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DNA Repair Kinetic of Hydrogen Peroxide and UVA/B Induced Lesions in Peripheral Blood Leucocytes from Xeroderma Pigmentosum Patients and Healthy Subjects

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ABSTRACT: The objective of the present work was to study the fine kinetics of DNA repair in xeroderma pigmentosum (XP) syndrome, a complex disorder linked to a deficiency in repair that increases cancer susceptibility. The repair process was evaluated by the comet assay (CA) in cells from 2 XP patients and 9 controls exposed to UVA/B (UVA 366/UVB 280 nm) and H_2O_2 (150 μM) at temperatures of 4, 15, and 37°C. Samples were taken at 2-min intervals during the first 10 min to analyze the "fine kinetics" repair during the initial phase of the curve, and then at 15, 20, 25, 30, 45, 60, and 120 min. CA evaluation of DNA repair activity points to BER/NER initiation in the first 30 min with both inductors at 37°C and 15°C, but final comet length showed differences according to treatment. Repair kinetics during 120 min showed a good correlation with clinical features in both XP patients. Differences in final comet length were less pronounced in XP cells treated with H_2O_2 than with UVA/B, probably because the peroxide produces mainly base oxidation but less bulky lesions; UVA/B generates a mixture of both. These findings reinforce the value of CA in testing in DNA repair ability or exposure monitoring.

KEY WORDS: comet assay; DNA repair mechanisms; base excision repair (BER); nucleotide excision repair (NER); xeroderma pigmentosum (XP); hydrogen peroxide; rUVA/B.

I. INTRODUCTION

DNA repair ability of white blood cells is an outstanding issue considering the wide acceptance of these cells as a target of primary DNA damage in different genetic monitoring studies.[1–5] Reparability of lesions must be considered in the monitoring study design so as to avoid mis-evaluations of damage level due to variations in the amount of lesions related to the action of excision repair mechanisms.

Xeroderma pigmentosum (XP) is an autosomic recessive disease characterized by nucleotide excision repair (NER) deficiencies; NER is a complex and versatile system that removes bulky lesions elicited by UVr or certain chemical agents. In XP cell fusion assays have allowed to identify eight complementation groups denoted with capital letters from A to G and a variant (XPV) that is NER proficient but deficient in a DNA polymerase involved in translation DNA synthesis. Affected people show a great sensibility to sunlight and a high incidence of skin and internal neoplasm. Neurological disorders are present in patients assigned to groups A, B, D, and F.[6–8]

There have been reported contradictory results in XP cells' DNA repair of UVr induced lesions; some XP cells are proficient while others are not. Moreover, XP processing of oxidative lesions have been recognized by others as similar to that of normal cells, a fact that can be attributed to redundancy in repair systems[9] and also to the increase in repair responses against oxidative damage.[10–12] The pheno-

typic evaluation of repair in cells from XP patients and normal subjects is useful in the repair test standardization to be used in genetic monitoring studies. Three aspects of the problem have been considered here: (a) the time interval when specific repair mechanisms are more active, (b) the ability to repair damage caused by either oxidation or ultraviolet radiation (UVr) in resting or unstimulated peripheral blood cells, and (c) the temperature dependence of repair kinetics according to the evaluated agent.

In order to address the above-mentioned issues, we followed the repair process after challenging peripheral blood lymphocytes with UVA/B radiation or H_2O_2. Alkaline single-cell gel electrophoresis, "the comet assay" (CA), was used to characterize the repair process. We have focused on the first minutes after recognition and cut off the lesion by evaluating the length of the comets' tails at short time intervals in order to characterize a possible differential repair pattern between XP and normal cells. The final comet length and the "cut and patch" kinetics during the first 30 min were considered since, to the best of our knowledge, they had not been reported by any other previous work, a procedure that we denominated "fine kinetics."

II. MATERIALS AND METHODS

Control subjects. The control group included 9 young healthy females and males, who gave their consent to be included in this group. These individuals did not have a history of chronic or acute viral or inflammatory diseases, they had not been exposed to anesthesia, nor to known genotoxics in their workplace, and all of them were nonsmokers. Their blood samples were treated in the same way as that of the XP patients.

Patients. Patients differed in age, gender, history of sun exposure, and severity of skin and ocular symptoms. Patient 1 (XP1) is an 8-year-old boy (body weight 36.6 kg, height 115 cm) who began to be protected from sunlight shortly after the initial skin modifications became apparent. In order to avoid sunlight, several measures were implemented and surveyed by health personnel: Sunscreen protective lotions of high SPF (sun protection factor), a home attendant teacher so as to avoid being outside during the daylight period, the use of two or more clothes simultaneously, and the coverage of every window in his home with heavy curtains. A skilled physician did a monthly examination that included a photographic "map" of lesions, nutritional counseling, and support. Patient 2 (XP2; body weight 52 kg, height 156 cm), a 27-year-old female, was almost completely unaware of the need of sunlight avoidance until she was 20. She was exposed on a daily basis to sunlight on her way to and from her job; she scarcely utilized sunscreen lotions and also used to dress in light cotton clothing that did not cover the chest, arms, or legs so as to avoid friction of fabrics over cutaneous lesions.

