THE ALKALINE COMET ASSAY AS A BIOMARKER OF PRIMARY DNA DAMAGE IN PERIPHERAL BLOOD LEUKOCYTES OF NUCLEAR MEDICINE PERSONNEL

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Abstract

The aim of this study was to assess whether occupational exposure to chronic low doses of ionizing radiation in nuclear medicine departments may lead to genotoxicity. The alkaline comet assay was selected as a biomarker of exposure to evaluate the levels of primary DNA damage in peripheral blood leukocytes of exposed and corresponding control subjects. Statistically significant differences were found between comet tail length and tail moment values measured in leukocytes from the exposed and control groups. Within exposed group significant inter-individual differences in DNA damage were assessed, indicating different genome sensitivity. In majority of exposed subjects the levels of DNA damage were in positive correlation with the duration of occupational exposure, while the influences of age and dosimeter readings could be excluded. However, the levels of primary DNA damage detected both in control and exposed subjects were significantly influenced by smoking. The present study indicates the possibility of genotoxic risks related to occupational exposure in nuclear medicine departments. Therefore, the exposed personnel should carefully apply the radiation protection procedures to minimize, as low as possible, radiation exposure to avoid possible genotoxic effects. According to results obtained, the alkaline comet assay could be usefully applied as a sensitive additional biomarker in the regular health screening of workers occupationally exposed to low doses of ionizing radiation.

Introduction

Ionizing radiation is an ubiquitous environmental physical agent whose DNA damaging effects are fairly well established. As a strong physical mutagen and a clastogenic agent, it causes the breakage of phosphodiester bonds in DNA, cross-linking as well as damage to nucleotide bases [1].

The exposure of patients and workers to radiation in medicine is a direct consequence of the use of radiation to improve the health of the individuals. Medical personnel employed in nuclear medicine departments is occupationally exposed to low doses of ionizing emissions from different radioactive isotopes such as ^{32}P, ^{67}Ga, ^{111}In, ^{201}Tl, ^{59}Fe, ^{57}Co, ^{51}Cr, ^{99}mTc, ^{131}I, ^{192}Ir [2,3]. Over the past two decades, medical occupational radiation exposure has decreased in spite of increased use of radiation in medicine. This decrease reflects primarily the introduction of new technologies in medicine. However, possible consequences from exposure to low radiation doses as in diagnostic radiology, occupational activities or in nuclear medicine investigations should be studied to determine potential deleterious genetic effects to exposed populations. Evaluations of the cytogenetic impact of chronic low dose radiation exposure are still scarce. It is very important to estimate absorbed doses from individuals occupationally exposed to ionizing radiation for carrying out radioprotection procedures and restrict the hazards to human health [4].

In order to obtain information concerning distribution and extent of radiation exposures, different biological methods for dose assessment have been developed. Human biomonitoring can be performed using various genetic markers which detect early biological effects. Among them, the most frequently used are evaluation of DNA mutations, chromosomal aberrations, the induction of micronuclei and sister chromatid exchanges. Under the many biological parameters studied, the most fully developed biological indicators of ionizing radiation exposure are unstable chromosomal aberrations (in particularly dicentrics) in peripheral blood lymphocytes [4,5]. This methodology usually complements data obtained by physical dosimetry. As a routine, it is used whenever the individual dosimeter shows an exposure to penetrating radiation above its limit of detection. One of the advantages of cytogenetic dosimetry is that this biological dosimeter can be assessed at any moment whereas physical dosimeters are not always present in the subject [4].

During the last decade, the single cell gel electrophoresis (SCGE) or comet assay was introduced as a useful technique for human biomonitoring studies [6,7]. While biomonitoring studies employing cytogenetic techniques are limited to circulating lymphocytes and involve proliferating cell populations, 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. In this assay, increased DNA damage is visualized on the individual cell level as an increased migration of genetic material from the nucleus in the direction of electrophoresis, and this displacement can be quantified using computerized image analysis. The comet assay is a rapid, simple and sensitive technique for measuring DNA breakage with a small number

of cells and detects intercellular differences in DNA damage [8,9]. Being sensitive, this assay seems particularly well suited for studying radiation-induced DNA damage. By using the comet assay positive results on the DNA-damaging effects of X-radiation on occupationally exposed subjects were reported previously [10].

