APPLICATION OF THE ALKALINE COMET ASSAY IN BIOMONITORING OF MEDICAL PERSONNEL OCCUPATIONALLY EXPOSED TO IONIZING RADIATION

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INTRODUCTION

Ionising radiation is a ubiquitous environmental physical agent whose DNA damaging effects are fairly well established. The effects of low-level exposure to ionizing radiation are of concern to large number of people, including workers receiving radiation exposure on the job. Medical radiation workers are employees of hospitals, clinics and private offices where radiation is used in the process of delivering health care to humans. These workers can be categorised into two groups "exposed employees" who receive at least a minimum detectable exposure during a one-year period, and "potentially exposed employees" who work in the vicinity of radiation but whose exposures are below detectable limits. 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. Trends in radiation exposure of both patients and workers are effected not only by developments in radiation protection, but also by dose in the practice of medicine [1].

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 [2]. The extent of health hazards is difficult to assess. Therefore, development of procedures that can be used to precisely identify health hazards in the exposed populations is a most significant approach towards establishing effective programs for disease prevention [3].

A wide range of methods is presently used for the detection of early biological effects of DNA-damaging agents in environmental and occupational settings. Currently, unstable chromosomal aberrations in peripheral blood lymphocytes, in particularly dicentrics, are the most fully developed biological indicators of ionizing radiation exposure [2,4]. 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 [2]. During the last few years, the single cell gel electrophoresis (SCGE) or comet assay was introduced as a useful technique for human biomonitoring studies [5,6]. 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 addition, 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 [7,8]. By using the comet assay positive results on the DNA-

damaging effects of X-radiation on occupationally exposed subjects were reported previously [9].

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

SUBJECTS AND METHOD

Subjects of study

The population studied comprised 40 volunteer blood donors: 25 of them had been occupationally exposed to ionizing radiation and 15 were unexposed control subjects. Each person completed a standardised 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 extraoccupational exposure to potential mutagenic hazards, such as smoking, alcohol and drug consumption, viral diseases, recent vaccinations, and radiodiagnostic examinations.

The exposed group consisted of 9 female and 16 male subjects working in the radiology units at Croatian hospitals. Eleven of them were physicians, 8 nurses and 6 radiological technicians. Their mean duration of occupational exposure was 18.4 years (range 5-31 years). The mean age of the whole exposed group was 45.2 (range 30-60 years). During their work they all wore individual dosimeters (film badges). The mean value of dosimeter readings for whole exposed group during one year prior the study was 2587.2 mSv (range 410-11336 mSv). Ten exposed subjects were smokers (4 female and 6 male), and 15 non-smokers (5 female and 10 male). One year prior to the beginning of the present study exposed subjects had not been subjected to diagnostic X-ray examinations.

Control subjects were healthy students and office employees (5 females and 10 males), chosen from the general Croatian population. Eight of them were non-smokers (3 female and 5 male), and seven of them were smokers (2 female and 5 male). The mean age of the control group was 45.2 (range 30-60 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 either to diagnostic X-ray or nonionizing examinations. None had received any therapeutic irradiation. They also were not taking any medications or oral contraceptives.

Blood sampling

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 period after collection.

The alkaline comet assay

The comet assay was carried out under alkaline conditions, basically as described by Singh et al. [5]. 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.

Comet capture and analysis

A total of 100 randomly captured comets from each slide were examined at 400 x magnification 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.

Statistical analysis

The comet parameters measured in the exposed and control groups were evaluated by using non-parametric Mann-Whitney *U* test. The level of statistical significance was set at $p < 0.05$.

Results

Results of the alkaline comet assay have been summarised in Tables 1 and 2. Within the exposed group marked interindividual differences regarding comet tail parameters were observed (Table 1). In the majority of exposed subjects the influence of age, dosimeter readings and duration of occupational exposure on the DNA damage could be excluded (Table 1). The highest level of DNA migration was recorded in exposed subject No. 22. No statistically significant differences were found between the mean values of comet tail parameters recorded within smoking and non-smoking individuals in the exposed group, independently on their gender ($p>0.05$, Mann-Whitney *U* test).

