

An aphidicolin-block nucleotide excision repair assay measuring DNA incision and repair capacity

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The objective of the present study was to develop a cellular phenotype assay for nucleotide excision repair (NER), using benzo[*a*]pyrene diol epoxide (BPDE) as model mutagen. Since *in vitro* exposure to BPDE may lead to DNA strand breaks resulting from both direct interaction with DNA and incisions introduced by the repair enzymes, we aimed to discriminate between both types of breaks using the comet assay and quantified the DNA strand breaks after *in vitro* challenge of peripheral blood mononucleated cells (PBMCs) with BPDE in the presence or absence of the DNA polymerase inhibitor aphidicolin (APC). The assay was performed with a low (0.5 μ M) and a high (2.5 μ M) BPDE concentration. The individual NER capacity was defined as the amount of DNA damage induced by BPDE in presence of APC, diminished with the damage induced by BPDE and APC alone. First, the assay was applied to a NER-deficient human fibroblast cell line (XPA^{-/-}) to validate the methodology. Lower repair capacity and a higher amount of BPDE-induced DNA adducts were observed for the XPA^{-/-} fibroblasts as compared to the wild-type fibroblasts. Repeated experiments on PBMCs from four donors showed low intra-individual, intra-experimental and inter-assay variation for both concentrations, indicating the reliability of the method. To assess the inter-individual variation, the assay was applied to PBMCs from 22 donors, comparing the repair capacity after exposure to 0.5 μ M ($N = 10$) and 2.5 μ M ($N = 12$) BPDE. The repair capacity showed a higher inter-individual variation as compared to the intra-individual variation. Moreover, this difference was more pronounced using the low concentration. All these results indicate the adequacy of the method using this low concentration. Further improvement, however, should be recommended by applying the study with low BPDE concentration in a larger population and taking into account the relevant genotypes for NER.

Introduction

It has been shown that a combination of given genotypes may be predictive not only for chromosome aberrations (CAs) (1,2) and micronucleus (MN) induction (3) but also for cancer (4) since many genes are involved in repair pathways for base excision repair (BER), nucleotide excision repair (NER), double-strand break repair and mismatch repair. Besides

genotyping, integration of the repair capacity (phenotyping) is becoming an attractive approach.

In recent years, our laboratory has developed and implemented a direct comet-based DNA strand break repair phenotype assay to assess the repair of oxidative DNA damage induced in peripheral blood mononucleated cells (PBMCs) of donors challenged *in vitro* with H₂O₂, X-rays or styrene oxide. This method was successfully applied by us in combination with genotyping to assess individual susceptibility in bio-monitoring of nuclear power (5,6) and styrene-exposed workers (7) and newborn daughter–mother pairs (8). Several other studies have used the same approach to investigate DNA repair capacity after challenging PBMCs with ionizing radiation (9–12), bleomycin (13–15) or H₂O₂ (16). The data show that for BER the method is quite sensitive, applicable for biomonitoring and a valuable alternative or complementary method to genotyping.

If BER phenotyping seems well developed, no equivalent methods are available for NER, double-strand break repair or mismatch repair. As far as NER is concerned, Langie *et al.* (17) proposed a modified alkaline comet assay to determine the ability of human PBMC extracts to perform damage recognition and incision. The assay was based on the adapted comet protocol developed by Collins *et al.* (18) to measure *in vitro* the BER capacity of cell extracts. Two other *in vitro* assays for NER have been proposed; the first is based on the comet assay (19) and the second adapted an alkaline unwinding flow cytometry assay for the measurement of NER-mediated breaks (20). The published assays, however, do not always allow discrimination between DNA breaks induced by the mutagen and those resulting from DNA repair incisions, or do not assess a cellular repair phenotype, but instead measure the repair capacity of DNA repair enzymes in cell extracts.

Within the Environmental Cancer Risk, Nutrition and Individual Susceptibility network of Excellence (www.ecnis.org), we aimed to develop and validate a methodology for NER, with benzo[*a*]pyrene diol-epoxide (BPDE) as model mutagen. Considering that *in vitro* exposure to BPDE may induce DNA strand breaks as a result of direct interaction with DNA and of incisions introduced by the repair enzymes as well, we aimed at discriminate between both types of breaks using the comet assay and developed the aphidicolin-block NER phenotype assay, which quantifies the DNA strand breaks after *in vitro* challenge of PBMCs with BPDE in the presence or absence of the DNA polymerase inhibitor aphidicolin (APC).