The aim of the present study was to assess and quantificate the levels of primary DNA damage in peripheral blood leukocytes of medical workers employed in nuclear medicine departments and corresponding non-exposed control subjects. As a sensitive biomarker of exposure the alkaline comet assay was selected.

Subjects and method

The population studied comprised 40 volunteer blood donors: 20 of them had been occupationally exposed to ionizing radiation and 20 were unexposed control subjects. Each subject completed a standardized questionnaire which included items concerning personal data (age, health status) and data on occupational exposure to ionizing radiation at the time of the study. The questionnaire also included items concerning extra occupational exposure to potential mutagenic hazards, such as smoking, alcohol and drug consumption, viral diseases, recent vaccinations, and radio diagnostic examinations. The exposed group consisted of 12 female and 8 male subjects employed in the nuclear medicine department. They are occupationally exposed to low doses of particular ionizing emissions from different radioactive isotopes (^{32}P, ^{67}Ga, ^{111}In, ^{201}Tl, ^{59}Fe, ^{57}Co, ^{51}Cr, ^{99}mTc, ^{131}I, ^{192}Ir). Three of them were physicians, 10 technicians, 2 engineers, 4 clerks and one cleaner. Their mean duration of occupational exposure was 15.5 years (range 1-35 years). The mean age of the whole exposed group was 40.9 (age range: 26-56 years). During their work they all wore individual dosimeters (film badges). Their dosimeter readings during one year prior the study were in range 0-970 μSv. Eight exposed subjects were smokers (5 female and 3 male), and 12 non-smokers (5 female and 7 male). Control subjects were healthy students and office employees (12 females and 8 males), chosen from the general Croatian population. Thirteen of them were non-smokers (7 female and 6 male), and seven of them were smokers (5 female and 2 male). The mean age of the control group was 40.9 years (age range: 26-56 years). None of them had ever had any contact with sources of ionizing radiation. They also had not been occupationally exposed to known genotoxic agents. None of the control subjects reported alcohol consumption. During the year prior to the blood sample collection, the control had not been subjected to diagnostic X-ray or nonionizing examinations. Peripheral blood samples of

the exposed and control subjects were collected by venipuncture into heparinised tubes. All samples were coded, cooled and processed within a maximum of 2 h after collection.

The alkaline comet assay

The comet assay was carried out under alkaline conditions, basically as described by Singh et al. [6]. Fully frosted slides were covered with 1 % normal melting point (NMP) agarose (Sigma). After solidification, the gel was scraped off from the slide. The slides were then coated with 0.6 % NMP agarose. When this layer had solidified a second layer containing the whole blood sample mixed with 0.5 % low melting point (LMP) agarose (Sigma) was placed on the slides. After 10 minutes of solidification on ice, slides were covered with 0.5 % LMP agarose. Afterwards the slides were immersed for 1 h in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1 % Na-sarcosinate (Sigma), pH 10) with 1 % Triton X-100 (Sigma) and 10 % dimethyl sulfoxide (Kemika) added fresh to lyse cells and allow DNA unfolding. The slides were then placed on a horizontal gel-electrophoresis tank, facing the anode. The unit was filled with fresh electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) and the slides were set in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4^oC under dim light. Electrophoresis was carried out for 20 min at 25 V (300 mA). After electrophoresis the slides were rinsed gently three times with a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 μg/ml) and covered with a coverslip. Slides were stored at 4^oC in sealed boxes until analysis. A total of 100 randomly captured comets from each slide were examined using an epifluorescent microscope (Zeiss) connected through a black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments Ltd., U.K.). A computerizing image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and then evaluates the range of derived parameters. To quantify the DNA damage, the following comet parameters were evaluated: tail length (TL), and tail moment (TM). Tail length (length of DNA migration) is related directly to the DNA fragment size and presented in micrometers. It was calculated from the centre of the cell. Tail moment was calculated as the product of the tail length and the fraction of DNA in comet tail. The data on comet parameters measured in the exposed and control groups were logarithmically transformed and evaluated by using non-parametric Mann-Whitney *U* test Differences were considered to be statistically significant when $p < 0.05$.

