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DNA damage induction and repair evaluated in human lymphocytes irradiated with X-rays and neutrons

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Key words: Comet assay; DNA damage; SCGE; DNA strand breaks

Abstract

The objective of this study was to evaluate the kinetic of the DNA damage induction and their subsequent repair in human lymphocytes exposed to various types of radiation. PBLs cells were isolated from the whole blood of two young healthy male subjects and one skin cancer patient, and than exposed to various doses of low LET X-rays and high LET neutrons from ^{252} Cf source. To evaluate the DNA damage we have applied the single cell get electrophoresis technique (SCGE) also known as the comet assay. In order to estimate the repair efficiency, cells, which had been irradiated with a certain dose, were incubated at 37^0C for various periods of time (0 to 60 min). The kinetic of DNA damage recovery was investigated by an estimation of residual DNA damage persisted at cells after various times of post-irradiation incubation (5, 10, 15, 30 and 60min).

We observed an increase of the DNA damage (reported as a Tail DNA and Tail moment parameters) in linear and linear-quadratic manner, with increasing doses of X-rays and ^{252}Cf neutrons, respectively. Moreover, for skin cancer patient (Code 3) at whole studied dose ranges the higher level of the DNA damage was observed comparing to health subjects (Code 1 and 2), however statistically insignificant (for Tail DNA p=0.056; for Tail moment p=0.065). In case of the efficiency of the DNA damage repair it was observed that after 1 h of post-irradiation incubation the DNA damage induced with both, neutrons and X-rays had been significantly reduced (from 65% to 100 %). Furthermore, in case of skin cancer patient we observed lover repair efficiency of X-rays induced DNA damage. After irradiation with neutrons within first 30 min, the Tail DNA and Tail moment decreased of about 50%. One hour after irradiation, almost 70% of residual and new formed DNA damage was still observed. In this case, the level of unrepaired DNA damage may represent the fraction of the double strand breaks as well as more complex DNA damage (i.e.-DNA or DNA-protein cross links), which needs a longer time for sufficient rejoining. For the slight increase of the DNA damage observed after 30 min of incubation (both, for X-rays and ^{252}Cf neutrons) the NER mechanisms might be responsible.

1. Introduction

The radiation sensitivity plays a very important role in the tumor therapy and radiation risk. The importance of these problems in man is clearly shown by the genetic disorder Ataxia-Telangiectesia, in which radiation hypersensitivity is associated with defects in repair of double strand breaks. Furthermore, it is well known that sensitivity of cells exposed to the ionizing radiation depends from one hand on the amount of initial DNA damage and on the other hand on the repair capacity. In this paper, we present the efficiency of induction of the DNA damage by various LET radiation as well as the efficiency of subsequent rejoining of those damage, studied in human lymphocytes *in vitro*.

In our study to evaluate the kinetic of the DNA damage induction and their subsequent repair we have applied the single cell gel electrophoresis technique. This method is considered to be a rapid and very efficient one for the evaluation of different type of DNA damage i.e. single and double strand breaks, alkali labile sites as well as for estimation of the repair of the DNA damage, at the level of individual cells [1]. This method was developed from such procedures as: nucleoid sedimentation and the halo assay [1, 2]. The comets are formed under electrophoresis following the migration of DNA pieces upon the principles of releasing damaged DNA from the core of nucleus. The use of alkaline conditions in this technique makes the single and double strand breaks, and alkali-labile sites of DNA more visible. The migrating fraction of DNA is visualized by staining with fluorophor and quantified using epifluorescence microscope. The image of the cells resembles "comets" with the brightly fluorescent head and a tail. The length of the "comet" tail and the percentage of the DNA presented at comet tail give the information about the degree of DNA damage in the individual cell [1, 2, 3].

The aim of this study was to investigate the differences in level of the DNA damage induced *in vitro* by low LET X-rays and high LET ^{252}Cf neutrons in lymphocytes isolated from two health subjects and one skin cancer patient. Moreover, the efficiency of cells to repair this damage was also evaluated.

2. Material and methods

Chemicals: normal melting temperature agarose (NMA), low melting temperature agarose (LMA) were purchased from GIBCO BRL, Paisley, Scotland, Triton X-100 from Aldrich Chemie, Steinheim, Germany), sodium sarcosinate, EDTA, ethidium bromide, DMSO, RPMI 1640, Tris and Histopaque from Sigma Chemicals,) sodium chloride, sodium hydroxide from POCH Lublin, Poland.