The distribution of comet tail parameters in control subjects was characterised by nuclei with small comets (Table 2). However, unexposed smokers had significantly increased levels of DNA damage compared to non-smokers. It was also observed that the basal levels of

DNA damage were significantly higher in unexposed female subjects, compared to male ($p<0.05$, Mann-Whitney *U* test).

Tail length

The comet tail lengths measured in exposed subjects were in the range 15.26 ± 0.20 μm to 22.56 ± 0.47 μm, with a mean value 17.57 ± 0.09 μm (Table 1). The observed values differed significantly ($p<0.05$, Mann-Whitney *U* test) from the values of tail length measured in control subjects, which were in the range 12.09 ± 0.22 μm and 15.08 ± 0.15 μm. For the control group the mean tail length was 14.07 ± 0.04 μm (Table 2).

Statistical significance of the results obtained for both experimental groups was evaluated by means of Mann-Whitney *U* test. While for the control group significantly elevated differences of comet tail lengths in smokers, compared to non-smokers, as well as in female compared to male were observed, similarly was not found in the exposed group.

Tail moment

The values of tail moments measured in the exposed group ranged between 12.82 ± 0.26 and 19.97 ± 0.44, and the mean value was 14.94 ± 0.08 (Table 1). The observed values differed significantly ($p<0.05$, Mann-Whitney *U* test) from the tail moment values measured in control subjects, which were in the range 8.65 ± 0.28 to 12.49 ± 0.16, with a mean value of 11.45 ± 0.05 (Table 2).

Statistical significance of the results obtained for both experimental groups was evaluated by means of Mann-Whitney *U* test. While for the control group significantly elevated differences of comet tail moments in smokers, compared to non-smokers, as well as in female compared to male were noticed, similarly was not found in the exposed group.

Table 1. The individual results of the alkaline Comet assay on peripheral blood leukocytes of medical personnel occupationally exposed to ionizing radiation expressed as mean values the measurements of 100 comets per each subject.

Subject No.	Gender smoking	Exposure (years)	Dose (mSv)*	Comet parameters evaluated					
				Min.	TL ± SE (μm)	Max.	Min.	TM ± SE	Max.
1	M^S	26	11336	12.18	17.47 ± 0.60	54.49	9.58	14.92 ± 0.59	51.35
2	M^N	31	8548	12.18	16.12 ± 0.31	30.13	9.77	13.69 ± 0.31	27.54
3	F^N	21	7240	10.90	16.03 ± 0.40	35.90	8.32	13.32 ± 0.39	32.77
4	M^N	13	6950	10.90	16.42 ± 0.59	57.69	8.33	13.80 ± 0.56	52.66
5	M^S	15	4210	13.46	17.99 ± 0.46	38.46	10.57	15.20 ± 0.43	33.16
6	F^N	23	4090	11.54	18.20 ± 0.51	38.46	9.05	15.53 ± 0.48	33.64
7	M^N	9	3340	14.10	21.65 ± 0.40	30.77	11.56	18.82 ± 0.39	27.92
8	M^N	28	2210	11.54	15.33 ± 0.27	26.28	9.11	12.82 ± 0.26	23.61
9	M^N	30	1900	12.18	17.26 ± 0.22	24.36	9.10	14.64 ± 0.22	21.58
10	M^N	15	1740	12.18	15.26 ± 0.20	24.36	9.73	12.84 ± 0.19	20.80
11	F^N	28	1500	12.18	16.09 ± 0.21	26.28	9.97	13.59 ± 0.21	23.20
12	M^S	12	1280	12.18	16.18 ± 0.20	22.44	9.92	13.66 ± 0.19	19.71
13	F^N	13	1160	11.54	16.14 ± 0.26	23.72	9.10	13.67 ± 0.26	21.28
14	M^N	5	1153	14.10	18.92 ± 0.30	26.92	11.08	16.16 ± 0.30	23.88
15	M^S	11	1133	14.10	20.09 ± 0.30	32.05	11.23	17.33 ± 0.29	28.23
16	M^S	29	1119	12.18	16.81 ± 0.35	32.69	9.66	14.27 ± 0.33	28.88
17	M^S	17	850	12.18	16.49 ± 0.41	32.05	9.20	13.98 ± 0.38	29.93
18	M^N	10	790	12.82	18.52 ± 0.32	32.05	10.31	15.79 ± 0.31	28.22
19	M^N	22	780	12.82	18.64 ± 0.55	55.77	10.01	15.85 ± 0.49	47.36
20	F^S	8	720	10.90	15.48 ± 0.23	21.79	8.78	13.02 ± 0.23	19.23
21	F^S	14	683	12.82	19.13 ± 0.51	37.82	10.20	16.44 ± 0.47	33.29
22	F^N	20	617	15.38	22.56 ± 0.47	44.87	12.25	19.67 ± 0.44	40.30
23	M^N	29	470	13.46	18.46 ± 0.51	48.72	10.82	15.72 ± 0.46	42.35
24	F^S	24	450	12.82	17.56 ± 0.35	31.41	10.21	14.87 ± 0.32	26.28
25	F^S	8	410	12.18	16.35 ± 0.29	35.26	9.38	13.79 ± 0.28	31.25
Mean (± SE)		18.4	2587.2	**17.57± 0.09↑**			**14.94± 0.08↑**		