In both cases skin cancer was present in spite of the different management of preventive measures. In XP1, the clinical record showed that 11 skin neoplasms on the face, shoulders, and arms had been removed at the moment of our study, while XP2 was surgically treated 8 times, including lesions in the right eyebrow and the nose tip. Both patients suffered from xerophthalmia and remarkable photophobia, they were free of neurological symptoms, and at the moment of the study remained of unknown complementation group.

Venous blood was taken in the laboratory at about 9:00 a.m. just before the beginning of the test procedures; samples were collected in heparinized disposable syringes and diluted with Ca^{2+} Mg^{2+} free PBS (Sigma).

A. Treatment of Peripheral Blood Cells

Hydrogen peroxide treatment. Lymphocytes were separated from whole blood as follows: Approximately 6 ml of blood from patients was drawn into heparinzed tubes and the peripheral blood mononuclear cells, including lymphocytes, were separated by centrifugation. Then, the cells were resuspended in 1 ml of culture medium RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% of fetal calf serum (FCS, GIBCO). An aliquot of 142 μl from the cell suspension was treated with

H_2O_2 (Merck Darmstadt, Germany) (150 μM) for 5 min in 1.5-ml microcentrifuge tubes at normal atmosphere and 25°C in the darkness. Cells were pelleted at 200*g* for 5 min at 4°C and resuspended in 1 ml of culture medium supplemented with 10% FCS. Cell viability was determined at the end of treatment with the Trypan blue exclusion method; then the cell suspension was incubated at one of the three test temperatures (4, 15, and 37°C) for 120 min in two different incubators and a cold bath. Whenever it was needed, samples of 10 μL were taken and mixed with 75 μL of low melting point (LMP) agarose.

UVA/B irradiation. Irradiation was performed in 6 well culture plates (Corning Costar). Cell suspensions were layered on the plates placed horizontally over an electrophoresis table in an ice filled tray. Thickness of the liquid column was 1 mm. A dual-wavelength lamp (UVA 366–UVB 280 nm, 6 W; Bioblock Scientific, France), was placed above the liquid surface, oriented so that the zone of homogeneous density of irradiation covered all the wells area. Cultured cells were irradiated at a distance of 10 cm from the lamp, and without any other illumination. The radiant energy was measured with a radiometer (VLX-3W; Cole-Parmer, UVB 280 nm, 10 J/cm^2; UVA 366 nm, 142 mJ/cm^2). After irradiation an aliquot was taken to determine cell viability with the Trypan blue exclusion method.

Incubation and repair assays. Cells were incubated for 2 h at three different temperatures: 4°C, reported as restrictive for global excision repair; 15°C, reported permissive for base excision repair (BER) but less for nucleotide excision repair (NER), and 37°C, permissive for both.[13,14] Samples for the CA were taken before the beginning of treatment (time 0) and at 2, 4, 6, 8, 10, 15, 20, 25, 30, 45, 60, and 120 min. Untreated cell suspensions were incubated at the three temperatures to serve as negative controls. Each event was performed twice in a period of 15 days.

The comet assay. Alkaline single-cell gel electrophoresis was performed according to Singh et al.[15] In brief, incubation was ended by mixing and centrifugation of 20 μl of incubation volume with 75 μl of 0.85% LMP agarose (BDH) dissolved in Ca^{2+} and Mg^{2+} free PBS, pH 7.4 at 37°C, and layered over a microscope slide, precoated with 100 μl of 1% normal melting point (NMP) agarose. LMP agarose was allowed to harden at 4°C for 5 min and another LMP agarose layer was added. After 5 min at 4°C, the slides were immersed in alkaline lysis solution (2.5M NaCl, 100mM Na_2EDTA, 10mM Tris, 10% DMSO, and 1% Triton X-100; pH 10 was set with NaOH pellets) for 1 h at 4°C. Slides were immersed in 4°C fresh alkaline electrophoresis buffer (0.3M NaOH and 1mM Na_2EDTA) for 20 min to allow DNA to unwind. Electrophoresis was done at 25 V (1 V/cm) and 300 mA for another 20 min. The slides were washed three times for 5 min each time with 0.4M Tris-HCL pH 7.5 at 4°C, excess humidity was removed, and the slides were kept in a dust free box until stained with propidium iodide (Sigma) at 2.5 μg/ml. The whole procedure was performed under dim light.

Analysis of slides. DNA damage was evaluated with a fluorescent microscope (Olympus BH2 fluorescence microscope (Olympus, Tokyo, Japan) at magnification 40×, according to total length of "comets" measured with an eyepiece scale. The length of DNA migration was the distance between the estimated leading edge of the head and the end of the tail in nonoverlapped comets.