2.1 Lymphocytes preparation and treatments

Lymphocytes were separated using Histopaque separation medium (Sigma Chemicals Co. Ltd.), from heparynized whole blood collected by venepuncture from two young male donors (Code 1 and 2), as well as from one skin cancer patient (Code 3) [4, 5, 6].

Cryopreservation

2 ml of heparinized whole blood collected from a male volunteers were diluted 1:1 with RPMI 1640 and kept at 4^0C for 10 min. Lymphocytes were separated by centrifugation over 4ml of Histopaque at 200 g for 30 min. Then the lymphocytes were removed and washed twice with RPMI 1640. The lymphocytes suspended in RPMI were counted in a hemocytometer. For cryopreservation, the cells were resuspended in a freezing medium consisting of 10% DMSO, 40% RPMI and 50% fetal calf serum. The cell suspension was transferred into plastic freezing vials in aliquots of 1.8-2 x10^6 cells. Vials were placed in -70^0C

Thawing

The vials were submerged in a 37^0C water bath until the last trace of ice melted. The thawed cells were quickly transferred to centrifuge tubes containing 15 ml of a thawing medium (50% fetal calf serum, 40% RPMI 1640, 10% Dextrose). Cells were centrifuged at 200 x g for 10 min and then resuspended in RPMI 1640. The mean viability of the cells examined with the application of trypan blue exclusion test was ≈97 % and 88% for health subjects and skin cancer patient, respectively.

2.1.1 **Irradiation**

X-rays irradiation

The X-rays irradiation was performed at the Institute of Nuclear Physics using Philips X-rays machine with an average dose of 1Gy/min. Lymphocytes resuspended in cold RPMI 1640 at the finale concentration of 100 000 cells per ml were exposed to different doses of X-rays (from 0 to 1.4 Gy). To avoid repair of the DNA damage, lymphocytes were irradiated at 4^0C.

^{252}Cf neutrons irradiation

Irradiated was done with ^{252}Cf source at the Faculty of Physics and Nuclear Techniques of the University of Mining and Metallurgy. For irradiation samples (100 000 cells/ml) were placed in the polyethylene chamber filled with distilled water to obtain a uniform distribution of fast and low energy neutrons. The exposition was performed for different times (0 to 5 h). Because of the technical reason, the irradiation was done at room temperature (about 21^0C).

2.2 **Study of repair of the DNA damage**

In order to estimate the repair efficiency, cells, which had been irradiated with a dose of 1.4 Gy of X-rays and 0.92 Gy of ^{252}Cf neutrons, were incubated at 37^0C for various periods of time (0 - 60 min). The kinetic of DNA damage recovery was investigated by an estimation of the residual DNA damage persisted at cells after various times of post-irradiation incubation (5, 10, 20, 30 and 60 min).

2.3 **Slide preparation**

Slides (two repetitions of each slide) were prepared according to the standard procedure described elsewhere [7, 8, 9, 10]. Slides were immersed for 1 h at 4^0C in a freshly prepared cold lysin solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate), with 1% Triton X-100 and 10% DMSO added just before use. Then the slides placed side by side in a horizontal gel electrophoresis tank filled with fresh electrophoresis buffer (1 mM EDTA, 300 mM NaOH) to a level of about 0.25 cm above the slides. After 20 min of unwinding of DNA, the electrophoresis was conducted at 4^0C for 30 min at V, 300 mA. All of the steps after lysis were carried out under yellow light to prevent any induction of additional DNA damage. After electrophoresis, the slides were washed three times for 5 min with a Tris buffer (0.4 M Tris, pH-7.5) and stained with 60 µl of 17 µg/ml ethidium bromide dissolved in distilled water. Before analysis, the slides were stored in a light proof box containing moist PBS at 4^0C.

