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Age, sex, and race influence single-strand break repair capacity in a human population

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Abstract

Recently, we developed an improved comet assay protocol for evaluating single-strand break repair capacity (SSB-RC) in unstimulated cryopreserved human peripheral blood mononuclear cells (PBMCs). This methodology facilitates control of interexperimental variability [A.R. Trzeciak, J. Barnes, M.K. Evans, A modified alkaline comet assay for measuring DNA repair capacity in human populations. *Radiat. Res.* 169 (2008) 110–121]. The fast component of SSB repair (F-SSB-RC) was assessed using a novel parameter, the initial rate of DNA repair, and the widely used half-time of DNA repair. The slow component of SSB repair (SSSB-RC) was estimated using the residual DNA damage after 60 min. We have examined repair of γ -radiation-induced DNA damage in PBMCs from four age-matched groups of male and female whites and African-Americans between ages 30 and 64. There is an increase in F-SSB-RC with age in white females ($P < 0.01$) and nonsignificant decrease in F-SSB-RC in African-American females ($P = 0.061$). F-SSB-RC is lower in white females than in white males ($P < 0.01$). There is a decrease in F-SSB-RC with age in African-American females as compared to white females ($P < 0.002$) and African-American males (nonsignificant, $P = 0.059$). Age, sex, and race had a similar effect on intercellular variability of DNA damage in γ -irradiated and repairing PBMCs. Our findings suggest that age, sex, and race influence SSB-RC as measured by the alkaline comet assay. SSB-RC may be a useful clinical biomarker.

Keywords

SSB repair capacity; SSB repair kinetics; DNA repair; Comet assay; γ -irradiation; Cryopreserved lymphocytes; Oxidative DNA damage; Reference standards; Biomonitoring study; Dispersion coefficient H; Coefficient of variation

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2008.08.031.

Introduction

DNA repair capacity in humans can be described as the repair or removal of many different types of lesions via several biochemical pathways. Analysis of DNA repair capacity may be an attractive biomarker for clinical investigators, as alterations in several DNA repair pathways are linked to both heritable and sporadically occurring age-associated diseases such as cancer [1,2]. Collins et al. have shown that the levels of 8-oxodeoxyguanosine, which may be dependent on multiple factors including capacity to repair of oxidative DNA damage, are associated with heart disease mortality rate [3]. A variety of factors may influence capacity of different DNA repair pathways in humans. The influence of age, sex, tobacco use, and environmental exposure on the capacity to remove different DNA damage in peripheral blood mononuclear cells (PBMCs) has frequently been studied using the comet assay and other methods. In most studies employing the alkaline comet assay, age did not affect the repair of γ -radiation- and hydrogen peroxide-induced DNA damage [2,4–7]. However, other investigators using the alkaline comet assay and the host-cell reactivation assay have found that repair capacity of γ -radiation- and UV-induced DNA lesions declines with age [8,9]. Studies evaluating the role of sex on DNA repair capacity have been inconclusive. Most reports employing comet assay methodology have shown no effect of sex on capacity to repair ionizing radiation- and bleomycin-induced DNA damage [4,5,7,10]. However, Wei and colleagues using the host cell reactivation assay found that capacity to remove the known activated tobacco carcinogen, benzo[a]pyrene diol epoxide, is decreased in females as compared to males [11,12]. There is no available information about DNA repair capacity in humans of different racial or ethnic origin. Most studies use samples derived from white population cohorts [5,6,13]. There are also contradictory reports about the influence of tobacco use on DNA repair capacity [5,10,14].

The alkaline comet assay is a sensitive, and relatively inexpensive and noninvasive technique used for the detection of DNA damage and DNA repair [15]. DNA damage detected by the alkaline comet assay includes single-strand breaks (SSBs), alkali labile sites (e.g., apurinic/apyrimidinic (AP) sites), double-strand breaks (DSBs), and modified bases as well as DNA–DNA and DNA–protein crosslinks. One of the major advantages of this assay is the capacity to analyze DNA damage and repair in individual cells. Furthermore, small numbers of cells are required for this assay which is particularly advantageous for analyses performed in clinical samples.

Several comet assay studies have shown that individuals with cancer have lower levels of DNA repair [2,10,16]. DNA repair capacity was also reported to be dramatically decreased in patients with hereditary DNA repair syndromes, including xeroderma pigmentosum, trichothiodystrophy, and Nijmegen breakage syndrome [1,17]. It is possible that the comet assay studies produce consistent findings for cancer and DNA repair syndrome-related investigations because of the magnitude in the decline of the ability to remove DNA damage in these patients. In normal humans, smaller levels of incremental change in capacity of different DNA repair pathways may be associated with aging, sex, tobacco use, or other conditions and exposures. However, investigations done using this technique did not provide clear evidence that these demographic and epidemiologic factors affect DNA repair. The failure to find this evidence could reflect the inability of the standard comet assay to detect more subtle changes. It is clear that improvements in this DNA repair assay should be considered to reevaluate the importance of the demographic and epidemiologic factors as predictive factors for DNA repair capacity and more extensive usefulness of the comet assay in epidemiologic studies.

We have recently implemented modifications in the alkaline comet assay which may increase the ability of the assay to find subtle differences in repair capacity of SSB and other

types of DNA damage detected by the alkaline comet assay in unstimulated cryopreserved PBMCs [18]. These include: (1) standardization of multiexperimental SSB repair data using negative and positive reference standards and (2) estimation of DNA repair parameters describing fast and slow components of SSB repair.

The ability of this assay to distinguish subtle variations in SSB repair capacity (SSB-RC) in healthy cohorts may also depend on the extent of interexperimental variability. The use of reference standards to reduce interexperimental variability was proposed at the International Workshop on Genotoxicity Testing Procedures (Washington, DC, March 1999) [19]. We have introduced a modified standardization formula [18]. This formula is based on the simpler formula derived by De Boeck et al. [20]. Our standardization procedure provides a way to separate interindividual variation in SSB-RC from the interexperimental variation and interindividual differences in DNA damage levels. Use of the Olive tail moment is a more accurate measurement in assessing the capacity to remove γ -radiation-induced DNA damage than the other comet assay parameters, tail DNA and tail length [18].