We defined the individual NER capacity as the amount of DNA damage induced by BPDE in presence of APC, diminished with the damage induced by BPDE and APC alone. This value corresponds to the incision capacity of the NER enzymes. All experiments were performed with a low (0.5 μ M) and a high (2.5 μ M) BPDE concentration. In order to validate

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the methodology, complementary experiments were performed on DNA repair-deficient XPA^{-/-} and wild-type human fibroblasts. Intra-individual and inter-individual variation of repair capacity were analysed in PBMCs isolated from young healthy non-smoking female donors. The low BPDE concentration revealed a lower intra-individual and a higher inter-individual variation for the repair capacity compared to the high BPDE concentration, which supports the reliability of the method when the low BPDE concentration is used.

Material and methods

Study population

PBMCs from 22 young healthy non-smoking female volunteers were used for the comparison of NER capacity after exposure to a low (0.5 µM, *N* = 10) or high (2.5 µM, *N* = 12) BPDE concentration. To assess the intra-individual variation of repair capacity, PBMCs from four donors were sampled three times at 1-week intervals. All participants signed an informed consent form. The protocol was approved by the ethical committee of St Pierre University Hospital in Brussels. Clinical characteristics, smoking, drinking and nutritional habits of the donors were collected by a detailed questionnaire.

Collection and processing of blood samples

Venous blood samples were collected in heparinized tubes (BD Vacutainer, Becton Dickinson, Plymouth, UK) and kept at 4°C until processing. Within 24 h, PBMCs were isolated using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and cultured in Ham's F-10 medium containing 25 mM L-glutamine (Gibco; Invitrogen, Paisley, UK) supplemented with 15% foetal calf serum (FCS; Gibco) and 2% phytohaemagglutinin A 16 (PHA; Murex Biotech Ltd, Dartford, UK). Samples were incubated in 5% CO₂ in a humidified incubator at 37°C.

Treatment of PBMCs and human fibroblasts

After 24 h PHA stimulation, PBMCs were transferred to serum-free medium and challenged with 0.5 or 2.5 µM BPDE (Sigma-Aldrich, St Louis, MO, USA) dissolved in dimethyl sulphoxide (DMSO) (Merck, Darmstadt, Germany) for 2 h. Control samples were exposed to 0.5% DMSO. All exposures (prepared in duplicate cultures) were processed in parallel.

Alkaline comet assay

The comet assay was performed as described previously by Singh *et al.* (21) with modifications according to De Boeck *et al.* (22). For each electrophoresis, a positive and negative internal standard was included which consisted of human K562 erythroleukemia cell line (23) treated or untreated with 2 mM ethyl methane sulfonate (EMS), respectively (24). The percentage of tail DNA (TD) was considered to be the most reliable DNA damage parameter (24). Slides were coded before analysis and 100 cells per culture were scored.

Cultivation of human fibroblasts

Xeroderma pigmentosum group A (XPA^{-/-}) and wild-type human fibroblasts were cultured in MEM (Gibco) supplemented with 20% FCS, 1% penicillin/streptomycin, 0.4% essential and non-essential amino acids and 0.02% minimal essential medium (MEM) vitamins. Cells were seeded and maintained in 25-cm² flasks in a humidified incubator at 37°C with 5% CO₂. Fibroblasts were allowed to attach for 24 h. After 24 h, the medium was replaced with fresh medium with or without BPDE and/or with or without APC. After treatments identical to those for the PBMCs, cells were trypsinized and comet assay was performed.

NER phenotype assay

To measure the repair capacity, duplicate treatments were performed in presence or absence of 0.5 or 2.5 µM BPDE (2 h) and with or without 30 min pre-incubation with 0.5 µg/ml APC (Sigma-Aldrich) dissolved in DMSO. Ten PBMC cultures per donor were prepared for the comet assay: two background controls, two exposed to DMSO (solvent), two exposed to APC alone, two exposed to BPDE alone and two exposed to APC + BPDE.

The repair capacity (RC) was calculated as follows: the extent of DNA strand breaks measured in the presence of the mutagen and APC diminished with the sum of the effects of the single compounds. [RC = %TD (APC + BPDE) – %TD (BPDE) – %TD (APC)] (Figure 1).