2.4 **Slide analysis**

The slides were examined at 200x magnification using an epifluorescence microscope (Olympus BX-50), equipped with a 100 W mercury lamp and an excitation filter of 515-560 nm, and a barrier filter of 590 nm. The CCD camera was used to get the image of the comet and automatic evaluation of the comet size was performed using Komet 3.0 software from Kinetic Img. One hundred fifty cells were measured per analyzed treatment point (25 cells in three different parts of the slide, two slides per dose). To evaluate a mean value of the damage 70-100 cells were measured per the agent dose (35-50 cells from each of two slides. From the various parameters describing “comet" and available through the Komet 3.0 software to estimate the DNA damage we have chosen the following measures:

- tail DNA - percentage of DNA in the tail of the comet,
- tail moment - fraction of DNA in the tail multiplied by the tail length

2.5 **Statistics**

From one hundred to one hundred fifty comets (50 - 75 from each of two replicate slides) were measured per dose (study of induction of the DNA damage) or per time point (repair studies). The last square best fit for the curves and the standard deviation were calculated with a use of Excel 5.0. Student's t analysis from Excel 5.0 was applied to determine the statistically significance differences between DNA damage.

3. **Results**

Figures 1 and 2 present the dose-response relationships for Tail DNA and Tail moment parameters observed in lymphocytes irradiated with different doses of X-rays (persons with code 1 and 2). At the whole dose range under the study an increase of the DNA damage with a linear-quadratic dose response relationship was observed.

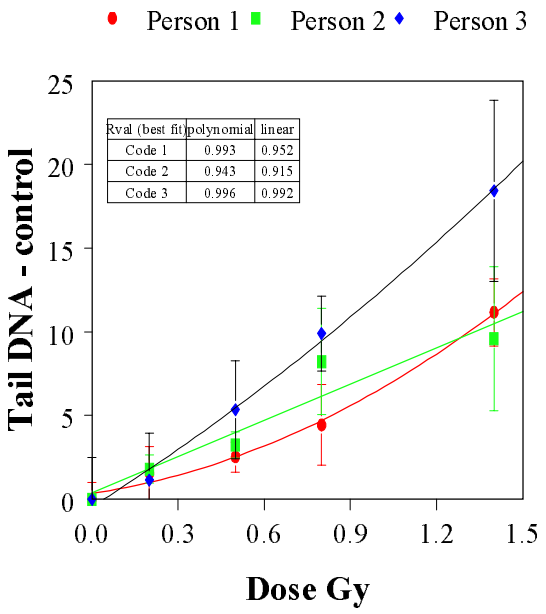

Fig. 1. The dose-response relationships observed in lymphocytes irradiated with different doses of X-rays.

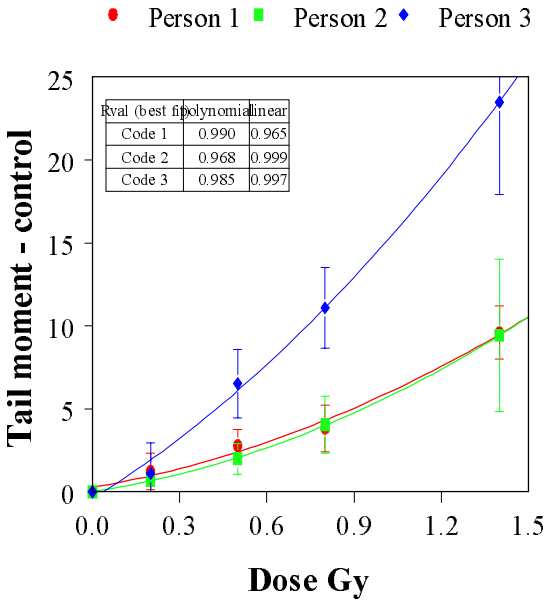

Fig. 2. The dose-response relationships observed in lymphocytes irradiated with different doses of X-rays.

The dose response curves observed for lymphocytes treated with neutrons from ^{252}Cf source are shown at Figure 3. There is a close to linear increase in the level of the DNA damage in the in the whole dose range.

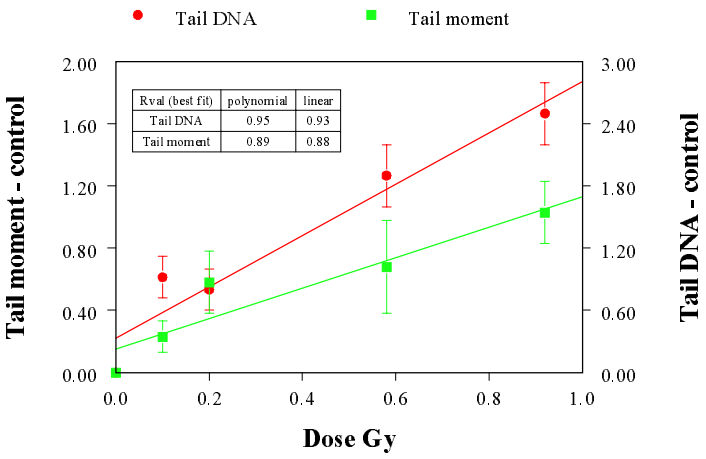

Fig. 3. The dose response curves observed for lymphocytes treated with neutrons from ^{252}Cf source.