Damage index was calculated by multiplying the value of a visual scoring damage category (from 0 to 4) by the number of comets classified in each category:

$$ID=n0\ (0)+n1\ (1)+n2\ (2)+n3\ (3)+n4\ (4)$$

where n=number of cells in the damage level.[16,17]

For each donor 1300 cells with comet were analyzed (50 comets/slide; one slide for each of 13 sampling time points; two independent experiments per condition). Means were calculated for each control group and for the two patients separately. The percentage of repair was calculated using the equation $100[(L_0–L_t)/L_0]$, where L_0 is the comet length at zero incubation time and L_t is that of the final sampling interval or 2-h incubation.

The comparative analysis of repair percentage was used in order to evaluate the kinetics of the processes.

III. RESULTS

Survival of cells. The proportion of cells alive at the end of each treatment was above 97.5% for H_2O_2 and 96.7% for UVA/B.

Control cells treated with hydrogen peroxide. H_2O_2 treated control cells were post-incubated at 37°C [Fig. 1(a); Table 1]; a longer DNA migration was seen from minute 0 to minute 2, then from minute 5 the comet tail increased to reach a peak around minute 20 (32.0% over initial length), then a continuous shortening began between minutes 20 and 60. At minute 120 the repair activity (in terms of shortening) is almost complete (4.6% over initial length). At 15°C the curve was almost flat; after minute 30 there was an increase in length that 90 min later reached 3.4% above the initial length [Fig. 1(b); Table 1]. At 4°C there were not remarkable variations during the first hour but in the second hour an increase was evident and at the end of the incubation period the comet length was on average 21.2% greater than that measured at the beginning of the treatment [Fig. 1(c); Table 1].

A. XP Patient Cells Treated with Hydrogen Peroxide

Patient 1(XP1). In cells incubated at 37°C there

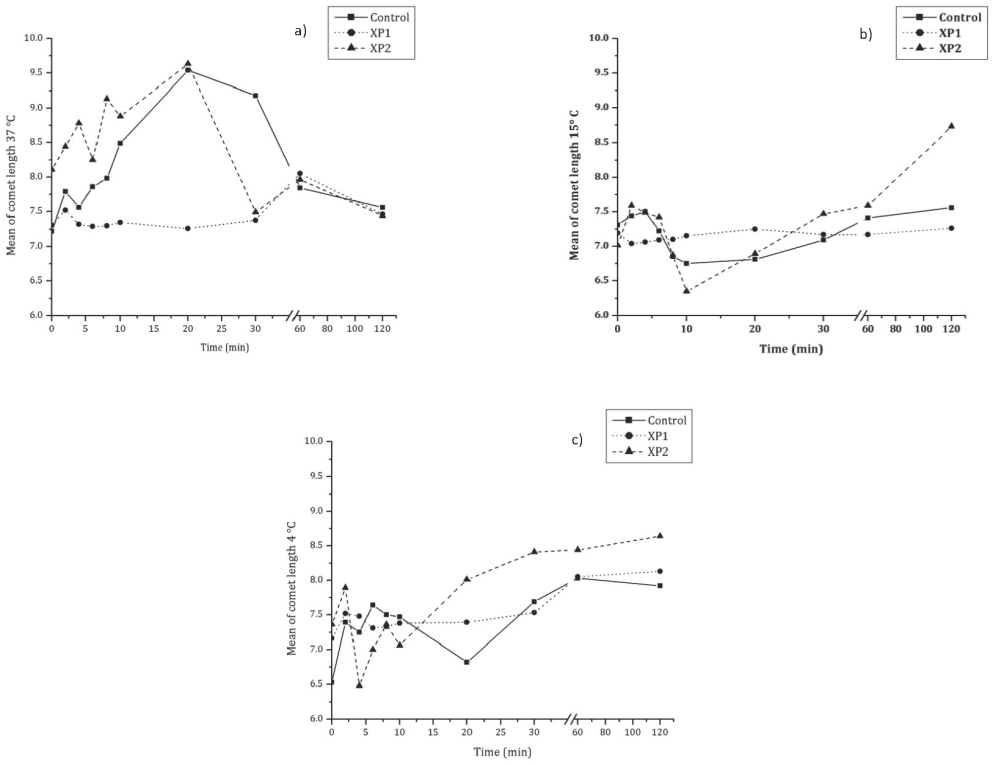

FIGURE 1: DNA repair kinetic of cells from control subjects and two XP patients after treatments with-H2O2 at three temperatures: (a) 37°C (permissive condition), (b) 15°C, and (c) 4°C.

TABLE 1: DNA repair kinetic* in cells from control subjects and XP patients after treatments with H_2O_2.