Results

Results of the alkaline comet assay have been summarized in Tables 1 and 2. It was found out that medical workers who were employed at nuclear medicine department for different periods of time showed a significant increase in DNA migration compared to the control values ($p<0.05$, Mann-Whitney *U* test). Within the exposed group marked inter-individual differences regarding comet tail parameters were also observed (Table 1).

The comet tail lengths measured in exposed subjects were in the range 18.17 ± 0.40 μm to 32.54 ± 0.66 μm, with a mean value 23.40 ± 0.17 μm (Table 1). Their tail moments ranged between 15.76 ± 0.46 and 28.82 ± 0.63, and the mean value was 21.01 ± 0.26 (Table 1). In majority of exposed subjects duration of occupational exposure was positively correlated with the extent of DNA damage recorded. The age did not significantly affect the observed DNA damage levels. Similar was found out for dosimeter readings recorded in the majority of exposed subjects. Moreover, in some exposed subjects without any registered dosimeter readings significant increases of primary DNA damage levels were observed (Table 1). It should be pointed out that the levels of DNA damage were significantly elevated due to smoking status. Taken together, exposed smokers had significantly increased values of both comet parameters compared to exposed non-smokers ($p<0.05$, Mann-Whitney *U* test, Table 1). Similar was found out for exposed female non-smokers compared to exposed male non-smokers.

Control subjects showed a low level of DNA damage in their leukocytes. The distribution of comet tail parameters in control group was characterized by nuclei with small comets (Table 2). Their comet tail lengths were in the range 12.59 ± 0.12 μm and 15.60 ± 0.14 μm with a mean value of 14.28 ± 0.04 μm, while the tail moment values were in the range 10.01 ± 0.13 to 13.09 ± 0.15, with a mean value of 11.77 ± 0.04 (Table 2). Unexposed smokers had significantly increased levels of DNA damage compared to non-smokers. It was also observed that the tail moment values in unexposed male subjects were significantly higher compared to female ($p<0.05$, Mann-Whitney *U* test). However, similar was not observed for the corresponding tail lengths.

Table 1. The individual results of the alkaline comet assay on peripheral blood leukocytes of medical personnel employed in nuclear medicine department expressed as mean values the measurements of 100 comets per each subject.

Subject No.	Gender Smoking	Age (years)	Occupation	Exposure (years)	Dose (μSv)*	Tail length (μm)			Tail moment		
						Mean ± SE	Min.	Max.	Mean ± SE	Min.	Max.
1	M^N	36	technician	15	970	18.26 ± 0.46	12.18	40.38	15.76 ± 0.46	9.38	36.60
2	F^S	31	technician	10	760	29.60 ± 0.73	14.74	51.28	26.33 ± 0.71	12.16	47.44
3	F^N	45	clerk	23	660	26.66 ± 0.78	15.38	70.51	23.55 ± 0.75	12.28	66.30
4	M^N	48	physician	17	500	23.42 ± 0.62	16.03	69.23	20.63 ± 0.61	13.41	65.39
5	M^N	26	technician	7	340	18.17 ± 0.40	12.18	41.02	15.78 ± 0.40	9.70	37.77
6	M^S	45	technician	24	330	18.87 ± 0.41	13.46	40.38	16.28 ± 0.38	10.90	34.97
7	F^S	51	engineer	32	320	18.21 ± 0.31	12.82	29.69	15.78 ± 0.31	10.02	26.60
8	F^S	30	technician	6	281	18.40 ± 0.34	13.46	35.26	15.90 ± 0.33	10.89	31.94
9	F^N	43	technician	16	250	24.46 ± 1.20	12.18	107.05	21.40 ± 1.13	9.64	100.20
10	M^S	56	physician	13	170	25.49 ± 0.85	15.38	75.00	22.42 ± 0.82	12.79	71.66
11	F^N	46	cleaner	8	100	18.64 ± 0.31	14.10	29.49	15.85 ± 0.29	11.40	26.68
12	F^S	45	clerk	9	0	32.54 ± 0.66	18.59	55.13	28.82 ± 0.63	15.49	48.53
13	F^N	26	technician	8	0	28.97 ± 0.72	16.03	57.05	25.82 ± 0.71	12.99	52.91
14	M^N	41	engineer	15	0	27.17 ± 0.62	17.95	45.51	23.98 ± 0.59	14.34	40.89
15	M^S	54	physician	27	0	26.02 ± 0.72	13.46	66.02	22.85 ± 0.68	10.63	60.63
16	F^N	53	clerk	35	0	25.86 ± 0.60	16.03	55.77	22.65 ± 0.58	13.09	51.89
17	F^N	29	technician	7	0	23.03 ± 0.44	13.46	35.26	20.11 ± 0.42	10.92	31.86
18	F^S	48	technician	28	0	22.54 ± 0.51	12.82	43.59	19.69 ± 0.49	9.90	38.51
19	F^N	35	clerk	1	0	22.53 ± 0.42	15.38	42.31	19.48 ± 0.40	12.75	39.13
20	M^N	31	technician	8	0	19.17 ± 0.48	12.18	41.02	16.58 ± 0.46	9.64	37.28
Mean values ± SE											
Smokers						**23.96 ± 0.28 -**			**21.01 ± 0.26 -**		
Non-smokers						**23.03 ± 0.21**			**20.13 ± 0.20**		
EXPOSED GROUP						**23.40 ± 0.17**			**20.48 ± 0.16**		

