DNA Damage in Hair Root Cells as a Biomarker for Gamma Rays Exposure

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ABSTRACT

The purpose of the present research is to examine whether human hair root cells can be used for dose assessment after in vitro exposure to ionizing radiation. Hair root samples plucked from random head regions were collected from 5 healthy human subjects. Some of these hair samples were used as control and some were irradiated with 0.5 - 5 Gy of gamma ray using a Cs-137 gamma irradiator at a dose rate of 0.14 Gy/sec. DNA damage (single strand break) were determined in hair root cells of these samples using the comet assay technique. The comet assay measurements, tail length (TL) and tail moment (TM) values showed that the irradiation significantly increased ($p < .05$) single-strand DNA breaks in hair roots cells of the exposed samples compared to control. A linear dose-effect relationship was observed when tail-moment or tail-length was plotted against the log of the radiation dose. This research suggests a possible usage of human hair root cells as a biomarker especially for low dose radiation exposure using the comet assay technique.

Keywords: gamma ray, radiation biomarker, DNA damage, hair root cells, Comet assay

1. Introduction

Today with the increased exposure to ionizing radiation (IR) with its medical, energy and military applications, the development of a rapid and sensitive method to assess exposure level to IR is of concern to a large number of people. The biological effects of radiation on people are strongly dependent upon the dose of radiation received. Ionizing radiation is a ubiquitous environmental physical agent whose DNA-damaging effects are fairly well established. IR produces a variety of primary lesions, such as single strand breaks (SSBs), alkali-labile sites, double strand breaks (DSBs), DNA–DNA and DNA– protein crosslinks,

and damage to purine and pyrimidine bases [1–4]. However, mammalian cells possess efficient enzymatic mechanisms for repairing these types of damage. Therefore, measurement of average DNA damage can be used for estimating the magnitude of IR exposure. In the past years, as a biomarker of exposure, DNA damage evaluated by the Comet assay has been used [5-10]. The Comet assay permits the detection of primary DNA damage and the study of repair kinetics at the level of single cells [11-13]. A variety of possible modifications of the assay facilitate the detection of SSBs, alkali-labile sites, DSBs, incomplete excision repair sites, and interstrand crosslinks. In addition to the above, DNA fragmentation associated with cell death or apoptosis can be evaluated with the Comet assay [13, 14].

Biological dosimetry is an important approach of dose estimation after radiation incidents or accidents. The main methods for dose estimation are the chromosome aberration assay, micronucleus rate, fluorescence in situ hybridation (FISH) translocation assay, lymphocyte counts and electron spin resonance on solid matrix components from for example (i.e. teeth dental enamel, fingernails, and extracted bone, etc). Other assays, such as the comet assay, use molecular endpoints to measure DNA damages, changes in the regulation of some sentinel genes, and the presence of protein biomarkers that are not radiation specific and working as a screening method. All these sources of information may be combined with biological dosimetry to obtain a clearer evaluation of the exposure.To assess the radiation dose received by a person, proliferating cell populations and peripheral blood lymphocytes were demonstrated to be the optimum sample matrix [15]. The comet assay can be applied to proliferating and non-proliferating cells and cells of those tissues which are the first sites of contact with genotoxic agents. The comet assay is the least time consuming, cheap and simple method and detects primary DNA lesions while the other cytogenetic techniques depend on

the detection of lesions left unrepaired or structural aberrations that could possibly occur as the result of the repair mechanism [16].

As well as the method, sampling strategy is also important for dose assessment. In this sense, the collection of hair roots could be an alternative sampling since it requires less skill, time, and money and less invasive for the subjects than collection of blood or biopsy samples. Moreover, the hair root undergoes rapid cell proliferation with short turnover time [17]. As a consequence, the matrix cells in an anagen hair root would be expected to be sensitive to genotoxic agents such as radiation and the damage would be rapidly manifested. About 85 – 90% of the hairs on one's head are in the anagen phase at a given time, so almost all of the hair pulled out would be in the anagen phase. In addition, the time factor between irradiation and sampling should be considered especially in proliferating cells since the damage would be repaired. In this sense, the comet assay should be applied on hair root cells in the shortest possible time after irradiation.