Condition		Time (min)									
		0	**2**	**4**	**6**	**8**	**10**	**20**	**30**	**60**	**120**
37°C	Control	7.22±0.62	7.79±0.02	7.56±0.53	7.86±0.89	7.98±0.43	8.49±1.61	9.54±0.99	9.18±1.64	7.84±0.51	7.56±0.46
	XP1	7.31±0.04	7.52±0.02	7.32±0.06	7.29±0.04	7.30±0.05	7.35±0.06	7.26±0.03	7.38±0.08	8.05±0.03	7.46±0.12
	XP2	8.11±0.21	8.44±0.30	8.78±0.16	8.25±0.59	9.13±0.33	8.88±0.17	9.63±0.62	7.49±0.37	7.96±0.25	7.44±0.55
15°C	Control	7.31±0.13	7.44±0.22	7.50±0.31	7.22±0.28	6.85±0.21	6.75±0.35	6.81±0.44	7.09±0.65	7.41±0.21	7.56±0.18
	XP1	7.19±0.02	7.04±0.09	7.06±0.37	7.09±0.27	7.10±0.14	7.15±0.07	7.25±0.03	7.17±0.04	7.17±0.06	7.26±0.06
	XP2	7.01±0.16	7.59±0.47	7.49±0.30	7.42±0.57	6.87±0.10	6.35±0.47	6.89±1.00	7.47±0.04	7.59±0.44	8.73±0.10
4°C	Control	6.53±0.59	7.39±0.91	7.25±0.68	7.64±0.62	7.50±0.63	7.47±0.93	6.82±0.03	7.69±0.60	8.03±0.03	7.92±0.35
	XP1	7.16±0.30	7.52±0.43	7.48±0.05	7.31±0.04	7.33±0.06	7.38±0.03	7.39±0.09	7.53±0.04	8.05±0.21	8.13±0.24
	XP2	7.36±0.10	7.89±0.33	6.48±0.34	7.00±0.21	7.36±0.02	7.06±0.05	8.01±0.21	8.41±0.01	8.44±0.06	8.64±0.42

* expressed as mean value of comet length ± standard deviation ($\bar{X} \pm SD$)

was a slight upward inflection in the curve between minutes 0 and 2 with a fast decline until minute 5; afterward the curve became flat until minute 30 when migration began to increase up to minute 60 (10.1% over initial length), then the comet length shortened and at 120 min it was 2.0% over the initial length. It is noteworthy that at the end of incubation the average comet length was less than 2% over that at minute 0. The absence of variation in DNA migration along the curve reveals that no incision/excision took place, an expression of no repair activity. In incubated cells at 15°C there was flat curve with a positive variation of 0.9% [Fig. 1(b); Table 1]. Comet length variations at 4°C were minimal in this patient, though a stepwise increase was observed to reach 13.5% over the initial length [Fig. 1(c); Table 1].

Patient 2(XP2). At 37°C the level of the cells' initial damage is higher than in controls but the repair kinetics resembled a normal behavior (incision and sealing activities were seen at intervals 2–20 and 20–40 min, reaching a maximum at minute 20 (18.6% over initial length), then migration decreased to reach 8.26% over initial length at 120 min [Fig. 1(a); Table 1]. At 15°C there is a peak between minutes 2 and 10 indicating DNA breakage (8.3% over initial length), but later between minute 30 and 120, comet length increases continuously indicating absence of sealing activity (final length 24% over initial length) [Fig. 1(b); Table 1]. Incubation at 4°C generated some small inflections in the first minutes followed by a continuous increase in DNA migration until 120 min to reach 17.4% over the initial length [Fig. 1(c); Table 1].

Control cells exposed to rUVA/B. Figure 2(a) shows kinetics of comet length variations at 37°C. The control curve is biphasic; the incision activity is observed as an increase in comet length from minute 0 to minute 10 (14.0% of positive variation). Afterwards, between minutes 10 and 20, a sealing activity is expressed to reach 13.2% over the initial length, and a second peak is reached at minute 60; then a slow decrease begins. The last sample taken at minute 120 reached a comet length that was 6.2% over the initial length. This is a measure of repair activity in terms of overall closure of breaks and consequent restriction to DNA migration during alkaline electrophoresis (see also Table 2). At 15°C the DNA migration curve shows a downward inflection between minutes 2 and 10 (16.8% below initial length), then increases slowly and at minute 30 is 0.9% over initial length). Then DNA migration decreases until minute 60 (11.2% below initial length) followed by a sharp increase that, at the end of incubation, represented 16.7% over the initial length [Fig. 2(b); Table 2]. There is no variation in comet tail length in cells incubated at 4°C indicating absence of repair activity [Fig. 2(c); Table 2].

B. XP Patient Cells Exposed to rUVA/B

Patient 1(XP1). There are no inflections in the curve as an expression of absence of any kind of repair activity at any of the assayed temperatures (Fig. 2, Table 2).

