DNA damage and repair in white blood cells at occupational exposure

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Abstract: The present work was aimed at finding appropriate biomarkers applicable in molecular epidemiological surveys of occupationally exposed individuals and/or population in order to prove low dose effects. Blood samples were taken from NPP workers, in the “strict regimen” area (exposed group) and from the administration staff (control group). The spontaneous and induced (exposed to a challenge dose of 2.0 Gy gamma irradiation) DNA repair synthesis in leucocytes, the level of DNA damage by single cell gel-electrophoresis in lymphocytes and the concentration of malondialdehyde in blood serum, were analyzed. A significant decrease of potentially lethal damage in leucocytes as well as reduction of DNA double strand breaks level in lymphocytes of persons with “mean annual dose” lower or equal to 5 mSv/a was found, compared to the control group. A higher repair capacity corresponding to elevated protein synthesis after a challenging dose of 2.0 Gy gamma rays and a significant decrease in the level of oxidative stress in the blood plasma were established in persons from the same group. The present investigation showed that annual doses not higher than twice the natural radiation background exert positive effects on DNA damage and repair, increase cellular resistance and decrease oxidative stress.

1. Introduction:

Some epidemiological surveys of radiation risk at occupational exposure have established statistically lower cancer death rate compared to no-exposed workers from the same enterprise [1, 2, 3, 4, 5]. There are no data correlating the dose to cancer death rate besides that for leukemia which have been statistically related to cumulated dose from external irradiation. These investigations allow defining the concept for hormetic effects that lead to reduction of the risk [6, 7]. Data supporting these phenomena could not be indisputably accepted because estimation of the real risk at low doses by the methods of classical epidemiology needs large cohorts of exposed person’s due to high spontaneous rates of cancer diseases and confounding factors. Because of this for the purposes of radiation protection the assessment of risk is based on extrapolation of data for high doses, so that the risk should not be underestimated. There is an ambiguity what happens at doses below 200 mSv, which are typical for occupational exposures [8].

Enough evidences are available to prove that defects in genes controlling DNA repair processes and genome stability are related to the increased risk for cancer development. Such an example is Ataxia Telangiectasia connected with an ATM-gene anomaly [9]. There are also data confirming that low doses of X- or gamma rays have some favourable effects persisting in the cell population. Such examples are the system for detoxification of free radicals, and reduction of chromosome aberrations after a consecutive irradiation with a high dose - the so-called adaptive response [10]. It has been shown that in cells cultivated with supernatant of irradiated with 2 cGy donor cells, and irradiated afterward with 4 Gy, the neoplastic transformation is twice reduced compared to directly irradiated with 4 Gy cells [11]. The transmission of adaptive response onto un-irradiated cells is called bystander effect. Two mechanisms of transmission of signals between irradiated and un-irradiated cells are suggested: by soluble extra cellular mediator-factors or by gap-junctions [12]. These effects are well expressed after single irradiation with X- or gamma rays whereas the effect depends on the cell type, usually in the range of 0.1 to 0.5 Gy, including changes in gene expression. When increasing the dose above 0.5 Gy these effects are not observed. If stimulating dose is separated into fractions, the critical factor appears to be the interval between single irradiations [10].
A considerable amount of experimental data in the last 10 years confirms the connection between the irradiation-induced gene expression, the adaptive response and hormesis [13, 14]. Latest achievements of molecular techniques give an opportunity to study the risk at a multidisciplinary perspective. This approach, called molecular epidemiology is based on combination of an epidemiological study with molecular methods and helps in estimation of risk not only for large cohorts, but allows distinguishing differences between single individuals [15].

The present investigation has been aimed to study the possibility of implementing a complex of molecular biomarkers to prove and characterize low dose radiation effects. The spontaneous and induced (exposed to a challenge dose of 2.0 Gy gamma irradiation) DNA repair synthesis in leucocytes, the level of DNA damage by single cell gel-electrophoresis in lymphocytes and the concentration of malondialdehyde in blood serum have been analyzed.

2 Methods and Materials

2.1 Study Design and Subjects

Blood samples of 80 exposed workers in the ‘strict regimen’ area and non-exposed NPP administration staff from the NPP Kozlodui in Bulgaria were studied. The recruited subjects gave informed consent to participate in the study and were assured of complete anonymity. Retrospective information about demographic characteristics, health status and occupational exposure (including age at initial exposure, age at sampling, radiation doses, etc.) was collected. Film dosimeters were used to measure gamma-ray exposure. The annual doses of irradiation in the exposed group were within the occupational safety limits (<50 mSv each year or <100 mSv for a period of five consecutive years). The study criteria consisted of: working conditions with predominantly gamma-ray exposure at a dose level ranging from 1 to 12 mSv/year (for 94% of the studied subjects), and no reported medical irradiation over the last three years.