M-male; **F**-female; **S**-smoker; **N**-non-smoker; *****cumulative dose measured in the last one year before blood sampling; **-** $p<0.05$ compared to control (Mann-Whitney *U* test)

Table 2. The individual results of the alkaline comet assay on peripheral blood leukocytes of control population expressed as mean values the measurements of 100 comets per each subject.

Subject No.	Gender Smoking	Age (yrs)	Tail length (μm)			Tail moment		
			Mean ± SE	Min.	Max.	Mean ± SE	Min.	Max.
1	M^N	36	14.63 ± 0.17	10.26	19.23	12.10 ± 0.17	7.88	15.89
2	F^S	31	14.55 ± 0.15	10.90	19.23	11.96 ± 0.15	7.91	16.43
3	F^N	45	14.63 ± 0.17	10.26	17.95	12.14 ± 0.16	8.26	15.39
4	M^N	48	14.00 ± 0.15	10.90	16.67	11.72 ± 0.14	8.57	14.47
5	M^N	26	14.62 ± 0.16	10.26	17.95	12.05 ± 0.16	7.86	15.54
6	M^S	46	15.03 ± 0.16	10.90	19.23	12.43 ± 0.16	8.56	16.71
7	F^S	51	14.04 ± 0.14	10.90	17.95	11.45 ± 0.14	8.05	15.70
8	F^S	30	15.60 ± 0.14	12.18	18.59	13.09 ± 0.15	9.47	16.07
9	F^N	41	14.72 ± 0.14	11.54	17.95	12.13 ± 0.14	9.16	15.35
10	M^N	56	13.81 ± 0.15	10.90	17.31	11.40 ± 0.14	8.39	14.47
11	F^N	49	14.41 ± 0.14	11.54	17.95	11.80 ± 0.14	8.34	15.18
12	F^S	45	13.37 ± 0.13	10.26	17.31	10.96 ± 0.12	7.98	15.14
13	F^N	26	13.70 ± 0.13	11.67	16.86	11.09 ± 0.13	9.06	14.33
14	M^N	41	14.04 ± 0.14	10.26	16.67	11.57 ± 0.15	7.47	14.71
15	M^S	50	15.01 ± 0.18	10.26	18.59	12.56 ± 0.17	8.09	16.32
16	F^N	53	14.17 ± 0.13	11.54	17.31	11.60 ± 0.13	8.70	14.68
17	F^N	29	12.59 ± 0.12	10.37	13.61	10.01 ± 0.13	6.76	11.34
18	F^S	48	13.81 ± 0.14	10.90	16.67	11.37 ± 0.14	8.39	14.14
19	F^N	35	13.99 ± 0.15	10.26	16.67	11.60 ± 0.15	7.72	14.47
20	M^N	31	13.76 ± 0.14	10.90	17.31	11.18 ± 0.13	8.20	14.66
Mean values ± SE								
Smokers			**14.49 ± 0.06 -**			**11.98 ± 0.06 -**		
Non-smokers			**14.16 ± 0.04**			**11.65 ± 0.04**		
CONTROL GROUP			**14.28 ± 0.04**			**11.77 ± 0.04**		