Repair of SSB induced by ionizing radiation can be characterized by the presence of at least of two DNA repair components. Separate repair components remove different types of DNA damage with various rates [21]. To our knowledge, there are no published epidemiologic reports in humans assessing the fast and slow components of SSB repair. We have established a procedure for estimating the fast and slow components of repair of SSB in clinical epidemiologic studies. The fast component of SSB repair can be assessed using a newly introduced DNA repair parameter, the initial rate of DNA repair, and a widely used parameter, the half-time of DNA repair. The slow component of SSB repair was estimated using the residual DNA damage after 60 min [18].

The aim of this work was to determine whether demographic and epidemiologic factors, such as age, sex, race, BMI, hypertension, and tobacco or alcohol use, affect the repair capacity of SSB in humans. We examined repair of γ -radiation-induced DNA damage in unstimulated cryopreserved PBMCs from four age- and sex-matched groups of whites and African-Americans between ages 30 and 64. We found that age, race, and sex affect the fast component of SSB repair, while the slow component was unaffected. We have also analyzed intercellular variability in DNA damage levels. We found similarities between intercellular variability in DNA damage and the rate of the fast component of SSB repair in irradiated PBMCs.

Materials and methods

Peripheral blood mononuclear cells isolation and cryopreservation

Blood donors were participants in the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study of the National Institute on Aging Intramural Research Program (NIA IRP). The purpose of this study is to disentangle the effects of race and socioeconomic status on the development of age-associated health disparities. The study has undergone IRB review and informed consent was obtained from all participants. The HANDLS subcohort used in this study consists of 24 African-American females, 24 African-American males, 24 white females, and 24 white males ranging in age from 30 to 64 years of age. These individuals were not included in other studies. The characteristics of the study population presented in Table 1 were collected from the self-reported medical history for each participant. Fasting blood samples were collected in 8-ml Vacutainer heparinized vials (BD, Franklin Lakes, NJ). Blood was transported at room temperature to the lab and the mononuclear cell isolation procedure was performed within 3 h of phlebotomy.

PBMCs were separated by centrifugation of blood diluted with RPMI 1640 medium over Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) at 200g for 15 min. The interphase layer was collected by pipette in a separate tube and was washed in RPMI 1640 medium. The pellet was resuspended to a final volume of 1.5 ml with freezing medium that consisted of 40% RPMI 1640 and 50% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO). Aliquoted vials were frozen in isopropanol-containing containers placed at -80°C .

Culture and cryopreservation of AG10097 cells

The nonmalignant lymphoblastoid cell line AG10097 was obtained from the Aging Cell Repository (National Institute of General Medical Sciences, Coriell Repository) and was grown at 37°C in 5% CO_2 environment in RPMI 1640 supplemented with 10% FBS (Invitrogen). For cryopreservation, cells in the exponential phase of growth were collected, suspended in freezing medium (40% RPMI 1640, 50% FBS, 10% dimethyl sulfoxide), and aliquoted to obtain samples containing 10^6 cells in 200 μl of freezing medium. Vials were frozen overnight in isopropanol-containing freezing containers (Nalgene, Rochester, NY) placed at -80°C . Samples were subsequently stored at -140°C .

Examination of SSB repair capacity in cryopreserved PBMCs

The alkaline comet assay was used to study repair capacity of SSB and other types of DNA damage detected by this assay in cryopreserved PBMCs. Two replicate experiments for each individual were conducted on different days. In each replicate experiment, two sample sets were prepared. A single sample set consisted of unirradiated and irradiated PBMCs that have undergone repair incubations ranging from 0 to 60 min. Each sample set was paired with one from another randomly chosen individual. Each set of samples was also matched with four negative (unirradiated AG10097) and four positive (irradiated AG10097) reference standards. Biological experiments include samples and reference standards for two individuals whose samples are run side by side. Thus, four replicates of kinetic data were obtained for each individual.

Frozen PBMC samples were thawed by submersion in a 37°C water bath. Cryopreserved PBMCs were suspended in tubes containing 10 ml of cold complete RPMI 1640 medium (medium supplemented with 20% FBS). Then cells were centrifuged at 200g at 4°C for 15 min. After washing, pellets were gently resuspended in complete RPMI 1640 medium. Cells were suspended in 0.5% low melting point agarose (Invitrogen, Carlsbad, CA) in PBS and spread on fully frosted microscope slides (A. Daigger & Company, Wheeling, IL) precoated with 0.5% normal agarose (Invitrogen). PBMCs from two individuals were embedded in two separate gels on each slide. On slides for the first set, gels with PBMCs from individual 1 were prepared near the slide marking area, whereas gels with PBMCs from individual 2 were placed at the opposite end of the slide. For the second set, the location of cell samples from two individuals was reversed. This was done to minimize the effect of gel position on the stretching out DNA from nucleus during electrophoresis. After a 20-min gel solidification period at 4°C , slides were placed in prewarmed complete RPMI medium in a CO_2 incubator for 30 min (37°C). Slides were then subjected to ^{137}Cs γ -radiation on ice at a dose 6.3 Gy and at a radiation rate of ~ 1 Gy/min using a Gammacell 40 Exactor ^{137}Cs γ -radiation source (Nordion, Ontario, Canada). In the next step, unirradiated and irradiated slides were immediately placed in cold lysis buffer (time point 0 min) or in complete RPMI medium and kept for 5, 15, 30, and 60 min in a 5% CO_2 incubator at 37°C . After the repair time had elapsed for each of the slides, DNA repair was stopped by placing slides in cold lysis buffer.

Reference standard samples were prepared from a single vial of cryopreserved AG10097 cells. After thawing, cryopreserved cells were washed in complete RPMI 1640 medium and

embedded in 0.5% low melting point agarose onto fully frosted microscope slides. After a 20-min gel solidification period at 4 °C, slides with embedded cells were subjected to ^{137}Cs γ -radiation on ice at the dose 6.3 Gy. For a single experiment containing two sample sets, 16 gels with AG10097 cells were arranged on eight slides. Four slides were γ -irradiated with dose of 6.3 Gy (positive reference standards) and the remaining four were not exposed to γ -radiation (negative reference standards). Finally, slides were placed in cold lysis buffer.