2.2 Blood Sampling, Ly Isolation and Irradiation

Blood samples were taken, transferred and stored at 4°C and the processing of probes were accomplished within 48 hours from the time of sampling. Lymphocytes were isolated by centrifugation of blood suspension with Ficoll for 40 min at 1800 rpm. The lymphocytes were pipetted and resuspended in phosphate buffer pH 7.4, followed by three times washing with the same buffer and centrifugation at 1100 rpm. The cells were then transferred in 0.5 ml RPMI 1640 and counted at light microscope. The cell suspension was diluted with PRMI to a concentration of \(5 \times 10^5\) cells/ml. Blood samples were irradiated at room temperature with 2Gy from \(^{137}\)Cs gamma-source at a dose rate of 92 cGy/min.

2.3 Single Cell Gel Electrophoresis (Comet Assay)

The pH of unwinding dictates the type of DNA damage detected by the Comet assay. Under neutral conditions (pH 7-8) mainly double strand breaks are detected. The method consists of several stages including embedding of cells into low melting agarose to reach a concentration of \(5 \times 10^4\)/slide, dipping in lysis solution (154 mM NaCl; 10mM Tris pH 7.5; 30mM EDTA pH7.5), washing of slides, dipping into TBE, pH 10 for 5 min, staining with EtBr and visualizing under fluorescence microscope. The DNA damage was evaluated by analysis of 250-cells/examined person. Cells were separated in 5 types: from I (without tail) to V (almost all DNA is in the tail) (Figure 1). For each examined person the Comet Index (CI) was calculated by the following formula:

\[
CI = n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5,
\]

where \(n\) was the relative part of cells pertaining to a definite type, and the coefficients from 1 to 5 reflected the level of damage. CI gave information about the level of DNA damage.

2.4 Determination of spontaneous and \(\gamma\)-induced DNA repair synthesis

Spontaneous (RSsp) and induced (RSind) after additional in vitro irradiation with 2 Gy DNA repair were estimated by unscheduled DNA synthesis (UDS) in peripheral blood leukocytes as published in details in a previous paper [16]. Both syntheses were defined in a short-term culture of whole blood. Hydroxyurea (HU, 10 mM) was used to suppress the normal DNA synthesis. The cells were then incubated for 2 hours at 37°C with 3H-thymidine, before or after in vitro gamma-irradiation with 2 Gy. The leukocyte population was separated and the activity of the incorporated marker was measured radiometrically. This methodological step has concerned the physiology of cells and allowed maintenance of both the original ratios of the cellular subpopulations and intercellular relations [17]. On the other hand, the blood possesses larger capacity (than separated lymphocyte cells) in
demonstrating radiation effects at low dose levels [18]. Measurement of normal DNA synthesis was performed in parallel for each individual on samples untreated with HU. The parameters were calculated as a ratio between 3H-thymine incorporations, measured as number of disintegrations per minute, in the presence or absence of HU or irradiation in relative units (Rel.U.).

2.5. Lipid peroxidation in blood plasma
Quantification of lipid peroxidation level was done by malon dialdehyde concentration, measured spectrophotometrically. Under heating and acid conditions lipid peroxidation products, mainly endoperoxides, underwent decomposition producing malonodialdehyde (MDA), which molecules reacted with thiobarbituric acid to form a colored complex. MDA concentration (in nmol/ml plasma) was estimated by the following regression equation: $C = 0.21 + 26.5 \Delta D$, where $C$ was the concentration of TBA active products (in nM MDA per ml plasma); and $\Delta D = D_{535} - D_{580}$ of the sample (in units “optical density”).

2.6. Statistical analysis
Experimental data were statistically analyzed by t-criteria of Student-Fisher method. Statistical significance of the results was assumed at p<0.05.

3. Results:
In order to analyze the effect of the dose the examined persons have been separated into 5 groups according to the annual doses, ranging from 5, 10, 15, 20 and above 20 mSv.y$^{-1}$, calculated by dividing cumulated dose to the length of service, which we called “mean annual dose”, and a control group from the administrative personnel of the NPP.

3.1. Single Cell Gel Electrophoresis
Over the past decade, the comet assay, or single-cell gel electrophoresis (SCGE) has become one of the standard methods for assessing DNA damage [19], with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as fundamental research in DNA damage and repair [20]. Peripheral blood lymphocytes are often used for this assay because of the high radiosensitivity of this cell population.