At figures 4 and 5 the efficiency of the DNA damage rejoining observed in lymphocytes exposed to a dose of 1.4 Gy of X-rays and incubated at 37^{0}C for various period of time is presented. For the post-exposure incubated cells we observed a rapid initial decline of the DNA damage with a half time of about 10 min, and a little increase of these damage following 30 min of incubation. After 1h of the incubation, the cells were able to repair about 65% - 100% of the initially induced DNA damage.

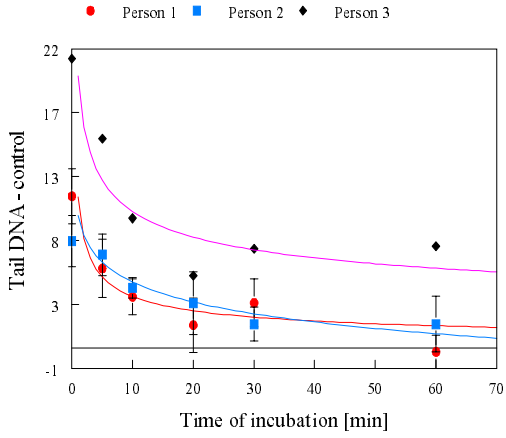

Fig. 4. DNA damage rejoining observed in lymphocytes exposed to a dose of 1.4 Gy of X-rays and incubated at 37^{0}C for various period of time.

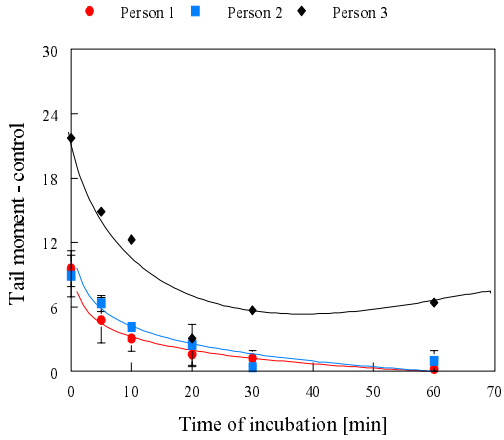

Fig. 5. DNA damage rejoining observed in lymphocytes exposed to a dose of 1.4 Gy of X-rays and incubated at 37^{0}C for various period of time.

The kinetic of the DNA damage repair observed for cells treated with neutrons is presented at Figure 6. For these cells it was observed much more slower decrease of the DNA damage, with the half time of about 30 min, and similarly as it was observed for X-rays irradiated cells, a little increase of the DNA damage in the following 30 min. After 1h of incubation, the cells were able to repair only about 24% of the initially induced DNA damage.

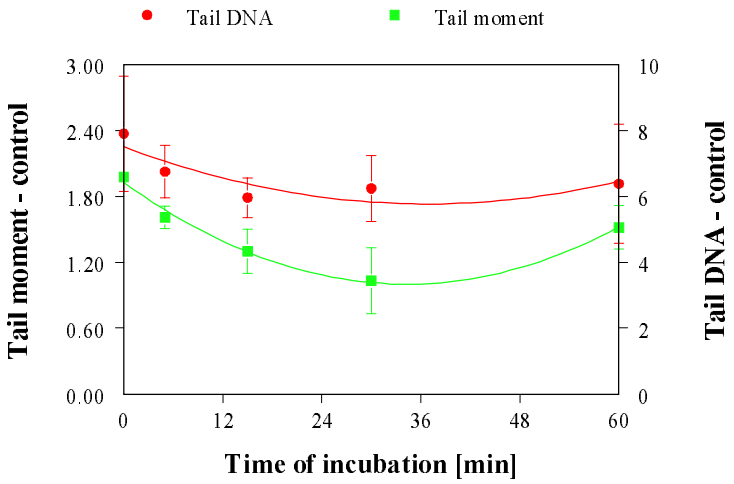

Fig. 6. The kinetic of the DNA damage repair observed for cells treated with neutrons.