Alkaline comet assay

The comet assay was performed under alkaline conditions following the procedure of Singh et al. [15] with some modifications. AG10097 cells and PBMCs embedded in low melting point agarose and subjected to γ -radiation and repair incubation were lysed overnight at 4 °C in lysis buffer (10 mM Tris-HCl, pH 10, 2.5 M NaCl, 100 mM EDTA, 5% DMSO, 1% Triton X-100). The unwinding step was performed for 40 min at 4 °C in unwinding/electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). Electrophoresis was performed at 8 °C for 30 min in unwinding/electrophoresis buffer at electric field strength 0.73 V/cm and current 343 mA. The slides were then neutralized (0.4 M Tris-HCl, pH 7.5), rinsed with distilled water, air dried, stained with 2 $\mu\text{g/ml}$ ethidium bromide, and covered with standard coverslips.

Comets were scored using an Eclipse E-400 fluorescence microscope (Nikon, Japan) attached to a Pulnix video camera (Kinetic Imaging LTD, Liverpool, UK) connected to the image analysis system Komet version 4.0 (Kinetic Imaging LTD). Olive tail moment was used here as a measure of DNA damage level [22,23].

Mathematical and statistical analysis

Assessment of SSB repair capacity in PBMCs using parameters describing SSB removal kinetics—Four sets of samples constituting two independent experiments with duplicate samples were prepared for cryopreserved PBMCs from each individual studied. Each of four sets of PBMC samples was subjected to γ -radiation and repair incubation and was processed with four negative and four positive reference standards through unwinding and electrophoresis steps. Fifty PBMCs and 25 AG10097 cells per sample were analyzed. Since conditions can differ slightly from electrophoresis to electrophoresis, the use of reference standards may minimize interelectrophoresis and interexperimental variation. Standardization was performed using the arithmetic mean values for the PBMC samples and reference standards. Negative and positive reference standards were used to standardize experimental values for irradiated and unirradiated PBMCs using the formula

$$y_{s,c}(D, t) = \frac{y(D, t) - C_n}{s_p - s_n}$$

where $y_{s,c}(D, t)$ is the standardized value for PBMCs being a function of γ -radiation dose (D) and time of repair (t), $y(D, t)$ is the experimental value for PBMCs being a function of γ -radiation dose (D) and time of repair (t), s_n is the value for negative reference standard, s_p is the value for positive reference standard, and C_n is the value for negative control being an average value of sample means obtained for unirradiated PBMCs subjected to 0- and 15-min repair incubations.

Standardized values obtained from the three or four data sets were taken together to calculate the means for each experimental SSB repair kinetics point. More information about the standardization procedure can be found in our methodological paper [18].

SSB repair kinetics was analyzed in SigmaPlot 8.0 (SPSS Inc., Chicago, IL) and Microsoft Excel 2003. SSB repair kinetics data for each individual were graphed in SigmaPlot on separate multiple line-scatter plots. Exponential curves with two exponential terms described by the formula $y = ae^{-bx} + ce^{-dx}$ were fitted to data showing removal of DNA damage with time in irradiated cells (Fig. 1). The estimated equation coefficients a , b , c , and d were then used in Excel to assess parameters describing SSB-RC in studied individuals. These parameters are the initial DNA damage, the initial rate of DNA repair, the half-time of DNA repair, and the residual DNA damage after 30 and 60 min. The initial rate of DNA repair, $v_0(DNAr)$, is the amount of DNA damage removed per time unit at the beginning of repair incubation and is expressed as percentage of total radiation-induced DNA damage removed per minute (%/min). The initial rate of DNA repair was calculated based on the slope coefficient of the equation of the straight line tangent to an exponential curve at the point for repair time 0 min,

$$v_0(DNAr) = -100A = 100(ab + cd),$$

where A is the slope coefficient of the tangent equation, and a , b , c , and d are the coefficients of the exponential equation.

The half-time of DNA repair, $t_{1/2}(DNAr)$, is the time in minutes necessary to repair half of the DNA damage induced by a genotoxic factor. Residual DNA damage is the fraction of DNA damage remaining at a particular time after γ -irradiation. The residual DNA damage was assessed after 30 min and after 60 min: $r_{30}(DNAd)$ and $r_{60}(DNAd)$, respectively. The values of both parameters were derived directly from the estimated exponential equation.

Estimation of relative intercellular variability in DNA damage levels in repairing PBMCs—The relative variability in DNA damage for each experimental SSB repair kinetics point was expressed using the dispersion coefficient (H) and the coefficient of variation (CV):

$$H = \frac{s^2}{\bar{x}} \\ CV = \frac{s}{\bar{x}}$$

Both parameters were determined based on the arithmetic mean ($\bar{x}$) and the standard deviation (s) calculated for each sample. The coefficient of variation or the dispersion coefficient obtained from three or four data sets from a single individual was taken together to calculate the geometric means for each experimental SSB repair kinetics point.

Analysis of SSB-RC and intercellular variability in DNA damage levels in repairing PBMCs in human population—We analyzed the strength of effect of demographic and health-related variables on SSB-RC and relative intercellular variability in DNA damage. Each relationship was graphed using SigmaPlot 8.0 and statistical analyses were performed using Statistica 7.1 (Statsoft Inc. Tulsa, OK). One-way and two-way ANOVA as well as t tests were used to detect significant differences in SSB-RC and intercellular variability in DNA damage between groups of human individuals characterized by independent variables including sex, race, age (stratified into groups), hypertension, current and past ethanol and tobacco use, and family history of cancer. Simple linear regression analyses were applied to investigate relationships between continuous independent variable (age, BMI, or pack-years) and dependent variable (SSB-RC or intercellular variability in DNA damage). Simple regression analyses were performed in

subsets of the 96-individual cohort characterized by sex or race or both sex and race. Regression analysis for the effect of age, sex, and race on SSB-RC is shown in Fig. 2.

Multiple regression analyses were done separately for males, females, whites, and African-Americans (Figs. 3-5, Tables 2 and 3). In multiple regression analyses, we tested simultaneously the effect of single discrete (sex or race) and single continuous (age, BMI or pack-years) independent variables on dependent variables (SSB-RC or intercellular variability in DNA damage). When necessary, interaction of sex or race with age, BMI, or pack-years was included in the regression. A significant test for the interaction coefficient indicates that the relationship between the dependent variable and one of the independent variables in the interaction is related to the value of the other variable in the product. Multiple regression models containing more continuous and discrete independent variables were also tested. Residual analysis was performed during all regression analyses to identify outliers. The residual analysis involved computing raw residuals, deleted residuals, and Cook's distances (Statistica).