The results of neutral comet assay are presented on Figure 2. Cells pertaining to a definite comet type were summed up for all 10 neutral samples for each examined group. In the group with annual doses lower than 5 mSv.y$^{-1}$ a higher number of normal cells were recorded compared to the control. In all studied groups normal cells were predominating over damaged cells. Increase of damaged cells was observed at annual doses above 15 mSv.y$^{-1}$. 

Figure 1. Neutral Comet assay – 6 undamaged cells, 2 comets type II and 1 comet type V.
Figure 2. Neutral comet assay. Distribution of comet types

In Table 1 the distribution of comet types in the neutral probes is presented. At annual doses below 5 mSv.y\(^{-1}\) the results are significantly different from the control value. In the group with annual doses up to 10 mSv.y\(^{-1}\) the results are not significantly increased compared to control or to the group with annual doses below 5 mSv.y\(^{-1}\). At annual above doses 15 mSv.y\(^{-1}\) a significant increase of CI was recorded.

Table 1. Mean values of evaluated parameters

| Parameter** | Control | <5 (1.93±0.92) | <10 (7.2±1.52) | <15 (12.72±1.28) | <20 (16.88±1.39) | >20 (23.58±1.87) |
|-------------|---------|----------------|----------------|------------------|------------------|-----------------|
| CI          | 1.24 ±0.09 | 1.16 ±0.006* | 1.23 ±0.12 | 1.24 ±0.05 | 1.41±0.28 | 1.55 ±0.38* |
| RSsp        | 0.70 ±0.07 | 0.54 ±0.15* | 0.65 ±0.05 | 0.73 ±0.08 | 0.81 ±0.12* | 0.91 ±0.20* |
| RSind       | 0.70 ±0.12 | 0.81 ±0.08* | 0.74 ±0.13 | 0.65 ±0.12 | 0.63 ±0.17 | 0.58 ±0.12* |
| MDA         | 3.90 ±0.24 | 3.58 ±0.30* | 3.97 ±0.09 | 4.07 ±0.19 | 4.15 ±0.13* | 4.22 ±0.17* |
| n           | 16       | 18             | 12            | 10              | 12               | 12              |

* p < 0.05 compared to control; **Mean ± SD

3.2 Determination of spontaneous and \(\gamma\)-induced DNA repair synthesis

Unscheduled DNA synthesis (UDS) is being successfully used to reveal mutagens in the occupational and/or natural environment [21, 22, 23]. Repair processes were assessed by measuring incorporation of labelled nucleotides into DNA of irradiated cells. A correlation was established
between the maximum rate of DNA single-strand breaks and the maximum rate of \( ^3 \)H-thymidine incorporation [24, 25].

We have used RSsp as a measure of Potential Lethal Damage (PLD) level, because after a low-dose exposure, a certain amount of un-repaired lesions remains in the cells, which become apparent through application of inhibitors of replicative DNA synthesis (HU and ara-C) [26]. The investigation of the radiation response of lymphocytes has shown that cells in \( G_0 \) are able to store information of the irradiation, which remains even after stimulation of proliferation. RSind (after additional in vitro \( \gamma \)-irradiation of blood samples with 2Gy) was used for adaptive response evaluation.

**Spontaneous DNA Repair Synthesis.**

The rate of spontaneous repair synthesis increased with the dose of gamma irradiation (Figure 3a). The spontaneous repair synthesis was statistically lower for the group that received up to 5 mSv.y\(^{-1}\) (annual mean dose 1.93 mSv.y\(^{-1}\)). In the other two groups with annual mean doses of 6.91 and 12.72 mSv.y\(^{-1}\) the spontaneous repair synthesis was close to the control values. The groups with annual mean doses of 16.88 и 23.58 mSv.y\(^{-1}\) respectively, showed an increase in the spontaneous repair synthesis, without statistical difference between them (Table 1).

**Induced DNA Repair Synthesis.**

In an attempt to understand and ascertain the stimulatory effect of low dose ionising radiation (so-called adaptive response), we compared the changes in the gamma-induced repair capacity (after 2Gy additional irradiation). The induced DNA repair synthesis was decreased with increasing the dose (Figure 3b). In the group with annual doses up to 5mSv.y\(^{-1}\) the induced DNA repair synthesis was statistically different to the control, while in the groups with annual doses up to 10 or 15 mSv.y\(^{-1}\) the
induced DNA repair synthesis was close to the control values. At annual doses up to 20 and above 20 mSv.y\(^{-1}\) the induced DNA repair synthesis was decreased, but statistically different were only the changes in the group with annual doses above 20 mSv.y\(^{-1}\) (Table 1).