Previously, we investigated radiofrequency radiation effect on genomic DNA of human hair root cells by applying the comet assay [18] and the comet assay protocol was firstly developed for hair root cells. In the present paper, possible usage of human hair root cell DNA damage in vitro as a biomarker for gamma rays exposure by the comet assay technique was investigated. In vitro dose and log dose response curves were constructed to determine the values of the mathematical coefficient that best fit the data points obtained in experiment. On the basis of curves, whether human hair root cells can be used as a model for biologic dose assessment was discussed.

2. Materials and methods

2.1. In vitro human hair root irradiation

Five human subjects (three women, two men, age 30 – 45 years) participated in the present study. This study was approved by the Ethics Committee of the Gazi University (Report no. 104). More detailed information of the subjects is given in Table I. Volunteers gave hair samples plucked from random head regions. Hairs were cut gently close to their roots and then placed into a 1.5-ml eppendorf microfuge tube (Greiner bio-one, Monroe, NC, USA) with the roots towards the bottom of the container. Immediately after the sampling, hair roots from each donor were divided in 6 specimens. One specimen was used as the control. The other five specimens were irradiated using a Cs-137 gamma source at ambient conditions at a dose rate of 0.14 Gy/sec at the Saraykoy Establishment of the Turkish Atomic Energy Authority in Ankara. Different specimens were irradiated at a dose of 0.5 Gy, 1 Gy, 2 Gy, 3 Gy or 5 Gy at different orders.

2.2. Comet Assay

The assay protocol for hair root cells was firstly developed in the study of Cam and Seyhan [18] by modifications from Singh et al. [19] and it was used in the present study. DNA single strand break measurement was carried out immediately after hair sample irradiation (within 30 min). In hair root cell preparation, 6 or 7 hair roots were immersed in a 1.5-ml Eppendorf microfuge tube containing 30 µl of a solution that contained 1 X Hanks' Balanced Salt Solution (HBSS Ca^{++} and Mg^{++} free, Life Technologies/GIBCO, Carlsbad, CA, USA), 20 mM Ethylenediaminetetraacetic acid (EDTA, Sigma Chemicals, St. Louis, MO, USA), 10 % Dimethyl sulfoxide (DMSO, Merck & Co., Whitehouse Station, NJ, USA). A plastic stick was used to loosen the cells from the hair roots. This cell suspension consisted of different types of hair root cells. Fifteen microliters of the cell suspension were mixed in 80 µl of low melting agarose (0.5 %, Sigma Chemicals) and pipetted onto a slide pre-coated (Sail Brand,

Shanghai, China) with 2 % normal melting agarose (Sigma Chemicals). Cells were spread with a cover glass, and maintained on ice for 15 min to solidify. After removing the cover glass, a layer of 0.5 % low melting agarose was added and solidified on ice. The slides were immersed for 1 hr in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Triton X-100, 10 % DMSO) at 4 °C. The slides were then placed in an electrophoresis unit (MSCHOICE, MP-250N omniPAC, Cleaver Scientific Ltd., Warwickshire, UK) containing a buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 40 min at room temperature to allow for DNA unwinding. Electrophoresis was then carried out for 30 minutes at 25 V (~300 mA). The slides were then neutralized with Tris-HCl buffer (0.4 M, pH 7.5) for 5 minutes and air-dried. This was repeated three times. 7.5 ul of SYBR Green (Lonza Group Ltd., Basel, Switzerland) solution was diluted with 600 ul of distilled water and 65 ul of this diluted solution was applied to each slide. Slides were coded before analysis and each slide was analyzed by using a fluorescent microscope (Olympus BX 51 fluorescence model (Olympus Corp., Tokyo, Japan) with 4',6-diamidino-2-phenylindole (DAPI)/fitc/Texas Red filter under blue excitation: wavelengths of excitation with a beam with half-width (400/15-495/15-570/25 nm) emission with a beam with half-width (460/20-530/30-625/650 nm) and dichroic (410-510-590 nm) filters and dichroic attachments at a resolution of 600×800 by digital color video camera (Pixera Corp., Los Gatos, CA, USA). All experimental set up and chemicals were purchased from company's distributors in Ankara, Turkey. From each sample, 100 cells (50 cells on each of two replicate slides) were analyzed by the comet assay software project (CASP, publicly available, http://www.casp.of.pl, Wroclaw, Poland) [20]. Cell capturing was applied manually to avoid overlapping cells with a total of 100 randomly captured comets per sample. The end points were calculated automatically. To quantify the DNA damage, tail length (TL) and tail moment (TM) were evaluated. Tail length is measured from the right border of the head area to the end of the tail in micrometer (μm). Tail moment was calculated as the

product of the tail length and the percent of DNA in the tail in arbitrary units (Tail moment = tail length x % of DNA in the tail).

