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IN VITRO STUDY OF RADIATION-INDUCED DNA DAMAGE*

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Abstract. This work presents the effects of low-LET radiations (electrons) on normal and modified cells DNA. The radiation-induced effects are assessed using the COMET-assay. The results show that modified cells are more sensible to exposure than the normal ones. The dose-effect relationship follows a linear-no-threshold model suggested by BEIR VII.

Key words: cell exposure, low-LET radiation, COMET assay, DNA fragmentation, radiation risk assessment.

1. INTRODUCTION

Biological Effects of Ionizing Radiation (BEIR) VII Report [1] develops the most up-to-date and comprehensive risk estimates for cancer and other health effects from exposure to ionizing radiation. This document supports previously reported risk estimates for cancer and leukaemia and focuses on the health effects of low levels of low linear energy transfer (low-LET) ionizing radiation such as electrons (beta rays and accelerated beams), X-rays and gamma rays.

It is known that ionizing radiations induce damages to living cells and, consequently, biological effects may occur following an exposure [2]. Injury to living cells results from the transfer of energy to atoms and molecules in the cellular structure. Ionizing radiation determines ionization or excitation of atoms and molecules, producing free radicals and break chemical bonds. New chemical bonds are created and cross-linkage develops between macromolecules. Damages

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to molecules that regulate vital cell processes (*e.g.* DNA, RNA, proteins) are more important that damages to other molecules.

Linear energy transfer (LET) describes the rate at which a type of radiation deposits energy as it passes through tissue [3]. Higher levels of deposited energy on the path length cause more DNA breaks. Therefore, it is expected that more cells to be killed by a given dose of ionizing radiation. Low-LET radiation deposits less energy in the cell along the radiation path and is considered less destructive per radiation track. For low-LET radiation, the number of ionizations per path length unit is low and DNA damages are mostly isolated, single break lesions. Different types of radiation have different levels of LET. For example, X-rays, gamma rays, and electrons are known as low-LET radiation. Heavy ions, neutrons or pions are classified as high-LET radiation. Generally, radiation sources have a mixture of high- and low-LET radiation.

When damaged, the DNA molecule acts in three different ways: it may repair itself, lead to the cell death or mutate [4, 5]. The most likely outcome for low doses is repair, while the probably events as mutations can lead to cancer [6]. Cancer caused by radiation is no different than that caused by other carcinogens. Radiation is not the only agent that can cause mutations in DNA. Some other mutagens are chemicals, heat, and ultraviolet light.

The BEIR VII report concludes that the current scientific evidence is consistent with the hypothesis that, at the low doses of interest in this report, there is a linear dose-response relationship between exposure to ionizing radiation and the development of solid cancers in humans. It is unlikely that there is a threshold below which cancers are not induced, but at low doses the number of radiation induced cancers will be small [1]. The BEIR VII committee also developed "risk models" for estimating the risk that an exposed individual will develop cancer. This task requires expressing the dependence of risk on radiation dose and also on sex and age at exposure. Data from epidemiologic studies were used to accomplish this task.

On average, the BEIR VII lifetime risk model predicts that approximately one individual in 100 persons would be expected to develop cancer (solid cancer or leukaemia) from a dose of 100 mSv. Lower doses would produce proportionally lower risks. For example, it is predicted that approximately one individual in 1000 would develop cancer from an exposure to 10 mSv [1]. At doses of 100 mSv or less, statistical limitations make it difficult to evaluate cancer risk in humans. A comprehensive review of available biological and biophysical data led the committee to conclude that the risk would continue in a linear fashion at lower doses without a threshold. This assumption is termed the "linear-no-threshold" (LNT) model [6].

There are two competing hypotheses to the linear no-threshold model. One is that low doses of radiation are more harmful than a linear no-threshold model of effects would suggest. The other hypothesis suggests that risks are smaller than

predicted by the linear no-threshold model. The BEIR VII report concludes that the preponderance of information indicates that there will be some risk, even at low doses, although the risk is small [1].

According to some epidemiological data, low exposures to radiation are beneficial to health, even if larger doses may be harmful [7, 8]. Hormesis model is based on two arguments. Firstly come epidemiologic studies, where there is a lack of proof for the LNT model combined with a number of studies indicating beneficial effects of radiation [9]. Secondly, cells irradiated *in vitro* with low absorbed doses, a few tens of mGy, show less damage as a result of a subsequent exposure within hours than do un-irradiated cells.