M-male; **F**-female; **S**-smoker; **N**-non-smoker; - $p<0.05$ compared to control (Mann-Whitney *U* test).

Discussion

Working in the presence of ionizing radiation is one of many managed risks within a hospital. However, over the past two decades, medical occupational radiation exposure has decreased in spite of increased use of radiation in medicine [11]. Occupational exposure in nuclear medicine departments is mainly related to low doses of particular ionizing emissions from radioactive isotopes such as ^{32}P, ^{67}Ga, ^{111}In, ^{201}Tl, ^{59}Fe, ^{57}Co, ^{51}Cr, ^{99}mTc, ^{131}I, ^{192}Ir [2,3]. These radioisotopes have unstable nuclei, and dissipate excess energy by spontaneously emitting radiation in the form of gamma and other rays. Increasing growth of nuclear medicine practice, the use of radioactive isotopes for diagnostic purposes (cell-labelling and tumor imaging) and the use of radiopharmaceuticals in therapy lead to an increase of potential genetic risks to medical personnel. Once a radiopharmaceutical has been administered, the patient becomes a mobile source of radiation exposure to several critical groups of individuals. This source of exposure consists of two distinct types: (i) exposure to photon radiation which is emitted by the radioactivity retained by the patient but has not been absorbed within the patient. (ii) contact with radioactive secretions, excretions or tissue from the patient [12]. Contrary to the patients, medicall staff is usually exposed to much lower doses, but for a longer period of time.

In order to obtain information concerning the distribution and extent of radiation exposures, as such data are lacking from physical dosimetry in many cases, the use of biological indicators has gain increasing importance. Among individuals occupationally exposed to low levels of ionizing radiation different cytogenetic changes, for example increased frequencies of chromosome aberrations, micronuclei and SCE are usually evaluated [2-5,10,13]. An important advantage of biological dosimeters is the fact that they can be assessed at any moment, whereas physical dosimeters are not always present in the subject. Another advantage is that subjects under study cannot intentionally modify the biological dosimeter [2].

Lesions induced by ionizing radiation in DNA can be also detected by the alkaline single cell gel electrophoresis or comet assay [10,13]. This sensitive, quick and accurate test has been widely applied to measure both *in vitro* DNA damage and repair following exposure to various genotoxic agents and for human biomonitoring [7,8,13]. Results of the present study using the alkaline comet assay revealed an increase of radiation-induced primary DNA damage in human leukocytes of occupationally exposed nuclear medicine personnel.

Physico-chemical interactions between ionizing radiation and DNA produce a broad spectrum of DNA lesions including damage to nucleotide bases, DNA-DNA and DNA-protein cross-links, alkali labile sites as well as DNA single- and double-strand breaks (DSBs) [14]. Using the alkaline modification of comet assay we were able to detect DNA lesions induced, because the endpoint measured by traditional comet assay is a mixture of direct strand breaks and DNA damage that is converted to strand breaks by alkaline treatment (alkali-labile lesions) [7-9].

