital analysis system Comet assay II (Perceptive Instruments Ltd., Suffolk, Halstead, UK), determining tail length and tail moment (tail length x tail % DNA/100).

Statistical analysis

Possible comet assay endpoints between control and exposed group were evaluated by using the Mann-Whitney *U*-test. The dose-response curve was obtained using the method of linear regression.¹¹

Results

As shown in Figure 1, tail length values for the control blood specimen varied between 10.37 μ m and 17.50 μ m (mean value 14.19 ± 1.49

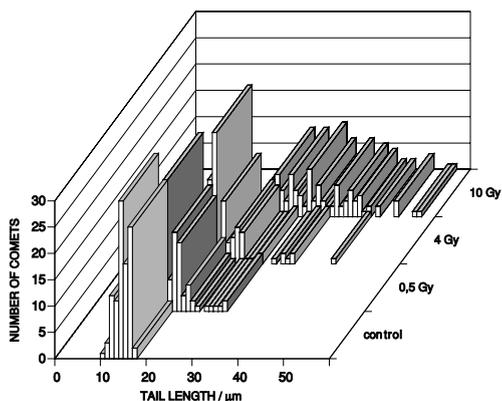


Figure 1. Distribution of the comets regarding their tail length.

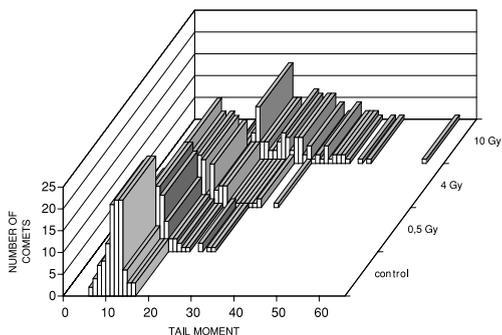


Figure 2. Distribution of the comets regarding their tail moment.

μ m). After the irradiation of the whole blood with the dose of 0.5, Gy the tail length ranged from 12.97 μ m to 27.88 μ m (mean value 17.81 ± 2.40 μ m). At the dose of 4 Gy, it ranged from 14.26 μ m to 44.73 μ m (mean value 21.12 ± 5.06 μ m) and at 10 Gy, from 21.39 μ m to 55.75 μ m (mean value 33.80 ± 7.80 μ m). Tail moment values (Figure 2) ranged for the control from 6.43 μ m to 14.62 μ m (mean value 11.04 ± 1.92 μ m), at 0.5 Gy, from 10.54 μ m to 23.84 μ m (mean value 14.81 ± 2.20 μ m), at 4 Gy, from 9.33 μ m to 34.10 μ m (mean value 16.91 ± 4.52 μ m), and at 10 Gy, from 15.04 μ m to 81.96 μ m (mean value 28.64 ± 8.74 μ m).

For both, tail length and tail moment, dose-effect relationship was found to be linear in a dose range of 0.5Gy and 10 Gy (Figures 3,4).

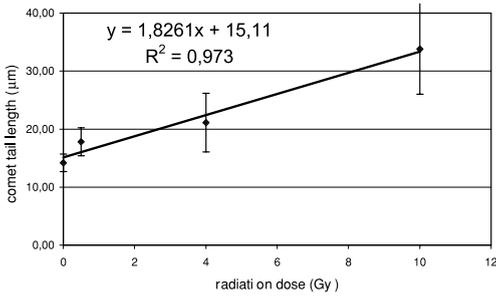


Figure 3. Tail length dose-response curve

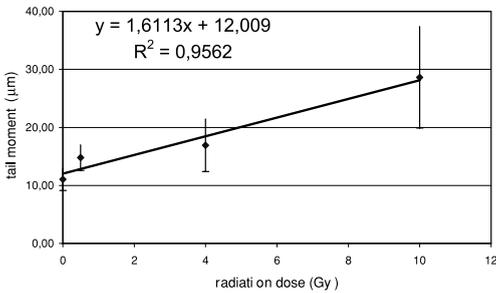


Figure 4. Tail moment dose-response curve.

Discussion

Our goal was to determine the extent of DNA breakage measured with the comet assay induced by ionizing gamma radiation as DNA damaging agent. Under the alkaline conditions used here, the comet assay detects double- and single strand breaks and alkali-labile sites that could occur as the result of physicochemical interaction of ionizing radiation with cellular DNA.¹² The comet values that increased with the applied irradiation dose indicate a dependence of the extent of radiation-induced primary DNA damage on the dose value.

Similar results, but only for the low-dose radiation, were obtained by Kormos *et al.*⁷, Olive *et al.*⁶, Vijayalaxmi *et al.*¹³, Plappert *et al.*⁸, Singh *et al.*⁵, He *et al.*². He *et al.* found the dose-response curve for tail length to be linear between doses of 1 Gy and 2 Gy.² They also assumed that the curve could remain linear at the higher radiation doses. Also for the low dose irradiation, some other authors

showed a good relationship between comet assay endpoints and results obtained by micronucleus assay.^{2,9} But due to the mitotic activity requirements by standard cytogenetic methods (micronucleus assay, chromosomal aberration assay) which is significantly reduced after the high dose irradiation, it is impossible to apply these techniques at dose levels higher than 5 Gy.^{8,14} As shown in the present paper, the comet assay does not require prior cell cultivation; it could therefore be used in the evaluation of the DNA damage even at the dose as high as 10 Gy which is essential in case of accidents involving ionizing radiation.

As shown in the Figures 1 and 2, the application of higher irradiation dose caused a shift of tail length and tail moment toward higher values. Regardless to the dose in all irradiated blood samples lymphocytes with comet endpoint, values were significantly higher than the sample mean value. According to Plappert *et al.* these cells were considered to express a deficiency in DNA repair efficiency.⁸

Beside the already mentioned absence of need for cell cultivation, there are many other advantages of the comet assay application in the ionizing radiation induced DNA damage risk assessment. The method is simple, rapid and it detects primary DNA lesions, whereas the other cytogenetic techniques are based on the detection of lesions left unrepaired and/or structural aberrations that could possibly occur as the result of the repair mechanisms.¹⁴

Comet assay also provides information on intercellular differences in the irradiation susceptibility. Because it does not involve cell cultivation, there is no need for the cell treatment with the chemicals as colchicine and cytochalasin B that are found to be mutagenic themselves and could possibly lead to false positive results.¹⁵ Therefore the comet assay could be widely applied in radiation biology and ionizing radiation risk assessment, espe-

cially in the studies of high dose effects on the DNA level.

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