In Tables 1 and 2 there are presented the percentage values of the repaired DNA, calculated as a decreasing with the time of incubation values of Tail DNA and Tail moment parameters, respectively (for X-rays irradiated lymphocytes).

Table 1.
Percent of repaired DNA, measured as a decreasing with time of post irradiation incubation value of Tail DNA parameter, in cells exposed to X-rays.

Time of incubation [min]	Percent of repaired DNA [%]		
	Code 1	Code 2	Code 3
0	0	0	0
5	47.49	12.61	27.62
10	66.13	43.51	55.17
20	84.95	58.07	74.93
30	70.25	77.48	65.67
60	100	77.61	64.85

Table 2.
Percent of repaired DNA, measured as a decreasing with time of post irradiation incubation value of Tail moment parameter, in cells exposed to X-rays.

Time of incubation [min]	Percent of repaired DNA [%]		
	Code 1	Code 2	Code 3
0	0	0	0
5	50.22	28.96	31.51
10	68.16	53.82	43.57
20	83.50	72.15	85.93
30	87.70	95.73	73.82
60	97.60	89.03	70.59

Percent of repaired DNA calculated for lymphocytes irradiated with ^{252}Cf neutrons (Tail DNA and Tail moment parameters) is presented in Table 3. Due to technical reason, the kinetic of ^{252}Cf induced damage repair is presented only for person with code 1.

Table 3. Percent of repaired DNA, measured as a decreasing value of Tail DNA parameter with time of post irradiation incubation, in cells exposed to ^{252}Cf neutrons (for person with code 1).

Time of incubation [min]	Percent of repaired DNA [%]	
	Tail DNA	Tail moment
0	0	0
5	15.21	18.51
13	25.03	34.78
30	21.01	47.8
60	19.24	23.14

4. **Discussion**

The radiation sensitivity plays a very important role in the tumor therapy and radiation risk. The importance of these problems in man is clearly shown by the genetic disorder Ataxia-Telangiectesia, in which radiation hypersensitivity is associated with defects in repair of double strand breaks. Furthermore, it is well known that sensitivity of cells exposed to the ionizing radiation depends on the amount of initial DNA damage and on the repair capacity. In this paper, we would like to present the preliminary data regarding evaluation of the DNA damage induction and their subsequent repair in lymphocytes from two normal subjects and one skin cancer patient.

The main goals of these studies were to evaluate the radiation sensitivity of the cells exposed to low LET X-rays and high LET neutrons from ^{252}Cf source, as well as an efficiency of these cells to repair introduced DNA damage. In our investigation we have applied the SCGE (single cell gel electrophoresis) method that is recognized as a very fast and useful tool for the analysis of the induction of DNA breaks and their subsequent repair [1, 2, 7, 8].

The level of the DNA damage (reported as a mean value of Tail DNA and Tail moment measures) increase with linear-quadratic manner for lymphocytes irradiated with X-rays and linear one, for cells exposed to ^{252}Cf neutrons. For lymphocytes isolated from the skin cancer patient (Fig. 1 and 2) the higher level of the DNA damage was observed, although statistically insignificant ($p>0.05$). Moreover, the viability of these lymphocytes was lower (88%) comparing with the viability of cells from health subjects (97%). It could results in higher DNA damage measured with the comet assay as well as with increasing number of the apoptotic cells.

In order to estimate the repair efficiency cells, which had been irradiated with a certain dose, were incubated at 37^0C. The kinetic of the DNA damage rejoining was investigated through an evaluation of the residual damage measured after various times of post-irradiation incubation (5, 10, 20, 30 and 60 min). It was observed that in lymphocytes irradiated with a dose of 1.4 Gy of X-rays, 50% of the initially induced DNA damage were repaired within first 10 min, and the bulk of the damage was rejoined within the first 20 min (about 70 %). Almost all the DNA damage reported as a value of Tail DNA and Tail moment parameters was repaired at the end of the 60-th min incubation period in case of subject with code 1 and 2 (Figures 3 and 4). For the skin cancer patient even after one hour of post-irradiation incubation only 64% (Tail DNA) to 70% (Tail moment) of the initial DNA damage were resealed.

Furthermore, the differences in efficiency of DNA damage repair observed between health subjects and skin cancer patient were statistically significant ($p<0.05$).