Patient 2 (XP2). At 37°C, the comet length increased from minute 0 to minute 6, when after a downward inflection at minute 8, it began to gain in migration length until minute 10 (18.6% over initial length) to return to baseline in minute 30 when the amount of breaks began to increase until minute 60; from this point the curve is almost parallel to the X axis and at minute 120 the comet length is a 17.8% greater than the initial length (Table 2). At 15°C there is an increase in length between 0 and 4 minutes (8.28% over the initial length), then a shortening until minute 8 followed by a continuous increase up to minute 20 (24.5% over the initial length). From this point to minute 30 the migration decreased to 3.5% below initial length. During the last incubation hour, the comet tail scarcely increased up to 1.3 over initial length [Fig. 2(b); Table 2]. At 4°C only small variations occurred until minute 60; from this point the migration increased to reach 16% over initial length [Fig. 2(c); Table 2].

DISCUSSION

Overall repair of H_2O_2 induced damage was efficient in the 9 control individuals and XP2 patient

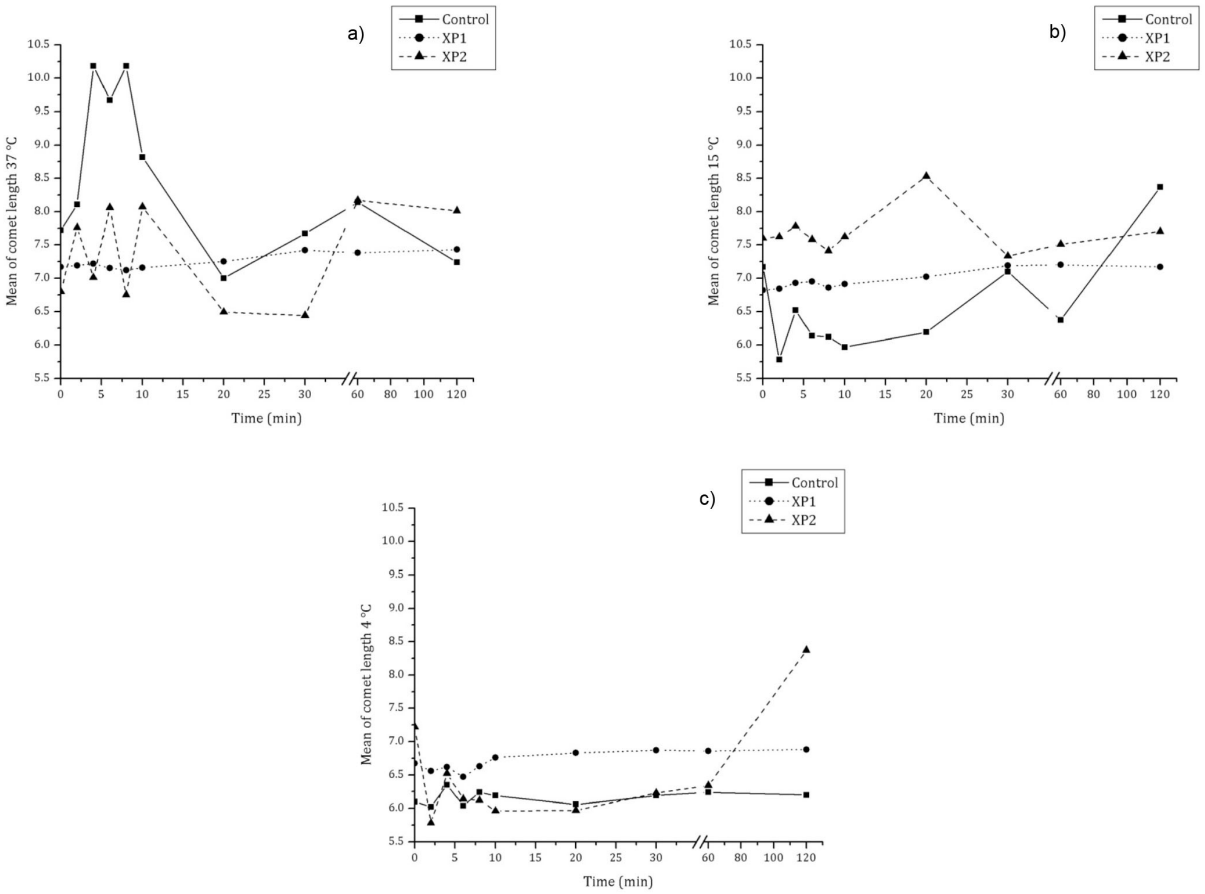

FIGURE 2: DNA repair kinetic of cells from control subjects and two XP patients after treatments with UVA/B at three temperatures: (a) 37°C (permissive condition), (b) 15°C, and (c) 4°C.

while XP1 did not repair DNA damage. Reduction in comet size showed a great variation in cells exposed to H_2O_2 (Fig. 1). H_2O_2 is a diffusible molecule that penetrates into the cell where, through Fenton reactions in the presence of transition metals, it increases the production of reactive oxygen species (ROS). One of these ROSs, the hydroxyl radical, is considered the ultimate effector of DNA damage. The oxidative burden can overwhelm the antioxidant capacity of the cell, which is related to many factors such as differentiation status, cell cycle position, growth rate, and cell age.[18,19] Variation is considered to be the result of the above-cited factors that influence the damage/repair in individual cells shaping the residual damage distribution in each subject.