In vitro cytokinesis blocked MN test

After 24-h PHA stimulation, PBMCs were exposed to APC. Cytochalasin B (Sigma-Aldrich) was added at 6 µg/ml culture at 44 h to the PBMC cultures. After 72 h, cells were put directly onto slides using cytospin (Shandon) at 700

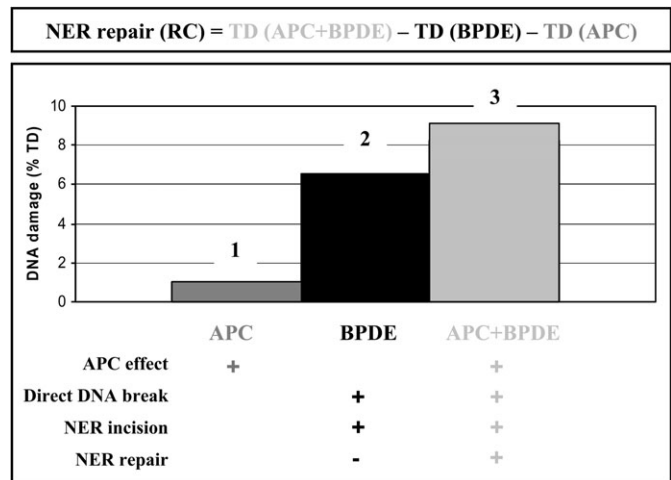


Fig. 1. Diagram depicting the contribution of the different treatments to the measured DNA strand breaks resulting in the NER capacity in the total population: (1) APC treatment alone results in a minor level of DNA damage; (2) DNA damage measured after treatment with BPDE consists of the direct DNA breaks and the NER incisions, a fraction of the induced DNA damage that is already repaired cannot be detected; (3) DNA damage measured after treatment with BPDE and APC consists of the APC effect, direct DNA breaks, the NER incisions and the remaining gaps resulting from the APC-blocked NER. The repair capacity (RC) was defined as the amount of DNA strand breaks damage induced by BPDE in presence of APC, diminished with the damage induced by BPDE and APC alone.

r.p.m. for 5 min and immediately fixed with methanol. Staining was achieved with 5% Giemsa (Merck) in Sørensen buffer, pH 6.8 (Prosan, Gent, Belgium) for 20 min.

Applying this method, no MN were scored. Two cultures for background, two for APC and two for solvent (DMSO) were analysed; 1000 cells were examined per culture; the percentage of binucleated cells, polynucleated cells and mononucleated cells were recorded. The slides were coded and analysed on a Zeiss transmitted light microscope at a magnification of $\times 1000$.

Cytokinesis-block proliferation index (CBPI) was calculated as follows:

$$\text{CBPI} = \frac{\text{number mononucleate cells} + 2 \times \text{number binucleate cells} + 3 \times \text{number multinucleate cells}}{\text{total number of cells}}$$

Immunodetection of BPDE–DNA adducts

After 2 h exposure to BPDE or DMSO, PBMCs were fixed in methanol:acetic acid (3:1), brought onto slides and kept at –20°C until immunodetection. Human fibroblasts were grown on microscope slides and after 2 h exposure fixed in methanol for 3 min (–20°C), followed by dipping in acetone (–20°C).

Cells were treated with RNase (Roche Diagnostics, Brussels, Belgium) (10 µg/ml in 2 \times SSC) for 1 h at 37°C, followed by proteinase K treatment (10 µg/ml) (Roche Diagnostics) for 10 min at room temperature for the PBMCs and 2% Triton in phosphate-buffered saline (PBS, pH 7) for the fibroblasts. DNA was denatured with 4 N HCl (10 min at room temperature), and the pH was adjusted to 7 with 50 mM Trisbase (5 min at room temperature). At the end of the denaturation step, non-specific binding of the antibodies was blocked by incubating the cells with blocking buffer (PBS/20% FCS) for 45 min at room temperature. Slides were incubated overnight at 4°C with monoclonal mouse antibody anti-BPDE–DNA (Clone 5D11) (1/50 in PBS/5% FCS) followed by incubation for 1 h with second polyclonal rabbit anti-mouse IgG–fluorescein isothiocyanate (FITC) antibody (1/100 in PBS/5% FCS) at room temperature. After dehydration in ethanol series (70%, 90%, 100%), cells were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics) in a 1,4-diazabicyclo[2.2.2]octane anti-fade solution.

The slides were analysed using a fluorescent microscope Zeiss axiscope (Carl Zeiss, Oberkochen, Germany) equipped with a triple band pass filter (n° 25, Zeiss), to visualize the FITC-labelled BPDE–DNA adducts and the DAPI-counterstained nuclei at a magnification of $\times 630$. Cells were classified according to the presence of BPDE–DNA adduct (yes/no). Per exposure condition, 500–1000 cells were scored.