Under these circumstances, we propose to determine the level of various molecular markers of DNA damage as a function of the dose of low-LET ionizing radiation. These studies allow tuning the dose levels in radiotherapy, for treatment efficiency and radioprotection of patients. We focus on the response of normal and neoplastic cells to the action of ionizing radiation. The purpose is to confirm the validity of risk models using in-vitro cell exposures. The irradiation effects are evaluated using the Comet test. We are also interested in assessing the DNA repair fidelity, especially double and multiple strand breaks, and whether repair capacity is independent of dose. Finally, we aim to evaluate of the relevance of adaptation, low-dose hypersensitivity, bystander effect, hormesis, and genomic instability for radiation carcinogenesis

2. *IN VITRO* ESTIMATION OF THE RISK OF BIOLOGICAL EFFECTS

2.1. MATERIALS AND METHODS

Cellular cultures of normal renal monkey (RM) cells were grown in a culture medium (Dulbecco's Modified Eagle Medium – DMEM), supplemented with 2% fetal bovine serum. Cellular cultures of neoplastic HeLa cells were also grown in a DMEM culture medium, supplemented with 10% fetal bovine serum.

Both culture cells were maintained on dedicated plates with a surface of 25 cm^2. The initial density of cells was of about 5×105 cells/plate (25 cm^2). After 24 h from initiating the cultures, when the cells are in the log phase of their development, the plates were exposed to a flux of electrons. After the treatment, the exposed and unexposed (control) cells were kept in an incubator to grow for another 48 h, in order to complete several cellular cycles. At the end of this interval, the cellular film was detached using trypsin and the cellular suspension was subjected to the COMET test.

Cells exposure was done using an electron beam generated by a radiotherapy linear accelerator Varian. The maximum value of the beam energy was 8 MeV. The cells were exposed to single doses between 1 and 20 Gy, calculated by the accelerator treatment planning system.

2.2. DNA DAMAGE ASSESSMENT

The COMET test is an uncomplicated and sensitive technique for the detection of DNA damage (single stranded breaks) at the level of the individual eukaryotic cell. The test can be applied to *in vitro*, *ex vivo* and *in vivo* systems. The methodology was developed by Singh *et al.* in the mid-1980s [10], and was modified later [11] by including unwinding under alkaline conditions. The COMET assay is a standard technique for evaluation of DNA damage/repair, bio-monitoring and genotoxicity testing. As a working method, it consists in including the studied cells into a low gelling point agarose, followed by the lysis of cells with a specific buffer on neutral or alkaline medium, unwinding of DNA and the electrophoresis of the cell lysed suspension. Electrophoresis is followed by a visual analysis of the DNA after staining with a specific fluorochrome. The DNA damage assessment is done by fluorescence intensity measurements. A cell with migrating DNA resembles a comet with a concentration of DNA at the "head" and a diffused trailing migration of DNA referred to as the ""tail". Studies using this assay have largely included those involving radiation and radiomimetic chemicals [12, 13].

In our experimental conditions, the COMET test was performed in alkaline condition. Images for DNA breaks assessment were taken with a epifluorescence microscope and a digital camera. The analysis software was CometScore v1.5. To quantify the level of DNA damage, the extent of DNA migration was defined using the 'Olive Tail Moment' (OTM), which is the relative amount of DNA in the tail of the comet multiplied by the median migration distance [14]. The statistical analysis of data was done using the Student's t test [15]. The minimum signification threshold was $p < 0,05$.

3. RESULTS AND DISCUSSIONS

The exposure of normal renal cells to different doses of low-LET radiations determined the variation of the number of registered comets. In the first case, when cells were exposed to 1Gy, the number of comets decreases by respect to the control (unexposed) cells. Greater doses (5, 10 and 20 Gy) lead to a gradual increase of the number of comets, as presented in Table 1.