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	Comet parameters evaluated					
		Min.	**TL ± SE (μm)**	**Max.**	**Min.**	**MT ± SE**	**Max.**
1	M^N	10.90	13.76 ± 0.12	17.31	8.26	11.29 ± 0.12	14.42
2	M^N	10.90	13.81 ± 0.15	17.31	8.39	11.40 ± 0.14	14.47
3	M^N	9.72	12.52 ± 0.13	13.61	5.78	10.12 ± 0.17	11.48
4	M^N	10.37	12.67 ± 0.18	14.91	6.30	9.09 ± 0.22	12.30
5	M^N	11.54	14.41 ± 0.14	17.95	8.34	11.80 ± 0.14	15.18
6	M^S	10.90	13.76 ± 0.12	17.31	8.26	11.29 ± 0.12	14.42
7	M^S	10.90	14.65 ± 0.16	17.95	8.88	12.12 ± 0.16	14.85
8	M^S	11.54	14.24 ± 0.14	17.95	9.27	11.58 ± 0.13	15.20
9	M^S	11.54	15.08 ± 0.15	18.59	8.67	12.49 ± 0.16	16.03
10	M^S	10.26	13.22 ± 0.15	17.31	7.72	10.64 ± 0.14	14.29
11	F^N	10.90	14.07 ± 0.15	16.67	8.35	11.57 ± 0.15	14.49
12	F^N	11.54	14.76 ± 0.14	17.95	9.53	12.16 ± 0.13	15.35
13	F^N	9.72	12.09 ± 0.22	17.50	4.67	8.65 ± 0.28	15.46
14	F^S	10.90	14.55 ± 0.15	19.23	7.91	11.96 ± 0.15	16.43
15	F^S	9.62	15.04 ± 0.15	17.95	7.06	12.42 ± 0.14	15.00
Mean (± SE)		**14.07 ± 0.04**			**11.45 ± 0.05**		

M-male; **F**-female; **S**-smoker; **N**-non-smoker.

DISCUSSION

Continued development of new technologies and procedures is anticipated in medicine, and these developments may continue to fuel the increased use of imaging procedures in medical diagnosis and therapy. 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 [1]. Provided that the worker follows all policies and procedures set forth by the institution, all workers will have personal exposures well within the boundaries of what is considered “safe”.