Statistical analysis

Statistical package SPSS 16.0 was used to analyse the data. Normality of the variables was evaluated by the Kolmogorov–Smirnov goodness-of-fit test. Since not all data were normally distributed, the non-parametric Mann–Whitney U-test was applied to assess significant differences between exposures and groups. The Kruskal–Wallis test was applied to assess significant differences between individuals and repeated experiments. The level of significance was set at $P < 0.05$ for all statistical analyses.

Results

Choice of medium, BPDE and APC concentration

In the first phase, different treatment conditions were tested to define the optimal experimental set-up. As far as the effect of serum for the BPDE exposure of isolated PBMCs is concerned, no statistically significant difference in DNA damage between mutagen exposure in serum and serum-free medium was detected ($\%TD_{BPDE_{serum}} = 13.8$; $\%TD_{BPDE_{serum\ free}} = 13.3$). To avoid uptake of BPDE as protein adduct by scavengers in the serum, exposure in the serum-free condition was chosen for all subsequent experiments.

In order to induce an adequate amount of repair incisions, a dose-range of BPDE concentrations (0.05, 0.5, 1.25, 2.5 and 5.0 μM) was tested. Figure 2 shows a clear increase in DNA damage with increased BPDE concentrations. APC treatment induced an increase in BPDE damage, indicating that the gap-filling process of NER is fully operating. This inhibitory effect of APC on the repair of DNA damage was found for all BPDE concentrations but is more pronounced with the low BPDE concentrations (0.05, 0.5 and 1.25 μM). In order to further investigate the optimal BPDE concentration for the NER capacity, a low (0.5 μM) and a high (2.5 μM) BPDE concentration were compared in the further experiments.

APC alone did not induce a statistically significant increase in DNA damage as compared to the solvent (DMSO) or the background (untreated) (data not shown). The choice of the APC concentration and exposure time was based on literature values (25). Nevertheless, we verified whether APC affected the cell proliferation in our test system. Therefore, we performed the *in vitro* cytokinesis-block MN (CBMN) assay on PBMCs exposed to the same APC treatment conditions (0.5 $\mu\text{g/ml}$ and 2.5 h) used in our repair assay. The CBMN assay allows the assessment of cell proliferation by calculating the CBPI based on nuclearity (26). In the presence of APC, no statistically significant change in cell proliferation of the PBMCs was observed as compared to the solvent (DMSO) or the background (untreated) (supplementary Table 1, available at *Mutagenesis Online*).

Repair capacity of XPA^{-/-} cells

To better validate the new repair assay, the methodology was applied to XPA^{-/-} and wild-type human fibroblasts. First,

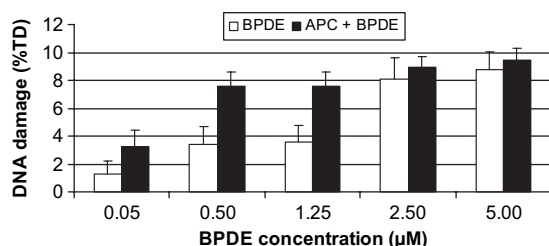


Fig. 2. Dose-range experiment showing BPDE-induced DNA damage measured with the comet assay (0.05, 0.5, 1.25, 2.5 and 5.0 μM BPDE), with or without 0.5 h APC pre-incubation (0.5 $\mu\text{g/ml}$).

different concentrations of APC (0.3, 0.6 and 1.5 μM) were tested and resulted in no remarkable difference or increase in APC-induced DNA damage measured by the comet assay (data not shown). Unlike the NER-deficient fibroblasts, wild-type human fibroblast showed statistically significant high repair capacity levels for the low BPDE concentration ($P < 0.05$, Kruskal–Wallis) (Figure 3). Although not statistically significant, higher repair capacity levels were detected in wild-type fibroblasts than in NER-deficient fibroblasts for the high BPDE concentration. The amount of BPDE–DNA adducts was higher in NER-deficient fibroblasts than in wild-type fibroblasts, although the difference was not statistically significant (Figure 4). All these observations indicate the specificity of our NER assay.