Results

We first examined the dose–effect relationship between the γ -radiation and the DNA damage level in fresh and cryopreserved AG10097 cells. Cells embedded in agarose were exposed to γ -radiation at the dose range 0–10 Gy and processed using the comet assay protocol described in our previous publication [18]. The observed DNA damage levels in cells measured as Olive tail moment (OTM), tail DNA, and tail length are shown in Fig. S1. The data are presented as arithmetic mean values and as standard error of mean. We analyzed OTM, tail DNA, and tail length for linearity in the quantification of the amount of γ -radiation-induced DNA damage in cells. As presented in Fig. S1, the OTM data show linearity for radiation doses 0–6 Gy with moderate saturation occurring in the range of 6–10 Gy. The tail DNA data show slight saturation-related departure from linearity in the dose range 0–6 Gy and moderate departure at higher studied doses. The tail length values increase rapidly between 0 and 2 Gy and then reach a plateau. The saturation of tail length likely occurs at a γ -radiation dose less than 2 Gy. In summary, the best results are obtained using OTM. The maximum dose at which no significant departure from linearity for this comet assay parameter appears can be estimated at approximately 6 Gy. The higher levels of single-strand breaks induced by radiation at a dose of 6.3 Gy and present at the beginning of the repair period (Fig. 1) are more accurately measured using OTM as compared to the tail DNA and tail length parameters. A detailed justification for use of the dose of 6.3 Gy as a radiation dose was provided in our previous paper [18].

SSB repair capacity was studied in 24-individual groups of African American males, African American females, white males, and white females. Standardized values obtained for 3–4 data sets were taken together to calculate the means for each experimental point. Examples of average standardized kinetics data obtained for two white males and two white females are shown in Fig. 1. The more rapid removal of DNA damage is clearly discernible during the first 15- to 30-min postirradiation period. DNA damage levels after 5 and 15 min of repair incubation were lower in individuals shown in Figs. 1b and 1d (high SSB-RC) than in individuals represented by Figs. 1a and 1c (low SSB-RC). Thus, PMBCs from individuals presented in Figs. 1b and 1d remove DNA damage more rapidly than individuals shown in Figs. 1a and 1c. DNA repair parameters estimating the early phase of damage removal (up to 15 min), the initial rate of DNA repair (represented in Fig. 1 by long-dashed straight lines), and the half-time of DNA repair confirm reported interindividual differences in SSB-RC. Both parameters are defined under Materials and methods. The values of the initial rate of DNA repair, $v_0(DNAr)$, are as follows: 6.8%/min (a), 13.7%/min (b), 5.7%/min (c), and 9.7%/min (d). The values of the half-time of DNA repair, $t_{1/2}(DNAr)$, were estimated to be

9. 8 min (a), 6. 1 min (b), 12. 1 min (c), and 7. 4 min (d). Lower values for the half-time of DNA repair correspond to higher SSB-RC. In addition, higher values for the initial rate of DNA repair correspond to higher SSB-RC.

Four DNA repair parameters, the initial rate of DNA repair ($v_0(DNAr)$), the half-time of DNA repair ($t_{1/2}(DNAr)$), the residual DNA damage after 30 min ($r_{30}(DNAd)$), and the residual DNA damage after 60 min ($r_{60}(DNAd)$), were measured for this 96-individual group. Three individuals were excluded from the analysis since they were identified as outliers as assessed by the residual analysis. The excluded individuals are characterized as follows:

- 33-year-old white female, $v_0(DNAr)=15.2\%/min$, $t_{1/2}(DNAr)=6.9$ min, $r_{30}(DNAd)=0.22$, $r_{60}(DNAd)=0.09$;
- 42-year-old white female, $v_0(DNAr)=1.7\%/min$, $t_{1/2}(DNAr)=34.5$ min, $r_{30}(DNAd)=0.47$, $r_{60}(DNAd)=0.25$;
- 58-year-old African-American female, $v_0(DNAr)=13.8\%/min$, $t_{1/2}(DNAr)=5.5$ min, $r_{30}(DNAd)=0.19$, $r_{60}(DNAd)=0.10$.

Thus, data for 93 individuals were used in further analyses. However, preliminary analysis for 96 individuals still results in significant conclusions. Furthermore, the assumption of homoscedasticity (homogeneity of variances of residuals corresponding to different values of independent variables) was not satisfied for the linear regression relationships where SSB-RC was expressed using the initial rate of DNA repair and the half-time of DNA repair. Logarithmic transformation of the initial rate of DNA repair and the half-time of DNA repair was applied to obtain homoscedasticity for these regression models.

The relationships between age and SSB-RC are shown in Fig. 2. SSB-RC was measured as the logarithm of the initial rate of DNA repair, the logarithm of the half-time of DNA repair, the residual DNA damage after 30 min, and the residual DNA damage after 60 min. Fig. 2 presents these relationships in groups of individuals characterized by sex and race. We found a moderate association between the age and the logarithm of the initial rate of DNA repair for white women ($r = 0.55$, $P < 0.01$; Fig. 2c), as assessed by simple linear regression analysis. We have also observed an inverse relationship between the age and the logarithm of the initial rate of DNA repair in African-American women ($r = 0.40$, $P = 0.061$; Fig. 2d). In white females and African-American females, similar changes in SSB-RC measured by the logarithm of the half-time of DNA repair are present ($r > 0.3$). There is no statistically significant age dependence of SSB-RC as measured by the logarithm of the initial rate of DNA repair for white males (Fig. 2a) and African-American males (Fig. 2b). There is no effect of race and sex on SSB-RC measured using the remaining DNA repair parameters: the residual DNA damage after 30 min and the residual DNA damage after 60 min.

We also analyzed the effect of age and race on SSB-RC in males (Fig. 3a) and females (Fig. 3b) and the effect of age and sex on SSB-RC in whites (Fig. 3c) and African-Americans (Fig. 3d). Results of this statistical analysis are presented in Table 2. Multiple linear regression analysis was performed to evaluate the effect of two primary independent variables and one secondary independent variable corresponding to their interaction on SSB-RC. If no interaction was found, the simpler regression with two independent variables was tested. A statistically significant interaction between age and race was found for females (Fig. 3b) when SSB-RC was expressed using parameters related to the fast component of SSB repair, the logarithm of the initial rate of DNA repair ($P < 0.002$), and the logarithm of the half-time of DNA repair ($P < 0.05$). The weak interaction between age and sex was detected in African-Americans (Fig. 3d) when SSB-RC was measured by the logarithm of the initial rate of DNA repair ($P = 0.073$) and the logarithm of the half-time of DNA repair

($P = 0.059$). We found no statistically significant effect of age and race in females (Fig. 3b), and age and sex in African-Americans (Fig. 3d) on the residual DNA damage after 30 min and the residual DNA damage after 60 min.