2.3. Data Analysis

The mean tail length and tail moment from the 100 cells measured for single strand breaks in each volunteer's control and exposed samples were used in data analysis. Data were analyzed using a SPSS Software Package for Statistical Analysis (SPSS for Windows, Version 16.0.0, SPSS Inc., Chicago, IL, USA). Both comet parameters measured in the exposed and control groups were evaluated by one-way ANOVA followed by Tukey HSD as a post hoc test. A level of difference at $p < .05$ was considered statistically significant.

3. Results

Tail moment data from individual subjects were presented in Table II. Average tail length and tail moment data versus dose and log dose are plotted in Figs. 1 and 2, respectively. Illustrative examples from the different doses are given in Fig. 3. A one-way ANOVA indicated that DNA single strand breaks measured as tail moment and tail length vary significantly with irradiation dose ($F(4)= 48.28$, $F(4)= 31.63$; $p< 0.05$). Post Hoc multiple comparisons (Tukey HSD) revealed significant differences between doses except for 0.5 Gy and 2 Gy doses compared to 1 Gy dose for both comet measurements. The Tukey test showed that differences between doses were at $p<0.01$. However, it has to be pointed out that significant inter-individual differences in tail moment measures were observed (table II). Dose-response curves were constructed by measuring in vitro responses of hair root cells to increasing doses (0 to 3 Gy) of gamma radiation in the comet assay. At 5 Gy, extensive damages were observed in most cells and could not be accurately measured. Only a maximum of 15 cells could be analysed for each of these exposed samples. Therefore, data from 5 Gy were excluded from the analysis. Quadratic, power and exponential functions of the applied

radiation dose were tested to describe experimental dose–response data without forcing the functions to pass through the origin. These functions have previously been used for the estimation of the absorbed dose in radiation processed food [21-24] and in radiosterilization of pharmaceuticals [25-28]. Parameter values and correlation coefficients calculated for these functions are presented in table 3. A power function of the applied dose D ($I = aD^b+c$) was found to correlate well with the experimental dose–response data relevant to both end points (table 3). Theoretical data derived using the parameter values given in table 3 for a power function were also presented in figs. 1 and 2 to give an idea about the degree of agreement between experimental and theoretical dose–response data. Moreover, dose response curves were constructed as plotting tail-moment or tail-length against the log of the radiation dose. A linear relationship was seen when the data using log dose, instead of dose. They were also presented in figs. 1 and 2.

4. Discussion

The risk of radiation over-exposure interests many people with the increasing application of ionizing radiation. While living with the effects of low-level ionizing radiation is one of the normal threats of life, occupational exposure is related to cumulative effects of radiation and often to the possible risks of radiation accidents. Considering accidents, in most cases just workers, in lesser extent several people can suffer from radiation over-exposure. Thus, to estimate the magnitude of exposure in individuals there is a vital need of biologic indicator as a complement to physical and clinical dosimetry or for cases which physical dosimeter is not carried. The biological indicators can have a further advantage in the way that the individual radiation damage is measured, which includes variability of individual radiosensitivity [51]. In the present study, the potential use of human hair root cells as a model for biologic dose assessment was investigated by using the alkaline comet assay. The level of DNA damage in

hair roots plucked from healthy donors exposed in vitro to a Cs-137 source was evaluated. The irradiation significantly increased ($p < .05$) single-strand DNA breaks in hair root cells of all exposed groups compare to control. The dose-effect relationship showed a linear increase at doses lower than 2 Gy and a slow down in increase of both comet parameters in the higher dose was observed. In addition, the endpoints at 0.5 Gy were found higher from expected (based on the rate of increase at each dose) as is seen from Fig. 1 and 2, that is because the dose rate of gamma source used for this study is not so low (0.14 Gy/sec). Similar results were also observed in the literature [29,30]. The reduction in the growth rate of tail moment seen at higher doses was thought to be a result of the loss of very small DNA fragments, and a question yet to be answered is to what extent small fragments are lost from analysis after high radiations [30]. Single-strand breaks, which are the initial damage induced by a genotoxic agent, can be used as a biomarker of exposure, whereas the more biologically relevant double-strand breaks can be analyzed to quantify the extent of damage [31]. It is well known that some background level of DNA breaks is always present in mammalian cells due to normal metabolism. Nature of spontaneous and induced DNA breaks is rather similar. IR-induced primary lesions could lead to genotoxic effects only in case if spatial distribution of the lesions along the chromatin and effectiveness of their repair is different from those spontaneously induced [32]. Inter-individual variations in comet measurements, independent of the irradiation doses, were observed in the present study. It may be related to individual differences in repair ability and radiation sensitivity and exogenous factors such as sex and age, air pollution, diet, infections, and other genotoxic agents. These factors that influence the level of DNA damage have been reviewed by Moller et al. [49]. In addition, inter-individual differences could be caused by endogenous factors such as cell position, waiting time, duration of measurement, number of selected cells, sample position in the slide changers, and