Intra-individual, intra-experimental and inter-assay variation in NER capacity

In order to assess the intra-individual and inter-assay variation, three samplings of four donors were performed at 1-week intervals and isolated PBMCs were challenged with both BPDE concentrations (0.5 and 2.5 μM). For each experiment, two parallel cultures per treatment condition were prepared and analysed, allowing evaluation of the intra-experimental variation. The results are summarized per concentration and per donor in Table I. No statistically significant difference between the three repeat experiments was detected for any of the four donors (Table I) nor between two parallel cultures for each treatment condition (supplementary Table 2, available at *Mutagenesis Online*). This indicates low intra-individual variation and inter-assay variation. The coefficients of variation (CVs = $SD/mean$) for the percentage of DNA damage induced by BPDE and defined by the comet assay were situated between 33 and 58% for the low BPDE concentration and between 4 and 67% for the BPDE concentration. As far as the CVs for BPDE-induced DNA adducts are concerned, CV values ranged from 9 to 36% for the low concentration of BPDE and from 14 and 20% for the high concentration. Except for Donor D, CVs for the repair capacity after challenging to 0.5 μM BPDE ranged from 16 to 26% and were in general lower as compared to the higher concentration (64–91%).

Inter-individual variation in repair capacity

The inter-individual variation of repair capacity studied in PBMCs isolated from 22 donors: $N = 10$ for 0.5 μM BPDE and $N = 12$ for 2.5 μM BPDE. As far as the DNA damage

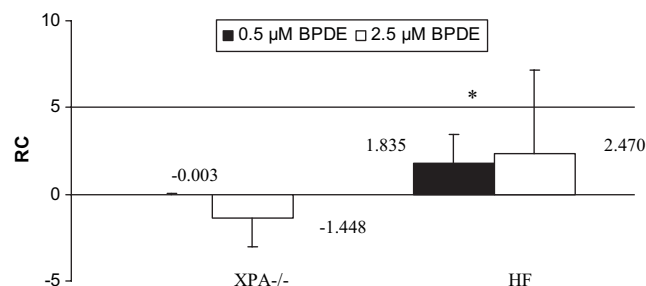


Fig. 3. Repair capacity (RC) of XPA^{-/-} and wild-type human fibroblast cells (HF) are presented for both low and high BPDE exposure. Per cell line and per treatment, two parallel cultures were processed and the experiment was repeated two times, median values are presented, * $P < 0.001$ (Kruskal–Wallis), HF versus XPA^{-/-}.

measured with the comet assay is concerned, APC alone did not induce a statistically significant increase of DNA damage as compared to the solvent (DMSO) or the background (untreated) among all individuals (data not shown).

For each individual, both low and high BPDE concentrations induced an increase in BPDE-induced DNA damage as compared to solvent; however, this increase was only statistically significant ($P < 0.05$, Kruskal–Wallis) for six donors exposed to the high concentration (Figure 5A and B). Although not always statistically significant, pre-incubation with APC induced an increase in BPDE-induced DNA damage as compared to BPDE alone for all donors exposed to 0.5 μM

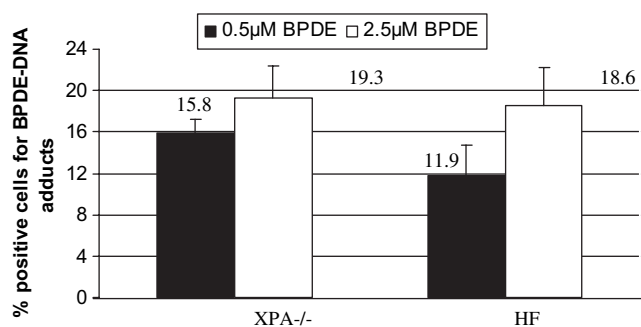


Fig. 4. Comparison of BPDE-induced DNA adducts of XPA^{-/-} and wild-type human fibroblast cells (HF) for both low and high BPDE exposure. Per cell line and per treatment, two parallel cultures were processed and the experiment was repeated two times; median values are presented.

BPDE (Figure 5A) and for almost all donors exposed to 2.5 μM (Figure 5B).

CVs for the percentage of DNA damage measured after exposure to APC, BPDE or APC + BPDE showed in general lower values after challenging with low BPDE concentration as compared to the high concentration (Table II). The DNA damage values after exposure to APC showed a statistically significant difference between the individuals for both concentrations ($P < 0.05$, Kruskal–Wallis). As far as DNA damage induced by APC + BPDE is concerned, a statistically significant difference was detected between individuals only for the high BPDE concentration ($P < 0.05$, Kruskal–Wallis).

The repair capacity values are shown in Figure 5C (0.5 μM BPDE) and Figure 5D (2.5 μM BPDE). At the low BPDE concentration, the CV among these donors was 72% (Figure 5C). As expected, a high variation (CV = 150%) was found among the 12 donors analysed at the high concentration (Figure 5D). The repair capacity values did not differ significantly between individuals, whatever the concentration used.