According to results obtained, the levels of DNA damage in majority of exposed subjects were positively correlated to the duration of occupational exposure. Observed heterogeneity in levels of DNA damage among exposed subjects could be addressed to specific modes of exposure as well as to different individual genome sensitivity. Because specialized workers often tend to perform the same tasks, it could be expected that some of them would exhibit higher levels of DNA damage. High levels of DNA damage recorded in leukocytes of occupationally exposed medical personnel suggest that working in area with continuous low level exposure may result in detectable primary DNA damage in circulating leukocytes caused either or/both by occupational contamination or by different clastogenic factors. These factors are responsible for the delayed effects of radiation. Among them there are superoxide radicals, that may induce genotoxic effects by indirect-acting mechanisms [15]. It could also be taking into account that nuclear medicine personnel is exposed to different radionuclides, that are characterized by different half-life and different emissions. Furthermore, some radionuclides are taken up in different amounts by different organs that might act as internal sources of irradiation. One of example is ^{131}I with an effective half-life of 8 and 3 days that might accumulate in thyroid gland [16]. It can be considered that nuclear medicine personnel is chronically exposed to small doses of different radionuclides which continuously causing lesions in DNA. Therefore, increased levels of primary DNA damage recorded by comet assay could be also related to accumulation of non-repaired DNA damage.

The accumulated dose range in nuclear medicine workers involved in our study was from 0 μSv to 970 μSv. However, we did not find a clear correlation between the DNA damaging effects and the doses recorded by the dosimeters. This result is in agreement with studies mentioned previously [10,17]. The authors discussed that it is difficult to establish dose-effect relationships for the low doses. It is also possible that certain parts of the body of the same subjects were nonhomogeneously exposed to higher radiation doses than those encompassed by the control of the dosimeter.

The comet assay allows determination of primary DNA damage, providing an assessment of current exposure. In addition to radionuclide exposure, other factors may contribute to enhanced levels of DNA damage, such as lifestyle associated factors. Our study confirms previous reports on smoking as a confounding factor that strongly influenced the basal levels of primary DNA damage both in exposed and control subjects. However, it has to be pointed out that the age of the individual had no significant effects on the mean basal level of DNA damage neither in exposed nor in control subjects of our study. These observations are also in agreement with previous reports of other authors [8,19].

Comet measurements may reflect both individual repair ability and DNA damage level. Because the measured damage level is a result of equilibrium between damage infliction and repair, a low damage level as assessed experimentally in an individual may be the result of an actual low number of lesions or of a high efficiency of repair. DNA damage detected with the comet assay is quickly repaired, usually within 2 h [20]. Considering that the DNA damage visualized by the comet assay is not permanent, high levels of DNA damage in exposed subjects could be ascribed to a general negative effect of exposure, such as an increased susceptibility to the activity of ionizing radiation or a decreased efficiency in recognition and repair of induced damage. The detection of damage would also suggest the presence of continuously active agents such as clastogenic factors in plasma.

It is well known that the some types of DNA damage (especially chromosome aberrations) in peripheral lymphocytes of the exposed subjects are accumulated during the long period of occupational exposure. The accumulation may be related to the populations of long-lived peripheral lymphocytes.

In the case of chronic exposure, a cumulative effect of ionizing radiation become more important and in these circumstances cytogenetic biomonitoring may provide different information on the DNA damage levels compared to comet assay. Because comet assay detects momentary DNA damage and/or repair activity, it reflects the current exposure and the actual levels of DNA damage present in peripheral blood leukocytes of the nuclear medicine workers at the moment of blood sampling.

Exposure to X-rays may result in effects that are both deterministic and stochastic in nature. Radiation workers are only at increased risk for deterministic effects if they work in violation of hospital safety policy and procedure [11]. Some stochastic effects to occupational exposure to X-rays would be cancer and genetic mutation. These are long term effects, which do not show in the exposed population until many years after the exposure. Under any circumstance, it is not acceptable for nuclear medicine workers to exceed their annual limit. It

is well documented that modern equipment and moderately aggressive radiation protection practices can keep occupational exposures at least an order of magnitude below the annual limit for the vast majority of medical workers. Rotation of workers through all tasks would also tend to distribute occupational exposures relatively uniformly over time.

In short, our study demonstrates the presence of increased primary DNA damage in nuclear medicine personnel exposed to low doses of ionizing radiation. Therefore, the personnel who work in nuclear medicine departments should carefully apply the radiation protection measures to minimize, as low as possible, radiation exposure to avoid possible deleterious genotoxic effects. The reported data also indicate that the standard alkaline comet assay protocol may be suitable for the biomonitoring purposes, as an additional complement to the standard biological dosimetry methods.

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