UV is known to elicit a response including NER and BER. Though in this study we were not focused in a strict dissection to define which one of the repair systems was acting at each time, we found that in XP1 the complete absence of tail length changes at 37°C after UV exposure reflects no activity of any of the above-mentioned mechanisms. A similar absence was evident after H_2O_2 treatment, where a predominance of nonbulky lesions triggering BER was expected.

These results are correlated with the more severe clinical features. XP1 suffered more skin neo-

TABLE 2: DNA repair kinetic* in cells from control subjects and XP patients after treatments with UVA/B

Condition		Time (min)									
		0	**2**	**4**	**6**	**8**	**10**	**20**	**30**	**60**	**120**
37°C	Control	7.72±0.25	8.11±0.06	10.18±0.81	9.67±0.98	10.18±0.46	8.82±0.76	7.00±1.41	7.67±0.24	8.14±0.83	7.24±0.13
	XP1	7.17±0.12	7.19±0.10	7.22±0.02	7.15±0.24	7.12±0.24	7.16±0.38	7.25±0.36	7.42±0.45	7.38±0.19	7.43±0.16
	XP2	6.80±1.53	7.76±0.17	7.01±0.18	8.06±1.75	6.75±0.47	8.07±1.49	6.49±0.13	6.44±0.17	8.17±0.10	8.01±0.07
15°C	Control	7.17±0.37	5.78±0.63	6.52±0.01	6.14±1.20	6.12±0.42	5.97±0.11	6.19±0.34	7.10±0.57	6.37±0.06	8.37±0.09
	XP1	6.82±0.24	6.84±0.08	6.93±0.08	6.95±0.36	6.86±0.47	6.91±0.66	7.02±0.76	7.19±0.13	7.20±0.28	7.17±0.38
	XP2	7.60±0.92	7.62±0.81	7.78±0.11	7.58±0.03	7.41±0.33	7.62±0.39	8.53±0.40	7.33±0.17	7.51±0.55	7.70±0.17
4°C	Control	6.10±0.64	6.02±0.54	6.35±0.46	6.04±0.57	6.24±0.70	6.19±0.54	6.06±0.41	6.19±0.33	6.24±0.54	6.20±0.21
	XP1	6.67±0.01	6.56±0.22	6.62±0.51	6.47±0.19	6.63±0.59	6.76±0.22	6.83±0.52	6.87±0.01	6.86±0.01	6.88±0.30
	XP2	7.22±0.57	5.78±1.10	6.52±0.33	6.14±0.28	6.12±0.85	5.96±0.18	5.97±0.23	6.23±0.63	6.34±0.47	8.37±1.03

* expressed as mean value of comet length ± standard deviation ($\bar{X} \pm SD$)

plasms in spite of his young age. He was exposed to a lower irradiation period than XP2 (8 years vs. 27 years) and the amount of sunlight that reached his skin was obviously less due to sun protective measures. XP2 did not repair UV lesions at 37°C, but she was efficient in coping with oxidative damage. In this case, BER activity could explain milder symptoms in spite of the higher sun exposure. BER has been reported as a repair pathway that could also remove bulky adducts.[20] It is a complex mechanism that includes short and long patch modalities. XP2 had a more proficient BER; there is a remarkable comet length variation over short periods of 2 or 5 min when cells are treated with H_2O_2 that could be interpreted as caused by heterogeneity in DNA damage and the two pathways of BER (Fig. 2). XP2 BER activity could be expected to be induced by a pro-inflammatory state linked to chronic actinic dermatitis, poor antioxidant intake, and higher exposition to UVA.[12,21,22]

Measurement of repair capacity with CA shows a good correlation with clinical features. This sensitive test could contribute to classifying patients according to the severity of the repair capacity impairment. Anyway, the approach that we called "fine kinetics" could be helpful in determining variations in specific zones of the repair curves related to variations in donor cells' repair capacity. Curves at all three temperatures in control cells reveal a different behavior in the time trend of comet length variation with both DNA damage inductors, H_2O_2 as well as UVA/B.