The percentage of background BPDE–DNA adducts ranged between 2.45 and 7.80% (data not shown). Although only statistically significant for one donor, a clear increase in the percentage of BPDE–DNA adducts was observed (Figure 5E) after exposure to 0.5 μM BPDE and showed a lower CV (21%) as compared to the comet assay results (Table I). A statistically significant increase in the percentage of BPDE–DNA adduct-positive cells was observed among all donors (except one) exposed to 2.5 μM BPDE ($P < 0.05$, Kruskal–Wallis) (Figure 5F) and showed a lower CV (25%) as compared to the CVs of the different comet parameters.

Table I. Intra-individual variation of the %DNA damage measured with the comet assay, the calculated repair capacity and the amount of BPDE-induced DNA adducts ($N = 4$)

	0.5 μM BPDE				2.5 μM BPDE			
	Median ^a	Q1–Q3	CV (%)	P -value ^b	Median ^a	Q1–Q3	CV (%)	P -value ^b
Donor A								
APC (%TD)	–0.123	–0.15 to –0.03	29	0.102	–0.076	–0.10 to 0.45	38	0.867
BPDE (%TD)	1.418	0.23–1.76	38	0.180	2.292	1.25–5.07	55	0.165
APC + BPDE (%TD)	3.695	2.38–4.58	24	0.156	5.581	4.74–12.21	50	0.156
RC	1.201	0.45–1.49	26	0.276	2.994	0.50–5.70	69	0.368
BPDE–DNA adducts ^c	5.758	5.10–7.44	9	0.565	18.196	16.87–26.15	18	0.368
Donor B								
APC (%TD)	0.001	–0.19 to 0.15	11	0.565	–0.143	–0.26 to 0.18	12	0.651
BPDE (%TD)	0.698	0.64–3.01	54	0.180	4.685	3.33–8.48	38	0.102
APC + BPDE (%TD)	4.013	4.00–5.63	15	0.180	8.018	7.07–9.02	9	0.867
RC	1.534	1.39–1.97	16	0.867	1.553	–0.50 to 2.11	91	0.102
BPDE–DNA adducts ^c	8.500	7.01–9.02	20	0.651	26.300	17.17–26.80	14	0.156
Donor C								
APC (%TD)	–0.169	–0.46 to 0.36	39	0.560	–0.118	–0.17 to 0.07	35	0.156
BPDE (%TD)	0.532	0.12–1.73	33	0.156	1.079	0.19–2.20	67	0.102
APC + BPDE (%TD)	4.089	3.97–5.35	11	0.741	3.966	2.99–5.60	29	0.156
RC	1.782	1.27–2.31	19	0.651	1.848	–0.22 to 3.09	64	0.180
BPDE–DNA adducts ^c	12.297	3.41–14.19	33	0.156	15.457	12.38–21.00	20	0.165
Donor D								
APC (%TD)	0.109	0.08–0.40	13	0.156	1.285	0.11–0.48	13	0.368
BPDE (%TD)	0.200	0.01–1.29	58	0.102	2.383	1.16–1.74	4	0.867
APC + BPDE (%TD)	2.354	1.13–2.50	18	1.000	4.398	2.68–6.03	30	0.368
RC	–1.115	–1.62 to 0.83	299	0.565	0.564	–1.03 to 2.66	126	0.276
BPDE–DNA adducts ^c	5.349	2.51–11.46	36	0.180	16.107	11.85–16.52	14	0.156

Two parallel cultures were processed per treatment and per experiment. Experiments were repeated three times with 1-week intervals. All given values are standardized with solvent. %TD, percentage of DNA damage measured in the tail of the comet; RC, NER capacity; Q1, 25 percentile; Q3, 75 percentile.

^aMedian values of three repeated experiments.

^bKruskal–Wallis tested on the three repeated experiments.

^cDNA adducts are measured as the percentage positive cells.