date of trial within the investigation. The impact of these assay-inherent factors on comet end points has been investigated by Rosenberger et al. [50].

The comet assay has been used in many times for the assessment of DNA damage in biomonitoring studies and genetic toxicology. Forchhammer et al. [33] addressed the issue of inter-laboratory variability of DNA damage measured by the comet assay. Twelve laboratories analyzed the level of DNA damage in same cells irradiated at the dose range of 1.5-7 Gy and detected dose-response relationships. The decline in the rate of increase in both comet end-points at higher doses was also observed in this study. Milkovic et al. [34] assessed DNA damage in peripheral blood lymphocytes of children prior to and following airway X-ray examinations of the chest using the alkaline comet assay and to compare data with the measured absorbed dose using thermoluminescent and radiophotoluminescent dosimetry systems. They observed qualitative (but not quantitative) correlation between the values of measured doses and the measurement outcome of the comet assay. Moreover, the ratio of DNA damage before and after irradiation showed that X-ray diagnostic procedures could alter DNA and highlights the importance of minimizing pediatric exposure to X irradiation. Vrhovac et al. [35, 36] showed the usefulness of the alkaline comet assay as an additional complement to standard biodosimetric methods. In one of their studies, they applied the comet assay in leukocytes of radiation worker accidentally exposed to gamma radiation and observed statistically differences in level of DNA damage at different times, one day-one week-one year, after the incident. Secondly, they compared the DNA damage level of 50 medical workers and 50 corresponding unexposed control subjects in their peripheral blood leukocytes with their dosimeter readings and occupation and observed significant differences between exposure periods and control subjects. However, they did not observe a statistically significant difference in comet parameters measured due to smoking and gender.

Lastly, Vrhovac and Zeljezic irradiated whole blood samples taken from two healthy, young donors with the doses of 0.5, 4 and 10 Gy of gamma radiation. They found a linear dose-effect relationship for both tail length and tail moment values, so pointed out possible usage of the comet assay in the detection of DNA lesions caused by high radiation dose, which is not possible by using standard cytogenetic methods [16]. However, we observed the linear dose-effect relationship in case we plotted comet assay measurements against the log of the radiation dose. Many studies assessed the effects of the Chernobyl radiation incident on exposed individuals and found increased levels of DNA damage [37-39]. However, there was one study on ionizing radiation that did not show occupational DNA damage [40]. Valverde reviewed data from 122 studies employed the comet assay for environmental and occupational biomonitoring, so reflected its success and paucity. Comet assay has high sensitivity but insufficient specificity since the results were not specific only for radiation or whatever the chemical/physical agent was. However, differences in individual radiosensitivity and laboratory set-up and procedures limit its applicability for dose assessment. In this context, the frequency of dicentrics in human lymphocytes is considered the most sensitive method to quantify exposures to ionizing radiation [41, 42], when using an appropriate in vitro calibration curve, according to the radiation quality. Despite these, use of comet assay over the past 20 years in the field of biomonitoring and the number of studies that continue to report its use are increasing [43]. McKenna [44] analyzed the comet assay as a test to predict the likely response of individual tumors to radiation. It was recommended that despite the advantages of assay, it should not be used in the management of cancer or any other disease unless fundamental issues relating to experimental validation, standardization and data interpretation are addressed.