We detected no interaction between age and sex in whites for any DNA repair parameter (Fig. 3c). However, when we analyzed the data using the simpler model with no interaction, we found that SSB-RC measured by the logarithm of the half-time of DNA repair and the residual DNA damage after 30 min are significantly affected by sex ($P < 0.01$ and $P < 0.05$). Sex also affects SSB-RC as measured by the logarithm of the initial rate of DNA repair in 46-year-old and younger white individuals (t test, $P < 0.02$); however, this relationship in whites is not statistically significant for the age span studied ($P = 0.149$). We also found that the logarithm of the initial rate of DNA repair increases with age in whites ($P < 0.01$). However, this relationship is mainly related to an increase in the logarithm of the initial rate of DNA repair in white females (Fig. 2c; $P < 0.01$). The relationship in white males is not statistically significant (Fig. 2a; $P > 0.05$). Finally, there is no effect of age on SSB-RC in whites and no difference in SSB-RC between white males and African-American males (Fig. 3a). We also found that there is no effect of body mass index (BMI), hypertension, alcohol or tobacco use, and family cancer history on SSB-RC in our 93-individual cohort as assessed by t tests, ANOVA, and regression analyses.

We studied the effect of age, race, and sex on relative intercellular variability in DNA damage levels in repairing PBMCs (Figs. 4 and 5). Both figures present examined relationships in groups of individuals characterized by sex and race: white males (Fig. 4a and Fig. 5a), African-American males (Fig. 4b and Fig. 5b), white females (Fig. 4c and Fig. 5c), and African-American females (Fig. 4d and Fig. 5d). The intercellular variability is expressed using the dispersion coefficient (H) in Fig. 4 and using the coefficient of variation (CV) in Fig. 5. The data are presented for selected experimental points: unirradiated PBMCs with no postirradiation incubation (row 1), or irradiated PBMCs subjected to 0 min (row 2), 30 min (row 3), and 60 min (row 4) of repair time. The results of the multiple regression analysis of age, race, and sex on intercellular variation in DNA damage are presented in Table 3 and Figs. 4 and 5. Table 3 shows analysis of the effect of two primary independent variables (age and race, or age and sex) and one secondary independent variable corresponding to the interaction on variation in DNA damage in single cells. The format of the data presented in Table 3 facilitated direct comparison of results of regression analysis for dispersion coefficient and coefficient of variation, since values of coefficient of determination R^2 are not affected here by the presence of different number of independent variables used in the regression model. Figs. 4 and 5 also present the results of the regression analysis done using no interaction terms when no interaction between primary independent variables was found.

Similar trends in the relationships among age, race, age, and intercellular variation in DNA damage are observed for dispersion coefficient (Fig. 4) and coefficient of variation (Fig. 5). The relationships for unirradiated and irradiated cells not subjected to repair incubation are statistically insignificant (Table 3 and Figs. 4 and 5 rows 1 and 2). All models presented in Fig. 4 row 4 and most of the models shown in Fig. 5 row 4 are affected significantly by sex or race or the effect of the interaction between age and race or age and sex. Similar relationships can be found in row 3 in both figures; however, both parameters for the intercellular variation in DNA damage are affected significantly only in the models for white and African-American females (Fig. 4b and Fig. 5b) and for white and African-American males (Fig. 4a).

The relationships presented in Figs. 4c and d and Figs. 5c and d in row 4 for irradiated cells subjected to 60-min repair incubation exhibit similarities to those presented in Figs. 3c and d

for SSB-RC measured by the logarithm of the initial rate of DNA repair and the logarithm of the half-time of DNA repair. Sex in the white study participants significantly affects the values of the dispersion coefficient (Fig. 4c; $P < 0.005$), the coefficient of variation (Fig. 5c; $P < 0.01$), and both DNA repair parameters (Fig. 3c). We also found an interaction between age and sex in African-Americans that was significant for the dispersion coefficient (Fig. 4d; $P < 0.05$), and close to significant for the coefficient of variation (Fig. 5d; $P = 0.080$) and both DNA repair parameters (Fig. 3d). We found an interaction between age and race in males for the dispersion coefficient (Fig. 4a; $P < 0.002$) and the coefficient of variation (Fig. 5a; $P < 0.002$). This interaction was not present for any DNA repair parameter (Fig. 3a). Finally, race in females affects the dispersion coefficient (Fig. 4b; $P < 0.02$) and coefficient of variation values (Fig. 5b; $P < 0.005$). Direct comparison of R^2 coefficients and other regression coefficients for the dispersion coefficient and the coefficient of variation shows that both parameters are comparable in measuring the intercellular variability in DNA damage in single cells (Table 3).

Discussion

We have examined capacity to repair SSB, alkali labile sites, and DSB (SSB-RC) in unstimulated cryopreserved PBMCs using the alkaline comet assay. We have found that cryopreserved PBMCs subjected to γ -radiation and repair incubation are able to remove induced DNA damage (Fig. 1). We observed that interindividual variation in the fast component of SSB repair is influenced by age, race, and sex (Figs. 2 and 3). A newly introduced DNA parameter, the logarithm of the initial rate of DNA repair was found to be a useful measurement for the fast component of SSB repair. Finally, we show that intercellular variability in DNA damage levels in single cells varies between individuals and also depends on age, race, and sex. Furthermore, the interindividual relationships for the rate of the fast component of SSB repair and for the intercellular variability in DNA damage are similar.

At least two components of SSB repair kinetics have been identified when studied using alkaline sucrose gradient sedimentation [24], alkali unwinding [25], alkaline elution [26], and the alkaline comet assay [21]. The presence of the fast and slow components is related to different rates of repair for various types of DNA damage. Most SSBs are repaired very rapidly in most cell types including PBMCs. They constitute the majority of DNA lesions removed during the first 5–10 min of repair incubation since they are removed by the fast SSB repair component [21]. DSBs are generated during γ -irradiation at a frequency approximately 40 times less than that of SSBs. Studies show that DSBs are responsible for a 5% increase in the alkaline comet assay-detected radiation-induced DNA damage [27]. Most DSBs are repaired by the nonhomologous end joining pathway (NHEJ) at the rates lower than SSB [28]. Complex DNA damage which may include DSBs or multiple SSBs may constitute a form of damage removed by the slow SSB repair component at much lower rates than most SSB. It has been estimated that leukocytes will rejoin fewer than 70% of induced DSBs 24 h after exposure to 75 Gy [29]. Oxidative base lesions are not detected directly by the comet assay. They are indirectly measured as these lesions are converted transiently into AP sites and SSBs before their complete repair. These transitory lesions may be detected by the assay as damage being repaired by slower SSB repair components.