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. 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. Radiation workers are predicted to have a greater percentage risk of developing detrimental effects over the general public because of their generally greater exposure.

From the experience with radiation accidents it is evident that biological indicators are needed in order to obtain information concerning the distribution and extent of radiation exposures, as such data are lacking from physical dosimetry in many cases. These biological indicators can have a further advantage in that way that the individual radiation damage is measured which includes the variability of individual radiosensitivity. Under the many biological parameters which have been studied in this connection chromosomal damage in lymphocytes is most promising [9]. Among individuals occupationally exposed to ionizing radiation different cytogenetic changes, for example increased frequencies of chromosome aberrations and micronuclei are well known [10,11]. Scoring of dicentric and ring chromosomes in metaphase preparation of peripheral lymphocytes is the method of choice for quantifying acute over-exposures to ionizing radiation [4]. 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. Another advantage is that subjects under study cannot intentionally modify the biological dosimeter [2].

Ionizing radiation is a ubiquitous environmental physical agent whose DNA damaging effects are fairly well established. It induces DNA damage directly (as result of deposition of energy in cells) or indirectly (as result of free radicals formation and oxidative damage). It has been long known that physicochemical 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). DSBs were originally assumed to be the critical cytotoxic lesions, whereas base damage, particularly thymine glycols were implicated in mutagenesis. It is now accepted, however, that misrepaired DSBs are the principle lesions of importance in the induction of both chromosomal abnormalities and gene mutations [12,13,14].

Lesions induced by ionizing radiation in DNA can be detected by the alkaline single cell gel electrophoresis or comet assay [7, 10]. The same method was evaluated in the present study on occupationally exposed medical personnel. The comet assay is an easy, quick and accurate test that has been widely applied to measure both *in vitro* DNA damage and repair following exposure to various genotoxic agents and for human biomonitoring [6,7,15]. In present study the alkaline comet assay revealed the heterogeneity in the level of DNA breakage induced in human leukocytes by occupational exposure to ionizing radiation. The accumulated dose range in radiation workers involved in our study was from 410 mSv to 11336 mSv. We did not find any correlation between the genetic effects and the doses recorded by the dosimeters. This result is in agreement with studies mentioned previously [10,16]. 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. Another problem is about that some X-ray workers often have other jobs in small hospitals with the same kind of exposure [10]. The distribution of medical occupational exposures over time is poorly understood. Individuals receiving high readings in one year probably receive relatively high readings in succeeding years because specialised workers tend to perform the same tasks year after year [1].

The increased comet values in peripheral blood leukocytes of radiological workers exposed to ionizing radiation in the present study indicate a higher level of radiation-induced primary DNA damage. However, the influences of smoking habit, age or duration of the

occupational exposure on the levels of DNA damage assessed by use of comet assay could be excluded. Although the basal levels of DNA damage in studied unexposed subjects were strongly influenced by smoking; exposed smokers had no significantly increased levels of DNA damage compared to exposed non-smokers. An increased resistance of smokers to DNA damage induced by carcinogens has been described previously [17]. Wojewodzka et al [18] also reported on the lack of adverse effect of smoking habit on DNA strand breakage and base damage, as revealed by the alkaline comet assay. 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. The high interindividual variation of comet measurements obtained in the present study, independently on the doses recorded, could be also related with the ability of comet assay to detect primary DNA damage. 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. Therefore, the concurrent and past exposure levels for subjects involved in the present study were not the same. 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 concurrent exposure and the actual levels of DNA damage present in peripheral blood leukocytes of the radiological workers at the moment of blood sampling.

Under any circumstance, it is not acceptable for radiological workers to exceed their annual limit. It is well documented that modern X-ray 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 radiological workers. Rotation of workers through all tasks in a radiology program would tend to distribute occupational exposures relatively uniformly over time.

In short, our study demonstrates the presence of increased primary DNA damage in medical personnel exposed to ionizing radiation. The reported data 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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