There are many advantages of the comet assay application in IR-induced DNA damage risk assessment. One of them is that it does not require cell cultivation as well as provide information on different radiation sensitivity of cells, so it could be widely applied in radiation biology and IR risk assessment [16]. The other is the feasibility of its application to a broad spectrum of cells for human biomonitoring. Proliferating and non-proliferating cells, as well as cells from tissues are all suitable for evaluation using the comet assay to give an estimation of risk of exposure. It is worth noting that in the past 20 years, the majority of studies have included data from mononuclear cells (58%), leukocytes (35%), buccal cells (3%), nasal cells (2%), sperm (1%), and exfoliated lachrymal duct cells (1%) [43]. For the first time in the present investigation, human hair root cells were evaluated using the comet assay to estimate the extent of IR exposure. The hair, or the hair follicles and its cells, might make a useful biological indicator for the following reasons: plucking hairs is noninvasive and fairly painless compared to other types of sample collection. Since it could be performed by non-clinical personnel, it may be well suited for large scale sample collection; hair covers most of the skin surface and so it represents the only system where local doses or dose distributions over the body surface could be estimated; the cells of the follicle are sensitive to relatively small doses.

There has been a great deal of discussion about the most appropriate parameters and statistical methods for the analysis of DNA damage [45]. In environmental and occupational studies, the most widely employed parameter was tail moment (35%) followed by the use of tail length in 28% of the studies, and the percentage of tail DNA in almost 20% of cases. The use of tail moment data eliminates useful information on the relationship between the extent of migration and the percentage of migrated DNA [46]. Tail length data has been criticized because it is only linear with respect to dose within a narrow interval. Thus the tail moment or

the percentage of tail DNA is preferred [47, 48]. We investigated both tail moment and tail length data with the increased dose and found best fit to their response curves. Usually, estimation of the dose absorbed by a person during exposure to radiation refers to the calibration curve of a dosimeter that fitted from *in vitro* data. The power function of the applied dose D ($I = aD^b+c$) was found to correlate well with the experimental dose–response data relevant to both end points. This function will be likely sufficient for a dependence of the extent of radiation-induced primary DNA damage on the dose value. In practice, materials with linear dose–response characteristics are in choice for dose estimation. A linear dose-effect relationship was observed when tail-moment or tail-length was plotted against the log of the radiation dose. Hence, in this case, hair root cells can be considered as a good material for practical dose assessment.

The present study indicates hair roots as a possible radiation biomarker by applying the comet assay technique in short time. The detection of momentary DNA damage and/or repair activity could reflect the concurrent exposure and the actual levels of DNA damage present in hair root cells of the exposed people at the moment of hair sampling. Thus, the in vitro study design used here seemed not to indicate a general usage of hair root cells for accidental radiation exposure. However, the results of the study give answers to some basic requirements for a dose reconstruction in general. We suggest that future studies should be in vivo and extending the tests to a higher number of individuals whose absorbed dose was known, i.e. during radiation therapy.

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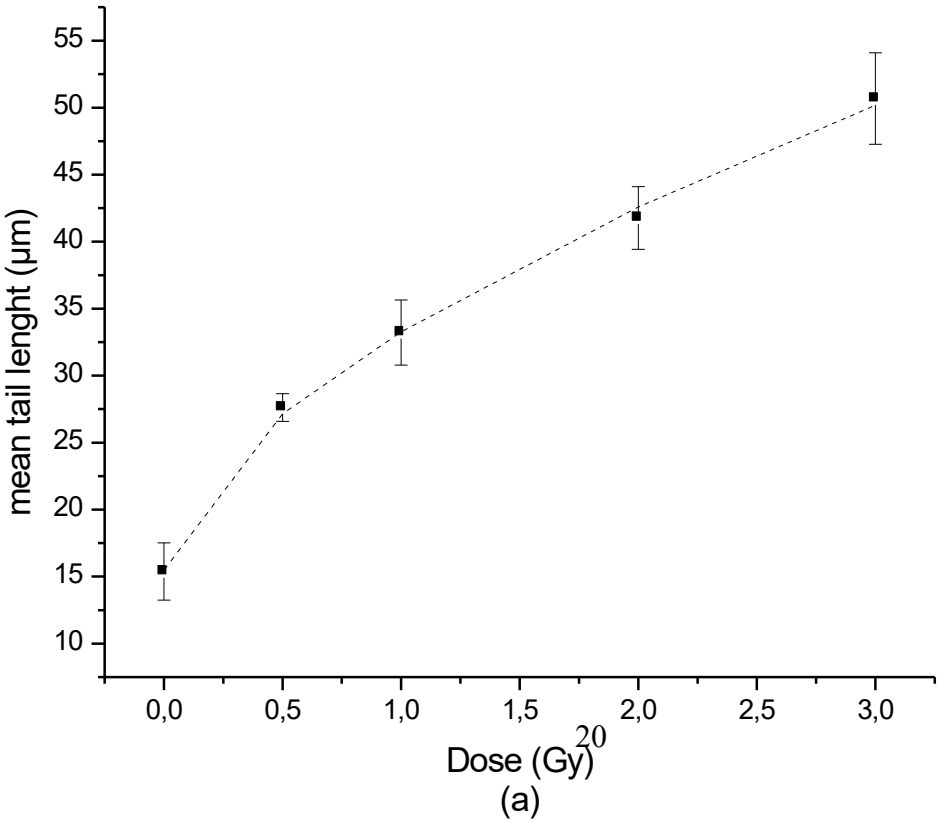
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Table 1
General information and confounding factors among volunteers.