We have demonstrated a set of DNA repair parameters that permitted us to examine both the fast and the slow component of SSB repair. The initial rate of DNA repair is the sum total of all repair components responsible for removing different types of repairable DNA lesions immediately after exposure to γ -radiation. Since the rate of the slow repair components is much lower compared to rate of the fast repair component [21], the value for initial rate of DNA repair is largely dependent on the initial rate of the fast component. The half-time of

DNA repair is less affected by the rate of the fast repair component and more by the rate of slow repair component. Another parameter used in our study, the residual DNA damage after 30 min, is dependent on both the fast and the slower SSB repair components. Finally, the residual DNA damage after 60 min is mainly affected by the slower SSB repair kinetics and also to the presence of unrepairable DNA lesions. Both parameters describing the fast component of SSB repair, the initial rate of DNA repair and the half-time of DNA repair, were logarithmically transformed. More detailed discussion of DNA repair parameters used in this study as well as approaches applied by others to assess SSB-RC using the comet assay can be found in our previous work [18].

We have analyzed the relationship between age and SSB-RC (Fig. 2). We found that there were age-associated differences in the rate of the fast component of SSB repair. In white women, the rate is faster in older ages; in African American women, the rate is slower in older ages. Statistically significant effects for white women and effects close to statistical significance for African-American women are observed for the logarithm of the initial rate of DNA repair. No significant increase in the fast repair component with age is present in white and African-American males (Figs. 2a and 2b). There is no available literature about SSB-RC and DNA repair capacity in humans of different racial or ethnic origin. All available reports show data on white population [5,6,13]. Furthermore, no changes in the fast component of SSB repair with age in white females were shown in literature reports. We also found no correlation between age and the residual DNA damage after 60 min related to the rate of slow repair component in any group (Fig. 2). There is no effect of age on the residual DNA damage after 30 min. A similar relationship for the residual DNA damage after 30 min and 60 min was obtained for a predominantly white male cohort (23 white males and 8 white females aged 25–91 years) by Singh et al. [9]. Repair of γ -radiation and H_2O_2 -induced DNA damage was not affected by aging in the other alkaline comet assay studies [2,4–7]. It can be concluded that assessment of the rate of the fast component is beneficial when performed as part of the comet assay procedure because it can provide additional information about interindividual variation in SSB-RC and DNA repair capacity in general.

We have examined the combined effect of age and race or age and sex on the fast and slow component of SSB repair (Fig. 3). We found that the rate of the fast component of SSB repair, associated mainly with DNA ligation and polymerization steps of BER, was lower in white females than in white males (Fig. 3c). Wei and co-workers reported that NER capacity to repair benzo[a]pyrene diol epoxide assessed using the host-cell reactivation assay was also lower in white females when compared to white males [11,12]. It was also found that lower nucleotide excision repair (NER) activity in females was associated with higher risk of lung cancer and sunlight-induced nonmelanoma skin cancer [8,11]. The observed differences in SSB-RC between groups in our study are small. However, there are several examples in the literature where subtle differences in NER capacity are associated with increased susceptibility to lung and nonmelanoma skin cancer [8,11,12]. It can be hypothesized that the activity of the DNA ligation and polymerization steps of BER and NER pathways is lower in white females than white males. The effect of age and sex in white individuals on the capacity of the BER pathway enzyme, OGG1, to remove 8-oxoguanine was measured by Paz-Elizur and colleagues [14]. In individuals 55 years of age and younger, they observed insignificant decrease in OGG1 activity in females when compared to males. The OGG1 activity levels were very similar in older individuals, mainly due to decrease in the OGG1 activity in males ($P < 0.01$). These findings indicate that relationships between white males and white females in the activity of different DNA repair pathways can be similar, although the effect of age on different pathways may vary. The observed variation in DNA repair capacity may be linked to different levels of induction by metabolism-related oxidative stress. Basal metabolism rate in a predominantly white

population was higher in males than females [30]. Furthermore, it has been suggested that an increase in DNA repair capacity with age may result from an age-related increase in release of reactive forms of oxygen by mitochondria [31].

We also found a decrease in the rate of the fast component of SSB repair with age in African-American females when compared with white females (significant effect, Fig. 3b) and African-American males (effect close to significance, Fig. 3d). In both cases, the effect of interaction is present. It is worth noting that no interaction was found when race- and sex-defined groups other than African-American females were analyzed by multiple linear regression. No changes in the slow component of SSB repair are found. The reasons for different age-related changes in the fast component of SSB repair in African-American females relative to other studied groups are unknown. It was found that African-Americans are subjected to higher levels of exposure to environmental pollutants than whites [32]. However, variation in exposure to environmental pollution does not explain obtained differences in SSB-RC, since African-American and white males respond similarly to γ -radiation. Furthermore, African-American and white males and females analyzed in our study resided in the same neighborhoods which suggests a similar pattern of residual genotoxic exposure. The intergroup variation in SSB-RC may be also associated, for example, with lifestyle and different age-related changes in detoxifying, antioxidant, and repair enzyme activities.