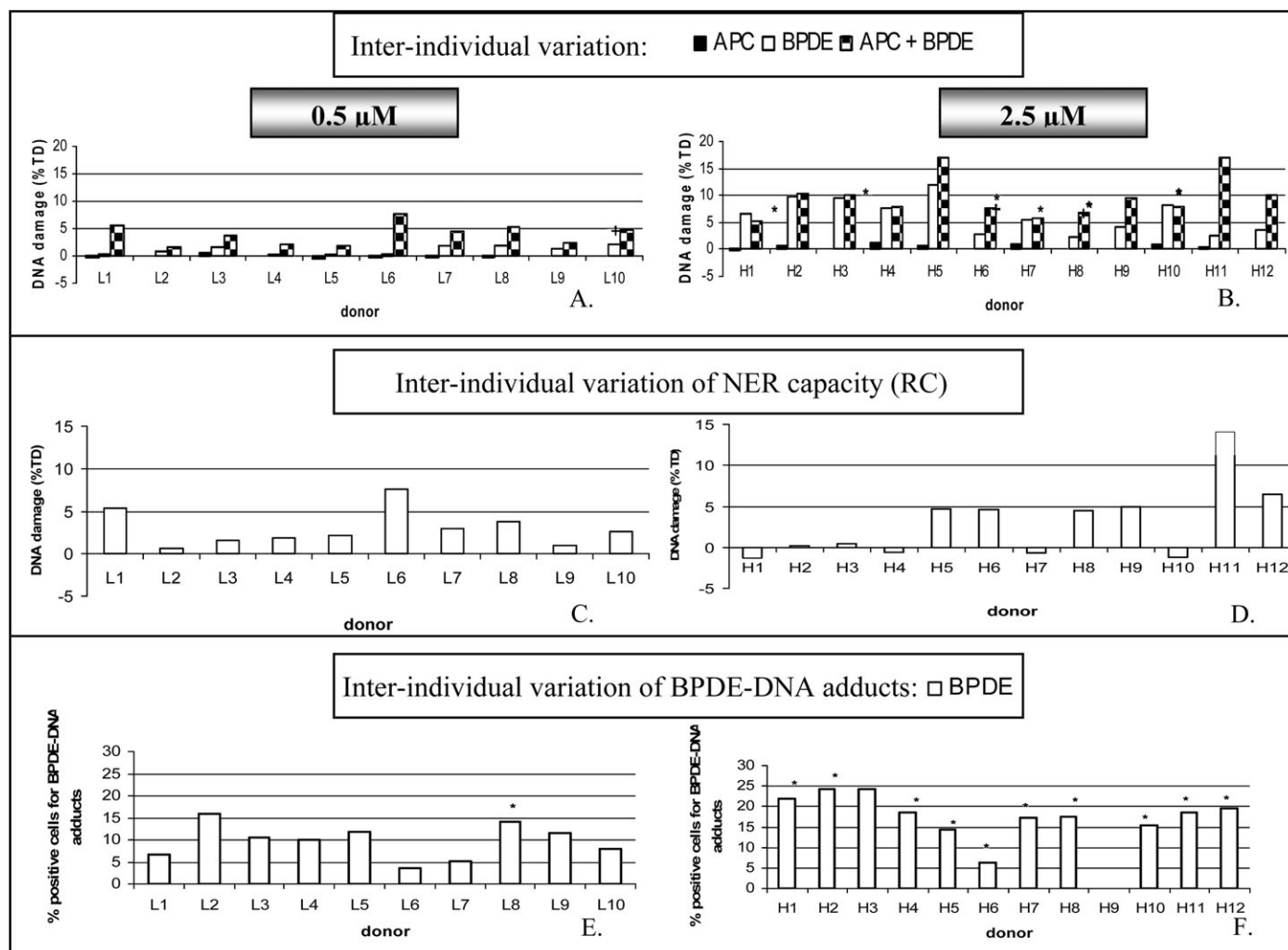


Fig. 5. Inter-individual variation after exposure to low BPDE concentration ($N = 10$) and exposure to high BPDE concentration ($N = 12$). Per donor, one experiment was performed with two parallel cultures for each treatment. (A) %DNA damage measured with the comet assay after exposure to low BPDE concentration. (B) %DNA damage measured with the comet assay after exposure to high concentration. (C) Repair capacity (calculated as the amount of DNA strand breaks damage induced by BPDE in presence of APC, diminished with the damage induced by BPDE and APC alone) for low BPDE concentration. (D) Repair capacity for high BPDE concentration. (E) Amount of BPDE-induced DNA adducts (low BPDE concentration). (F) Amount of BPDE-induced DNA adducts (high BPDE concentration) * $P < 0.05$ (Kruskal–Wallis) for BPDE compared to solvent (DMSO); + $P < 0.05$ (Kruskal–Wallis) for BPDE compared to APC + BPDE.

Comparison of low versus high BPDE concentration

For both concentrations tested, a statistically significant increase ($P < 0.05$, Mann–Whitney) of BPDE-induced DNA damage was observed after pre-incubation with APC compared to the BPDE-induced DNA damage and this increase was higher for the low concentration (Figure 6).