	Age	Gender	Occupation	Smoking	Alcohol	Chemical	Medication	Radiation exposure
1	45	Male	Medical Doctor	+	+	-	-	-
2	37	Female	Chemical Eng.	-	-	+	-	+
3	37	Male	Veterinarian	+	-	-	-	-
4	45	Female	Biologist	+	-	-	-	-
5	30	Female	Physicist	-	-	-	-	+

Table 2
Hair root cell **Tail moments** of individual volunteers exposed to gamma (Cs-137) radiation.
Each value is the average of 100 cells

Sample	**control mean±se(n=100)**	**0.5 Gy mean±se(n=100)**	**1 Gy mean±se(n=100)**	**2 Gy mean±se(n=100)**	**3 Gy mean±se(n=100)**
1	0.90 ±0.02	2.70 ±0.21	3.17 ±0.19	3.38 ±0.25	4.10 ±0.27
2	1.70 ±0.09	2.80 ±0.22	3.10 ±0.21	3.50 ±0.24	4.40 ±0.30
3	1.73 ±0.08	2.40 ±0.14	2.80 ±0.15	3.28 ±0.23	4.30 ±0.28
4	1.38 ±0.05	2.55 ±0.15	2.79 ±0.16	3.04 ±0.23	3.30 ±0.23
5	0.73 ±0.02	2.50 ±0.13	3.40 ±0.19	3.70 ±0.21	4.15 ±0.24
mean±SE(n=5)	**1.29±0.21**	**2.59±0.12**	**3.05±0.11**	**3.38±0.11**	**4.05±0.19**



(a)

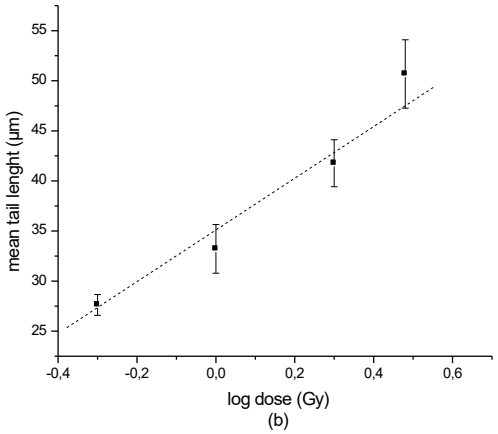
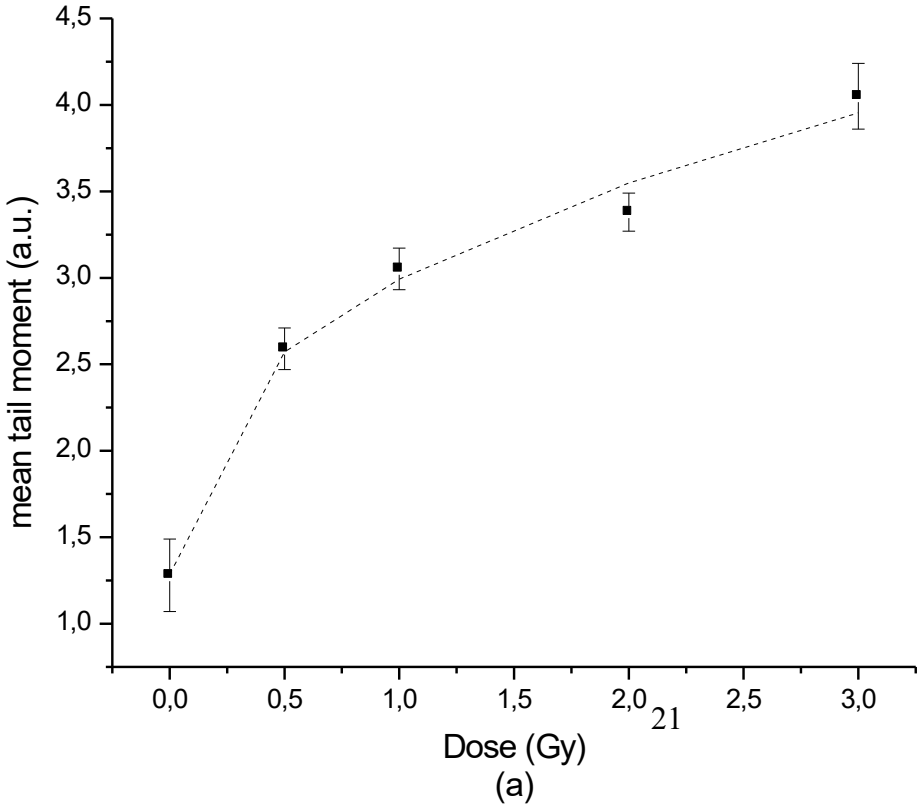