### 3.3 Lipid peroxidation in blood plasma

Overproduction of free radicals and their toxicity is one of the problems that cells encounter upon irradiation with ionizing radiation. Free radicals induce significant late health effects and lead to an accumulation of various quantitative and qualitative changes in the cells, commonly described as oxidative stress.

In our experiments quantification of lipid peroxidation level was done by malon dialdehyde concentrations in blood plasma of occupationally exposed persons. The dependence between MDA concentrations and the mean annual dose is presented at Figure 3b. The straight line represents the mean value of the control group. At doses below 5 mSv.y\(^{-1}\) the MDA values are below the mean control values.

The mean values of malon dialdehyde concentrations in the examined groups of occupationally exposed persons are presented in Table 1. Statistical decrease of MDA values compared to control was established only for the group with annual doses below 5 mSv.y\(^{-1}\). MDA was significantly increased in groups with annual doses 10 and 15 mSv.y\(^{-1}\) as compared to the group with 5 mSv.y\(^{-1}\). The other two groups with annual doses below and above 20 mSv.y\(^{-1}\) showed statistically different values from that of the control.

### Discussion

The main problem for both medical and non-medical use of ionizing radiation is the possible carcinogenic risks associated with small doses of ionizing radiation. These eventual risks are also of great importance with regard to natural irradiation.

The rapidly growing knowledge in molecular biology and radiobiology during the last decade has led us to examine effects of low doses (<100 mSv) and a fortiori of very low doses (<10 mSv). The physico-chemical events caused by an irradiation trigger a series of signals and reactions that can profoundly alter the fate of the DNA lesions. The defense mechanisms induced in a cell depend on number and nature of the cellular damage. Modern transcriptional analysis of cellular genes using microarray technology reveals that, without modification of the genome, numerous genes are activated or inhibited following doses much lower than those for which mutagenesis is observed [27]. Moreover, depending on the dose and the dose rate, different signaling systems are activated in cytosol (MAP kinases), mitochondria, and nucleus (protein kinases). In the nucleus, different levels of DNA damage lead to activation of different families of genes [28, 29, 30, 31].

In our study a well expressed directly proportional relationship between the mean annual dose and the level of potentially lethal damage (PLD) was established. A significantly lower level of PLD in comparison to the control was registered at annual doses below 5 mSv.y\(^{-1}\) (Table 1).

The formation of comets in neutral gel electrophoresis was mainly due to DSB that produce DNA fragments and free ends. The results showed that in all studied groups the undamaged cells were predominant, their number being reduced with increasing of the dose (Figure 2). At annual doses below 5 mSv.y\(^{-1}\) undamaged cells were more than those in the control, and all types of damaged cells, including slightly damaged ones were less than in the control group. At annual doses above 15 mSv.y\(^{-1}\) the number of type IV comets was considerably increased. The slight variation in the number of type V comets was due to the so-called effect of saturation of comet tail. The initial increase in the number of breakages led to elongation of the tail, but after a certain level of damage, started an increase of the quantity of DNA in the tail without change of its length [20]. This was the reason to accept type IV comets as a more reliable indicator for heavily damaged cells. It could be suggested that the initial formation of SSB at annual doses below 5 mSv.y\(^{-1}\) has induced repair processes leading to lowering the level of spontaneous lesions.

The effectiveness of DNA repair systems is evidenced by the lack of reduction in the mutagenic and lethal effect as the dose rate decreases in the cell lines in which the signaling or the DNA repair systems are impaired or blocked, for example, in hereditary diseases with defects in repair systems. At equal doses, the mutagenic effect varies markedly with the dose rate [32, 33]. A limited
number of lesions induce a reversible arrest of the cell cycle, which enhances repair. A high amount of lesions prolongs the cell cycle arrest, which can lead to apoptosis. The time taken by repair depends on the complexity of the damages and the repair systems operating. It is known that most of the lesions induced by low doses of ionizing radiation are small lesions, which are repaired by BER that could not be always recorded by the spontaneous repair synthesis (one in every four cases). Furthermore, the size of newly repaired sites depends on the type of damage, thus the level of spontaneous repair synthesis is defined not only by the quantity but also by the heaviness of the damage. Thus a correlation has been searched for the spontaneous repair synthesis and the neutral comet assay when DSB have an important role in the formation of comets. The results from the spontaneous repair synthesis and the comet assay were in accordance with data that quickly repaired DNA damages such as DSB, stimulate repair systems and could be connected with cell radioresistance. The stimulated cell protection led to statistically different decrease of cells with DSB (at doses below 5 mSv.y\(^{-1}\)) in comparison to the control group (Table 1). The favorable effect of low doses established in this study was probably due to activation of cell safeguard systems increasing the radioresistance of the cell to subsequent irradiation. Thus common mechanisms might be involved in the radiation hormesis and the adaptive response. To clarify this suggestion we conducted an additional in vitro irradiation with 2 Gy and analyzed the induced repair synthesis. The chronic occupational exposure of workers has been accepted as “initiating exposure” according to adaptive response classical scheme.