The assessment of intercellular variation in DNA damage using the dispersion coefficient H in response to genotoxic exposure was proposed earlier [9,33,34]. The coefficient of variation (CV) is another parameter that has been used in the comet assay to assess intercellular variation in DNA damage for each examined human participant [35], and the reproducibility of the assay [36,37]. We analyzed the intercellular variation in DNA damage levels in repairing cells from humans characterized by age, race, and sex in addition to sample mean values. We performed analysis using dispersion coefficient (Fig. 4) and coefficient of variation (Fig. 5). We found that similar trends in the relationships among age, race, age, and intercellular variation in DNA damage are observed for dispersion coefficient and coefficient of variation. Race or sex or the effect of the interaction between age and race or age and sex significantly affects the intercellular variation in irradiated cells after 60 min of repair incubation (Fig. 4 row 4; Fig. 5 row 4). Similar intersex relationships between white males and white females or between African-American males and African-American females are observed for intercellular variation in DNA damage (Figs. 4c and d row 4; Figs. 5c and d row 4) and for the fast component of SSB repair as measured by the logarithm of the initial rate of DNA repair and the logarithm of the half-time of DNA repair (Fig. 3 rows 1 and 2). Similar age-, race-, and sex-related trends in the fast repair component and the intercellular variability are caused by the fact that faster SSB repair component in PBMCs may result in lower intercellular variability in the DNA damage levels 60 min after exposure to γ -radiation. Intercellular variation in DNA damage in repairing cells in whites (23 males and 8 females aged 25–91 years) was also analyzed earlier using the dispersion coefficient by Singh et al. [9]. They observed similar effects of age on the dispersion coefficient for unexposed lymphocytes and for irradiated cells subjected to 0, 15, 30, and 60 min repair incubation as in our 24-individual white male group (Fig. 4a). However, they did not detect a link between the intercellular variation and the fast DNA repair component.

While no single test can reveal all the complexities of DNA repair capacity in individual humans, the comet assay when performed using reference standardization procedures can serve as a general screening method assessing capacity of intact cells to repair AP sites, SSB, and DSB. The application of DNA repair parameters measuring the rate of the fast repair component, the initial rate of DNA repair, and the half-time of DNA repair can increase the capacity of the comet assay to detect interindividual variation in SSB-RC.

When paired with modified comet assay protocols that utilize DNA glycosylases, assessment of oxidative base lesions may also be examined. Other DNA repair methodologies such as extract-based enzyme assays [14], the reactivation assay that analyze fidelity of DSB repair [38,39], BER [40,41], and NER [11], and cytokinesis-block micronucleus cyto (CBMN Cyt) assay [42] can augment the assessment of DNA repair capacity in clinical studies. However, the comet assay as shown by our results provides the opportunity to accurately assess the interindividual variation in the fast and slow SSB repair components using a limited amount of clinical material which can constitute easily accessible PBMCs. Cryopreserved PBMCs can be stored for a prolonged time with no need for stimulation of their growth using mitogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP sites	apurinic/aprimidinic sites
BER	base excision repair
BMI	body mass index
DSB	double-strand break
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
NER	nucleotide excision repair
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
SSB	single-strand break
SSB-RC	single-strand break repair capacity

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Fig. 1.

Repair kinetics of γ -radiation-induced DNA damage in human PBMCs. Cellular DNA damage levels are expressed as standardized Olive tail moment. Data for PBMCs are expressed as mean and standard error of mean. Exponential curves with two exponential terms $y = ae^{-bx} + ce^{-dx}$ (continuous lines) are fitted to each set of standardized values representing DNA damage decline in irradiated PBMCs during postirradiation incubation (open triangles). Solid circles represent unirradiated PBMCs. Values 0 and 1 are represented by dotted lines that correspond to negative and positive reference standards. The straight line tangent to exponential curve at the point for repair time 0 min corresponding to the initial rate of DNA repair is shown as long-dashed line.

Fig. 2.

Effect of age on SSB repair capacity (SSB-RC) in white males (a), African-American males (b), white females (c), and African-American females (d). The effect of age (X) on SSB-RC (Y) was analyzed using simple linear regression. Each relationship was described by linear regression curve (dashed line) and the correlation coefficient r with its corresponding P value in parentheses. The statistically significant ($P < 0.05$) value for correlation coefficient is marked in bold, whereas the value that is close to significance is underlined.

Fig. 3.

Effect of age, sex, and race on SSB repair capacity (SSB-RC). Graphs in panels a and b show the relationship among age (X_1), race (X_2), and SSB-RC (Y) in males (a) and females (b). Graphs in panels c and d present the relationship among age (X_1), sex (X_2), and SSB-RC (Y) in whites (c) and African-Americans (d). Data used to generate these graphs were also statistically analyzed in Table 2. Independent variables significantly related to SSB-RC are shown on graphs together with corresponding P values in parentheses. Statistically significant ($P < 0.05$) variables are marked in bold, whereas the values that are close to significance are underlined. SSB-RC was measured as the logarithm of the initial rate of DNA repair, the logarithm of the half-time of DNA repair, the residual DNA damage after 30 min, and the residual DNA damage after 60 min. Each point corresponds to a single individual. Dashed and continuous lines represent curves for simple linear regression describing the relationship between age and SSB-RC and performed separately for each group of white males (blue dashed lines), African-American males (blue continuous lines), white females (red dashed lines), and African-American females (red continuous lines).

Fig. 4.

Effect of age, sex, and race on variation in DNA damage in single cells measured by the dispersion coefficient. Variation in DNA damage in single cells was measured by the dispersion coefficient for the unirradiated and irradiated cells subjected to 0, 30, and 60 min of postirradiation incubation. Refer to Fig. 3 for the description of more details.

Fig. 5.

Effect of age, sex, and race on variation of DNA damage in single cells measured by the coefficient of variation. Variation in DNA damage in single cells was measured by the coefficient of variation for the unirradiated and irradiated cells subjected to 0, 30, and 60 min of postirradiation incubation. Refer to Fig. 3 for the description of more details.

Table 1

Clinical characteristics of the cohort

	Whites		African-Americans	
	Females	Males	Females	Males
	(n=24)	(n=24)	(n=24)	(n=24)
Age (in years; mean±SD)	45.5±11.0 (32.7–64.7)	47.7±10.4 (32.1–64.7)	47.3±10.4 (30.5–64.8)	48.5±10.4 (31.3–64.5)
BMI (mean±SD)	28.1±5.4 (21.4–39.4)	29.6±7.3 (19.3–47.6)	30.8±10.8 (17.2–61.9)	28.3±9.1 (18.7–56.6)
Hypertension (%)	26.3	43.5	36.8	42.9
Coronary atherosclerotic heart disease (%)	5.3	4.3	0.0	23.8
Diabetes mellitus, type 2 (%)	10.5	13.0	10.5	23.8
Current ethanol use (%)	57.9	77.3	52.6	55.0
Past ethanol use (%)	84.2	87.0	78.9	95.2
Current tobacco use (%)	58.3	22.0	50.0	66.7
Past tobacco use (%)	75.0	25.0	75.0	75.0
Pack-years (mean±SD)	14.8±14.9 (0–50)	6.5±12.0 (0–39)	8.9±12.1 (0–40)	12.6±13.0 (0–40)
Active cancer (%)	0.0	0.0	0.0	0.0
History of cancer (%)	10.5	8.7	10.5	0.0
Family history of cancer (%)	63.2	60.9	33.3	38.1