The efficiency of the resealing of the DNA damage observed in present study might suggest an existence of two phases in the kinetics of the DNA damage repair. The first one, which is a very fast, and almost 70% of the introduced DNA damage are rejoined (first 15 - 20 min), and the second one, when the repair is much slower and the cells need a longer time to reseal residual DNA damage. The first phase might represent the restoration of the DNA damage that could be rejoined by the simple action of ligases or the BER (base excision repair) mechanism. On the second one, the NER (nucleotide excision repair) mechanism might took place and much more complex DNA damage might be repaired (i.e. DNA double strand breaks or DNA cross links). Thus, the nucleotide excision repair mechanism could (might) be responsible for the slight increase of the DNA damage (due to excision of the damage nucleotides) observed in lymphocytes after 30 min of post-irradiation incubation (Fig. 1 and 2). However, in case of person with code 1 this effect is less pronounced especially for Tail moment parameter.

The efficiency of repair of the DNA damage observed in our study for human lymphocytes (both, normal and skin cancer patient) exposed to low LET radiation occurred to be slower than noticed for V79 or CHO cells lines by Olive at al. [11]. In V79 and CHO cells, almost 60% to 65 % of the initially induced DNA damage was rejoined within first 5 min, respectively. Also the efficiency of repair of the DNA damage observed by Wojewodzka at al. [12] in stimulated to proliferation human lymphocytes was slightly higher (70 % of the initial DNA damage was repaired within first 5 min) than observed in our study for non-stimulated to proliferation human lymphocytes. The letter difference, observed between stimulated [12] and non stimulated to proliferation human lymphocytes could be explained by the smaller poll of the dNTP presented in non proliferating lymphocytes [13].

Moreover, our data may suggest that, although, in a first phase of DNA repair (first 20 min) the efficiency of repair process observed in lymphocytes from skin cancer patient is comparable to the efficiency of the DNA repair in lymphocytes from normal subjects, a second phase (30 – 60 min) is much slower. An even after one hour of post irradiation incubation almost 30% to 35 % percent of the DNA damage is still present at these cells (Tables 1 and 2). The unrepaired DNA damage might represent double strand breaks, or other type of complex DNA damage. Thus, slower second phase of the DNA repair, in which the NER (nucleotide excision repair) mechanism took place as well as the DNA double strand breaks might be repaired, observed for lymphocytes isolated from skin cancer patient could indicate that these cells have some disturbance in NER or in double strand break repair mechanisms. However, the further studies with a larger group of skin cancer patient are needed to confirm these founding's.

The efficiency of rejoining of the DNA damage introduced in lymphocytes by exposure to ^{252}Cf neutrons was much slower then noticed for X-rays irradiated cells (Fig. 1, 2 3). At first 5 min decrease in initial DNA damage was minimal (Table 3). The fifty-percent of the DNA damages was restored after 30 min of incubation (Tail moment parameter). One hour after exposure 67% of the DNA damage was still unrepaired. The slower decline of the DNA damage noticed for cells which had been exposed to the neutrons, could have following reasons:

- the irradiation was performed at the room temperature (5 h of irradiation) and the first rapid phase of the DNA rejoining might be omitted;
- neutrons caused mainly the double-strand breaks and the dsb are rather slower repaired than the single-strand breaks [14].

Our results show that one-hour after the post-irradiation incubation most of the DNA damage induced with the ^{252}Cf neutrons was still observed, and these unrjoined damage may represent the double strand breaks (neutrons caused mostly the double strand breaks [10, 14, 15]. Moreover, Mayer *at al* (1990) has observed that in human lymphocytes double strand breaks decrease only for the short time after irradiation and then stopped [14]. It could suggest that lymphocytes need longer time for the sufficient rejoining of this type of DNA damage.

In conclusion, the kinetic of induction of the DNA damage did not differ significantly between normal subjects and skin cancer patient. Furthermore, the kinetic of the resealing of the DNA damage in the first 20 min. of post-irradiation incubation was found to be similar in lymphocytes from normal and skin cancer patient. However, the proportion of the residual damage at 60 min post-irradiation was significantly higher in patient than in control ($p<0.05$). Finally, the comet assay method occurred to be a very useful tool in such kind of study, and might be applied to detection of the susceptible individuals, as well as individuals with the defect in repair mechanism. This method could be very useful in radiation therapy especially for identification of people with hypersensitive phenotype to ionizing radiation or with disturbance in DNA damage repair.

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