Hydrogen peroxide treatment in controls. These controls showed that a complete repair was achieved between 60 and 90 min at 37°C, which is in agreement with different CA reports using H_2O_2 in the concentrations reported here in different cellular systems.[23–27] Hydrogen peroxide induced lesions could elicit NER even without helix distortion, which is the signal for NER holopolypeptide recruitment.[20,28] Besides, there are reports of a particular kind of oxidative lesion, the 8,5-cyclopurine-2-deoxynucleosides (CyPudNs), that can be repaired exclusively through NER action.[29]

There are evidences that redundancy is an outstanding feature of repair response in mammal cells, while a set of lesions remained unrepaired at 120 min. This slow repair of H_2O_2-induced breaks could be attributable to a continuous input of oxidative damage in the cells while repair is proceeding.[30–32] In line with this assumption there is a report on the persistence of H_2O_2 in thyroid cultured cells revealing a 23% remnant at 15 min and a 1.6% remnant after 1 h of incubation.[33] Another explanation for the slowness of repair can be ascribed to partial inactivation of repair enzymes by H_2O_2.[34]

The H_2O_2 curve pattern at 37°C could be related to the combined action of BER and NER systems. Differences in the 15°C curve relative to the curve at 37°C (a stepwise increase in comet length is observed at 15°C) could be explained by the effect of depression in NER activity.[35] Due to overlap between both systems, assigning to one of them the H_2O_2 repair curves is difficult.

There are many repair pathways involved in single-strand damage elimination but not all of them are restrained to "cut and patch" mechanisms, which can be monitored by the CA, and the fact that cells evaluated here are unstimulated G_0 lymphocytes excludes a post-replicative repair.

As mentioned in the literature, 15°C would act as a cutoff temperature for NER, allowing more BER activity. With this approach it is possible to distinguish between nucleotide and base excision repair activity by their temperature dependence.[14] Therefore if BER is still functioning at 15°C, comet length variations due to incision and sealing could be expected. Actually, size variations in the comet tail took place in the first 20 min, suggesting that BER was more active at this time interval.[34] Absence of further shortening in comet length after minute 20 could be attributable to inhibition in NER activity since it is responsible for the slower elimination of more voluminous lesions.[36,37]

There is a report of slower DNA repair rate in cells irradiated and allowed to recover at 20°C when compared with those incubated at 37°C.[38] Lower incubation temperatures could hamper helix unwinding that is needed for the positioning and assembling of exci-nuclease polypeptide complex.[28] On the other hand, impairment in repair

efficiency was expected as temperature is shifted from the optimal for enzyme reactions.[39,40]

The curve at 4°C did not show any inflection in DNA migration at the same sampling times where a significant length variation at 37°C was seen. Temperature of 4°C has been considered inhibitory for repair enzymes.[13,14] As variation in length at 4°C was almost totally absent, there was a cold repair until minute 60.[41] From this point there was increase in single-strand breaks that remained unsealed at the end of incubation.

Hydrogen peroxide treatment in XP patients. XP syndrome cases have been reported with a variable repair capacity between complementation groups and even between individuals.[42] The fact that XP2 presents a response to H_2O_2 treatment at 37°C that is not different from controls suggests that the individual possesses an ability to cope with oxidative lesions. XP2 shows a higher repair capacity for H_2O_2 attaining a reduction in comet length in the range of controls. On the contrary cells from XP1 revealed almost no response to oxidative treatment.

Several authors reported dissimilar responses in various cell model systems and explained the differences found as the result of disease heterogeneity at the molecular level, expressed as variations in the repair capacity for different categories of injuries.[12,43,44] Normal or increased repair of oxidative damage in XP cells could be related to an adaptive response to chronic exposition to oxidants.

XP is a disease where oxidative stress has been recognized as an outstanding biochemical feature.[45] Apurinic endonuclease (APE), the major enzyme that initiates the removal of apurinic sites in the BER pathway, has been found to be induced through HSP 70 in oxidative stressed cells with a consecutive increase in their repair capacity.[46] XP-V defines patients with the clinical symptoms of XP, which shows normal NER but defective *trans*-lesion DNA synthesis.[47,48]

Our results also coincide with those of Peak et al.[49] who found that XP cells exposed to H_2O_2 and evaluated with the alkaline elusion assay repaired more efficiently single-strand breaks than human epitheloid P3 cells. In the latter, repair was completed in 50 min for H_2O_2 treated cells but 25% of single-stranded breaks generated by 365-nm UVA treatment remained unsealed at minute 60.

Gopalakrishnan et al.[28] in a series of experiments using Epstein-Barr transformed diploid B lymphocytes from normal subjects and XP patients, found that an XPB deficient lymphoblastoid cell line, evaluated with the CA, showed a higher reduction in tail moment than its normal counterpart after treatment with 20μM H_2O_2 and 22 h of recovery.

In H_2O_2 treatments the curve at 15°C is depressed between minutes 10 and 20 as can be seen in Fig. 1(b). Reduction of excision activity is larger than that of normal cells at the same temperature, as should be expected if NER already affected by the disease is further impaired by suboptimal temperature. The remarkable difference between curves at 37°C and 15°C reported here further supports the assumption that at the latter, there is a change in the NER repair of H_2O_2 induced lesions. If BER and NER are needed together to repair this damage, then reduction of NER in XP cells could be expressed as an even more pronounced reduction in activity than in normal cells under the same circumstances.