Recent data have also shown that the effectiveness and rapidity of repair depend on the time, type of tissue and its proliferative status. Biological data show that there are two safeguard mechanisms of the cell genome: DNA repair and programmed cell death. The temporal abundance of radiation-induced damage is a major factor in the efficiency/fidelity of DNA repair and hence the frequency of induced mutations [34].

The probability of error-free or error-prone repair does not vary with dose and dose rate [32]. The data show variations in the efficacy/fidelity of DNA repair, that could be due to several mechanisms such as activation of some biochemical phenomena [35, 36], cell cycle arrest which allows additional time for repair, variations in the efficacy of repair associated with the temporal abundance of damages within a cell [37], or the existence of a threshold in doses or dose rate below which the radiation damage sensor ATM is not activated [38].

The repair activity fell regularly by increasing the dose, while only at doses below 5 mSv.y\(^{-1}\) it is statistically higher (Figure 3b). These results were indicative for a relation between the increased repair activity and the lower number of DSB detected by the neutral comet assay. Only at annual doses above 20 mSv.y\(^{-1}\) a statistically different decrease of repair activity was recorded. This correlated to the recorded increase of Comet Index in the same exposure group. The tendency of a decrease in the induced DNA repair synthesis corresponded to increase of PLD assessed by the spontaneous repair synthesis. A possible reason might be the balancing between the rate of cell safeguard stimulation and the compensated quantity of radiation-induced PLD, that could not give an opportunity to decrease damages below the rate of the spontaneous ones. Some authors have also established adaptive response in the lymphocytes of occupational exposed persons [39, 40, 41]. The repair mechanisms could be activated by doses 30-40% above the natural background, a condition that could be fulfilled only for annual doses below 5 mSv.y\(^{-1}\) (mean annual dose for the group 1.93 mSv.y\(^{-1}\)). There is a direct correlation between the oxidative and genotoxic stress. Taking into account that DNA is the most important and best-protected macromolecule in the cell it could be concluded that health effects of low dose irradiation are a result of membrane injuries, which are the initiating event of the cell damage [42]. Lipid peroxidation products such as MDA have been shown to exert carcinogenic properties. DNA adducts produced by the interaction with MDA are connected with malignancy. The damage of cell membrane might be the first step to explain the relation between cancer and low radiation doses. A significant increase of MDA concentrations in the blood serum of occupationally exposed NPP workers has been reported by Đurović et al [43]. Similar results were further interpreted as a direct effect and/or insufficient functioning of anti-oxidative systems [44, 45].

The results obtained in this study gave the reason to suggest that occupational exposure provoke dose dependent damage of the cell membrane. Only at annual doses below 5 mSv.y\(^{-1}\) the level of damage was lower than that in the control (Table 1). Anti-oxidative systems are the first safeguard neutralizing large part of free radicals. Un-scavenged free radicals reach DNA and give rise to SSB, which activate repair systems, supporting thus the low level of DSB in the cell. The
statistically lower level of oxidative stress at annual doses below 5 mSv.y" sup>−1" was probably due to the radiation-induced synthesis of anti-oxidative enzymes. So that it might be assumed that low doses are in favor of the lower level of oxidative stress.

Our investigation showed that an increase in PLD and changes in the redox regulation (statistical increase in MDA concentrations) have been established in the dose interval between 15 and 20 mSv.y" sup>−1". It is still not clear how disturbances in the function of anti-oxidative systems lead to accumulation of damages induced by free radicals [14]. Accumulation of free radicals at annual doses above 15 mSv.y" sup>−1" was not connected with the increase of DSB, suggesting that other mechanism might be involved in this case.

The results of the present study showed that exposure to annual doses below twice the dose of the natural background had favorable effect on DNA damage and repair, increased resistance of the cell, and decreased the level of oxidative stress.

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