The effect of age, sex, and race on SSB repair capacity (SSB-RC)

Table 2

DNA repair parameter	Regre- ssion coeffi- cients	White males and African- American males		White females and African- American females		White males and white females		African-American males and African American females	
		X_1 : age (years); X_2 : race; X_3 : $X_1 \cdot X_2$		X_1 : age (years); X_2 : sex; X_3 : $X_1 \cdot X_2$		X_1 : age (years); X_2 : sex; X_3 : $X_1 \cdot X_2$		X_1 : age (years); X_2 : sex; X_3 : $X_1 \cdot X_2$	
		Estimate	(Standard error)	Estimate	(Standard error)	Estimate	(Standard error)	Estimate	(Standard error)
Log ₁₀ (initial rate of DNA repair)	R^2	0.057		0.243^e		0.195^e		0.132	
	b_1	0.0029	(0.0020)	0.0079	(0.0028) ^e	0.0058	(0.0020) ^e	0.0019	(0.0028)
	b_2	−0.032	(0.041)	0.60	(0.19) ^f	−0.060	(0.040)	0.29	(0.20)
	b_3	–	–	−0.013	(0.004) ^g	–	–	<u>−0.0075</u>	(<u>0.0041</u>) ^g
Log ₁₀ (half-time of DNA repair)	R^2	0.077		0.107		0.184^d		0.115	
	b_1	−0.0022	(0.0016)	−0.0034	(0.0022)	−0.0026	(0.0016)	−0.0026	(0.0022)
	b_2	0.041	(0.032)	−0.32	(0.15) ^e	0.087	(0.032) ^e	−0.24	(0.15)
	b_3	–	–	0.0068	(0.0031) ^e	–	–	<u>0.0059</u>	(<u>0.0031</u>) ^h
Residual DNA damage (after 30 min)	R^2	0.048		0.030		0.146^c		0.037	
	b_1	−0.00023	(0.00054)	0.00053	(0.00057)	0.00082	(0.00062)	−0.00054	(0.00047)
	b_2	0.016	(0.011)	−0.007	(0.012)	0.029	(0.013) ^c	0.0059	(0.0096)
Residual DNA damage (after 60 min)	R^2	0.035		0.005		0.043		0.027	
	b_1	−0.00037	(0.00040)	0.00021	(0.00049)	0.00032	(0.00041)	−0.00049	(0.00047)
	b_2	0.0069	(0.0080)	0.002	(0.010)	0.0093	(0.0083)	0.0036	(0.0096)

Multiple linear regression was used to analyze the effect of two primary independent variables (X_1 and X_2) and one secondary independent variable corresponding to the interaction between X_1 and X_2 ($X_1 \cdot X_2$) on SSB-RC (Y) as described by the equation $\hat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i} + b_3 \cdot X_{1i} \cdot X_{2i}$. If no interaction was found, significance of the alternative relationship $\hat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i}$ was tested. Statistical significance:

Statistically significant values (c–f) are marked in bold, whereas values at a significance slightly lower than $P=0.05$ (a, b) are underlined. All reported regression coefficients are in bold or underlined for the relationship $\hat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i}$ while only coefficient of determination R^2 and regression coefficient b_3 corresponding to the relationship describing the interaction $\hat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i} + b_3 \cdot X_{1i} \cdot X_{2i}$ are in bold or underlined.

^a $P=0.073$
^b $P=0.059$
^c $P<0.05$

d $P<0.02$

e $P<0.01$

f $P<0.005$

g $P<0.002.$

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The effect of age, sex, and race on variation in DNA damage in single cells measured by dispersion coefficient H and coefficient of variation

Table 3

Experimental point	Regression coefficients	X_1 : age (years); X_2 : race; X_3 : $X_1 \cdot X_2$			X_1 : age (years); X_2 : sex; X_3 : $X_1 \cdot X_2$		
		White males and African-American males	White females and African-American females	White males and white females	African-American males and African-American females	Dispersion coefficient	Coefficient of variation
γ -radiation dose: 6.3 Gy repair incubation: 30 min	R^2	$R^2=0.106$	$R^2=0.113$	$R^2=0.156$	$R^2=0.155$	$R^2=0.102$	$R^2=0.101$
	b_1	$P=0.171$	$P=0.149$	$P=0.071$	$P=0.072$	$P=0.207$	$P=0.211$
	b_2	$P=0.056$	$P=0.048$	$P=0.523$	$P=0.395$	$P=0.083$	$P=0.067$
	b_3	$P=0.052$	$P=0.092$	$P=0.118$	$P=0.093$	$P=0.079$	$P=0.058$
γ -radiation dose: 6.3 Gy repair incubation: 60 min	R^2	$R^2=0.236$	$R^2=0.213$	$R^2=0.158$	$R^2=0.204$	$R^2=0.218$	$R^2=0.260$
	b_1	$P=0.008$	$P=0.014$	$P=0.068$	$P=0.023$	$P=0.015$	$P=0.005$
	b_2	$P=0.047$	$P=0.018$	$P=0.511$	$P=0.524$	$P=0.192$	$P=0.037$
	b_3	$P=0.001$	$P=0.001$	$P=0.175$	$P=0.140$	$P=0.049$	$P=0.013$
		$P=0.002$	$P=0.002$	$P=0.400$	$P=0.388$	$P=0.160$	$P=0.051$

Multiple linear regression was used to analyze the effect of two primary independent variables (X_1 and X_2) and one secondary independent variable corresponding to the interaction between X_1 and X_2 ($X_2 \cdot X_1$) on variation in DNA damage in single cells as described by the equation $\hat{Y}_i = b_0 + b_1 \cdot X_{1i} + b_2 \cdot X_{2i} + b_3 \cdot X_{1i} \cdot X_{2i}$. Statistically significant ($P < 0.05$) values for coefficient of determination R^2 and regression coefficient b_3 are marked in bold, whereas the values at a significance slightly lower than $P = 0.05$ are underlined. The regression relationships were not significant for PBMCs at the other experimental points, including unirradiated and irradiated PBMCs with no repair incubation.