Table 1

Values of "Olive tail moments" obtained after COMET assay of normal monkey renal cells, for control and low-LET radiation exposed cultures

Exposure (Gy)	X±ES	p	variation (%)
Control (unexposed)	1.08 ± 0.20	-	100
1 Gy (RM1)	0.45 ± 0.09	< 0.01	41
10 Gy (RM10)	3.12 ± 0.63	< 0.01	288
20 Gy (RM20)	6.96 ± 1.52	< 0.001	645

The decrease of the number of comets, evidenced for low-dose exposures (1Gy), may be determined by minor modification at DNA macromolecule level, followed by the activation of DNA repair mechanism. These mechanisms ensure a more efficient monitoring and error repairing activities of DNA. This behavior is similar to hormesis phenomena described in the introductory part.

Higher doses determine an increase of DNA breaks in the macromolecule, as evidenced by the increase of the amount of DNA fragmented comet tails. We note a gradual increase in dose-proportional number of comets used. Even if the number of experimental data in small, the increase seems to follow a linear dependence, suggesting that the linear model is suitable to describe this behavior.

Another cell line used was the HeLa neoplastic (cancer) cells. Their main characteristic is a high rate of proliferation, favouring a certain fragility of the DNA macromolecule.

Compared with control cells (Table 2), cultures exposed to electron beam with different doses lead to a progressive increase in the frequency and quantity of DNA comets expelled from exposed cells.

Table 2

Values of "Olive tail moments" obtained after COMET assay of HeLa neoplastic cells, for control and low-LET radiation exposed cultures

Exposure (Gy)	X±ES	p	variation (%)
Control (unexposed)	1.21 ± 0.34	-	100
1 Gy (RM1)	2.03 ± 0.35	NS	167
10 Gy (RM10)	31.19 ± 2.40	< 0.001	2,583
20 Gy (RM20)	51.87 ± 4.54	< 0.001	4,294

This increase in the number of comets for HeLa cells (Fig. 3) can be attributed to a combination of three factors: the breaks generated by the electrons, the malfunction of DNA repair mechanisms in cancer cells and the high rate of proliferation. The result is a more pronounced response of these cells to the exposure.

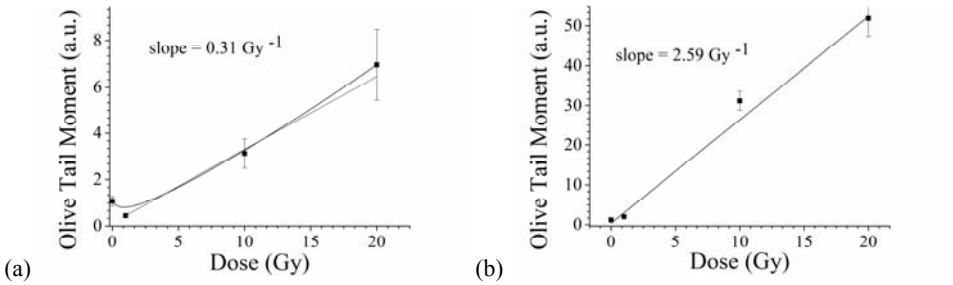

Fig. 1 – Variations of the OTM for: a) nornal; b) neoplastic HeLa cells, for control and low-LET radiation exposed cultures.

The graphs presented in Fig. 1a and b support the statement that a dose-risk relationship could be determined. In the case of normal cells, a hormesis effect is evidenced, followed by a linear dependence of the dose-effect. Neoplastic cells show a linear-non-threshold like behaviour.

The slopes of the two dependences, calculated by linear interpolation, are 0.31 Gy^{-1} for normal cells and 2.59 Gy^{-1} for neoplastic HeLa cells. These values suggest that normal cells are less damaged by radiations than neoplastic ones, which is a good aspect in case of cancer treatment using electron beams.

4. CONCLUSIONS

Exposure to low-LET radiations of normal and neoplastic cells shows that DNA damages occur. The evaluation of DNA breaks was assessed using the COMET test and Olive Tail Momentum. For normal renal cells, a hormesis effect seems to be activated at low doses. For higher doses, the dose-effect relationship seems to be linear. In the case of neoplastic HeLa cells, a dose-effect relationship can also be established. The genetic material fragility of neoplastic cells is increased compared with normal cells.

At this stage of research, we conclude that the effect of electron beam is more pronounced on cancer cells compared with normal. The confirmation of dose-effect relationships allows more investigation in order to establish a dose-risk model. The studies have to be developed and extended on the effects of high-LET radiations.

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