Fig.1. a) tail length - dose response curve. b) tail length- log dose curve (Experimental :(■), theoretical: dashed lines, a) power function, b) linear function).



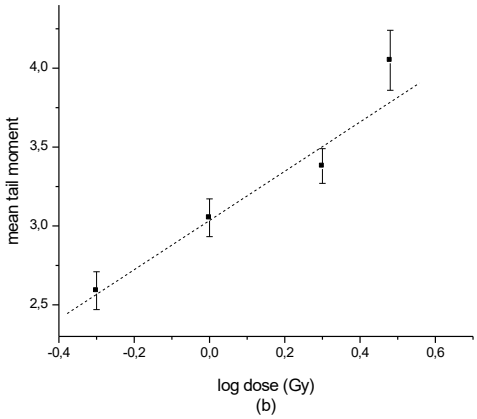


Fig. 2. a) mean tail moment - dose response curve. b) mean tail moment- log dose curve. (Experimental :(■), theoretical: dashed lines, a) power function b) linear function).

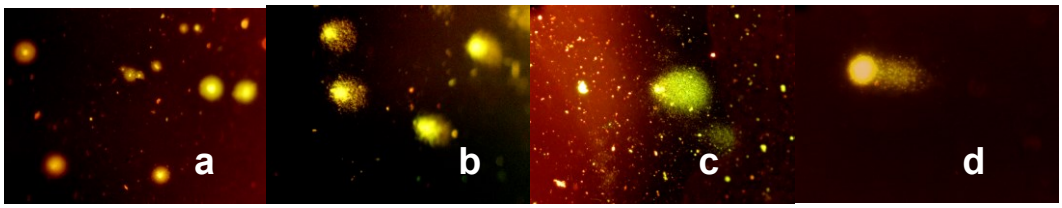


Fig. 3. Representations of comet images from hair root cells;

a) control, b) 1 Gy, c) 3 Gy, d) 5Gy.

Table 3
Functions tried to describe the variation of tail length and tail moment with absorbed radiation dose

Function		Measured quantity for TL	Measured quantity for TM
polynomial $I = a+bD+cD^2$ (a)	a b c R^2	16.94 18.11 2.37 **0.9837**	1.77 1.38 0.26 **0.9051**
power $I = aD^b+c$ (a)	a b c R^2	17.77 0.61 15.49 **0.9984**	1.71 0.41 1.28 **0.9904**
exponential $I = a(1-e^{(-bD)}) +c$ (a)	a b c R^2	42.82 0.51 16.28 **0.9890**	2.61 1.08 1.34 **0.9667**
linear I = a+bD (b)	a b R^2	35.10 25.82 0.9865	3.03 1.56 **0.9662**

Table captions

Table 1
General information and confounding factors among volunteers.

Table 2
Hair root cell Tail moments of individual volunteers exposed to gamma (Cs-137) radiation. Each value is the average of 100 cells

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Figure captions

Fig.1. a) tail length - dose response curve. b) tail length- log dose curve (Experimental :(■), theoretical: dashed lines, a) power function, b) linear function).

Fig. 2. a) mean tail moment - dose response curve. b) mean tail moment- log dose curve. (Experimental :(■), theoretical: dashed lines, a) power function b) linear function).

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