At 4°C there were no evidences of "cold repair"[41] predominantly until minute 20 where comet length variations are not apparent. This curve is very similar to that of the control but at a higher average in comet length.

UVA/B treatments in controls. UVA represents 95% of the UV radiation that reaches earth surface; the remaining 5% is UVB which is considered to be related with 80% of photoinduced neoplasms. Exposure to UVB causes promutagenic DNA modifications as *cis-syn* cyclobutane pyrimidine dimers or 6-4 pyrimidine-pyrimidone. On the other hand, UVA mutagenicity is related to photochemical reactions that generate reactive oxygen species (ROS) leading to DNA oxidative adduction that produces lesions like the 8-deoxiguanosine or glycol thymine.[50]

In normal cells, exposition to UVA/B and incubation at 37°C generated curves with an initial

increment in comet length as a result of excision nuclease NER plus BER activity (2 to 30 min) followed by a decrease in comet length that is the expression of polymerization and ligation (time interval from 30 to 45 to 120 min). The overall repair was efficiently performed reaching a final comet length that was 1.5% lower than the initial. At 15°C there was an upward inflection in the first 10 min, a behavior that could be related to the expression of BER as a pathway involved in oxidative repair. From that time point, the filling and sealing phase proceeds until minute 60 where a further increase is observed, probably due to a slow component of BER repair. Slow repair has been explained as the result of the combined UVA/UVB radiation[49] cross-linking and clusters of oxidative lesions elicited by UVA.[51] Another remarkable feature at 15°C is a negative inflection in the curve between 15 and 30 min, which is in concordance with the previously mentioned NER impairment at this nonpermissive temperature.[14]

Because ultraviolet light B is the cause of lesions as cyclobutane pyrimidine dimers mainly repaired by NER, the curve pattern in UVA/B exposed cells suggests that NER activity begins at about minute 2 in post-treatment incubation and it takes hours to eliminate the most complex lesions. At the end of our observation interval of 2 h, the comet remained 4.49% over the initial length, revealing the persistence of unrepaired lesions.

UVA/B treatments in XP patients. XP patients show a high sensitivity to UV light expressed in low cell survival and recovery after irradiation that is related to constitutive impairment in the NER mechanism. NER impairment is present in 7 out of 8 XP complementation groups.[6]

At 37°C the curve between minutes 2 and 30 is lower in XP patients. Considering that the cyto-physiological alteration in this disease is NER impairment, the difference between control and XP curves is in agreement with our preliminarily identification of this zone in the kinetic curve as an expression of NER. At 15°C there is evidence of repair in the first 8 min, an in-time response that can be explained on the basis of enhanced BER activity in oxidative stressed XP cells. However, after minute 30 (where NER is more active) comet size increases continuously, leading to an uncompleted repair. At 4°C there were slight variations in comet size in the first 10 min of post-incubation but with no consequence in the overall repair given the increased comet length at minute 120 (Fig. 2; Table 2).

XP cells exposed to UVA/B generate comet length curves with more inflections than control ones, a feature that could be the consequence of the impairment of NER, the main mechanism involved in the processing of UV lesions, and the partial participation of other pathways in repair due to a wide lesion spectra of the inductor.[50,52,53]

Diversity in the lesions induced by UVA/B could result in the overlap of NER and BER giving different curve shapes according to the relative production of each type of DNA lesion. It is evident that cells from XP2 conserve a reduced ability to repair UVA/B induced damage, which is in agreement with the fact that ROS generation by UVA is an important contributor to this wide lesion spectra.[9,52] The fact that XP1 did not respond in any way could be attributed to heterogeneity between XP complementation groups and even among individual patients. Neither of them was assigned to a complementation group at the moment of being studied so it is not possible to discuss differential repair capacity in relation to XP classification.

CONCLUSIONS

- Unstimulated lymphocytes repaired a wide spectrum of DNA lesions generated by both damage inductors studied here.
- In certain XP patients there is an ability to cope with oxidative lesions. Our results are in agreement with reports in the literature that indicate a variable repair capacity between complementation groups and even between individuals.
- Time distribution of excision and ligation phases in both treatments, as revealed by comet length variations, could be helpful to assign a specific repair mechanism to a region in the repair curve. Upon this assumption, shapes of H_2O_2 and UVA/B curves at 37°C and 15°C, point to BER/

NER initiation in the first 30 min.

• Differences in final comet length were less pronounced in XP cells treated with H_2O_2 than with UVA/B, probably due to the nature of the lesions. While H_2O_2 produces mainly base oxidation but less bulky lesions, UVA/B generated a mixture of both. Reduction in NER activity is expected to allow more unrepaired lesions in XP UV treated cells.

• Measurements of initial and final comet length as an indicator of repair efficiency could lead to mis-appreciation of the repair process. Variations in DNA migration along time are indicative of incision, resynthesis, or ligation. Lack of synchrony in repair between cells could be the explanation of high variability in the fine kinetics.

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