The percentage of BPDE-induced DNA damage and DNA damage induced by BPDE with APC pre-incubation was statistically significantly higher for the high BPDE concentration as compared to the low concentration ($P < 0.05$, Mann–Whitney) (Table I). The repair capacity for both concentrations is very similar (Table II). A statistically significant higher amount of BPDE–DNA adducts was detected ($P < 0.001$, Mann–Whitney) after exposure to the high BPDE concentration as compared to the solvent (Figure 7). Significantly higher amounts of BPDE–DNA adducts were formed at the high concentration compared to the low concentration (Table I), the latter showing no statistically significant increase as compared to the solvent (Figure 7).

Discussion

The objective of this study was the development of a cellular DNA repair phenotype assay for NER using the comet assay and comparing the repair capacity after exposure to a low (0.5 μM) and a relatively high (2.5 μM) BPDE concentration.

The comet assay is a sensitive, reliable and rapid method for DNA strand break detection at single cell level and is also being increasingly used in human biomonitoring (reviewed in ref. 27). In addition to the measurement of initially induced DNA damage, the comet assay can also be used to assess DNA strand break formation during excision repair, which also leads to an increase in DNA migration and makes the comet assay a useful tool to study repair capacity.

The relationship between DNA migration and excision break formation was first studied by Gedik *et al.* (28), who challenged HeLa cells with ultraviolet (UV)-C and analysed them over a period of time for DNA repair incision with the comet assay in presence of APC. Incomplete repair sites of

Table II. Inter-individual variation of %DNA damage measured with the comet assay, the calculated NER capacity and the amount of BPDE-induced DNA adducts

	0.5 μM BPDE ($N = 10$)				2.5 μM BPDE ($N = 12$)				P -value ^a (high versus low concentration)
	Median (abs value)	Q1–Q3	CV (%)	P -value ^b	Median (abs value)	Q1–Q3	CV (%)	P -value ^b	
APC (%TD)	−0.1 (0.8)	−0.2 to 0.1	37	0.036*	0.3 (1.1)	0.1–0.8	46	0.034*	0.140
BPDE (%TD)	1.1 (2.0)	0.3–1.7	39	0.179	6.1 (6.6)	3.0–9.3	48	0.299	<0.001**
APC + BPDE (%TD)	4.1 (5.0)	2.1–5.4	39	0.057	8.7 (10.4)	7.1–10.3	37	0.025*	<0.001**
RC (%TD)	2.4 (1.3)	1.5–4.2	72	0.081	2.5 (2.3)	−0.7 to 4.9	150	0.455	0.582
BPDE–DNA adducts ^c	10.2 (17.1)	6.4–12.5	21	0.120	18.4 (21.8)	15.4–21.9	25	0.317	<0.05*

Two parallel cultures were processed per treatment and per experiment. All given values are standardized with solvent. S.D., standard deviation; Q1, 25 percentile; Q3, 75 percentile; %TD, percentage of DNA damage measured in the tail of the comet; RC, NER capacity; N , total number of individuals; abs, absolute.

^aMann–Whitney U.

^bKruskal–Wallis.

^cBPDE–DNA adducts are measured as the percentage positive cells.

* $P < 0.05$, ** $P < 0.001$.

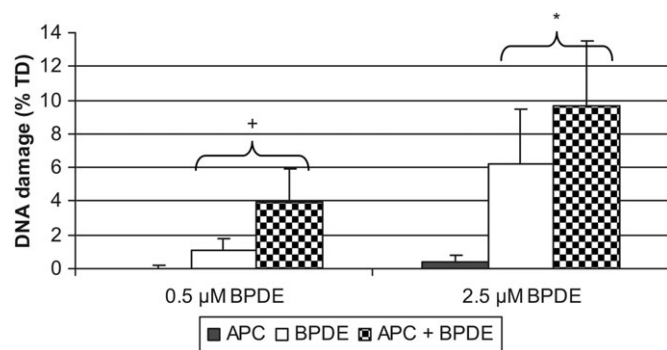


Fig. 6. Comparison of DNA damage induced by APC, BPDE and APC + BPDE for both low ($N = 10$) and high ($N = 12$) BPDE concentration. Per individual and per treatment, two parallel cultures were processed. * $P < 0.05$ and + $P < 0.001$ (Mann–Whitney U) for BPDE compared to APC + BPDE.

UV-C lesions accumulate in the presence of APC and lead to an additional increase of DNA migration, thus the DNA damage measured with the comet assay reflects NER-dependent incision at sites of UV-C lesions (28). This method has been applied in many studies (25,29–31) and the use of repair inhibitors has been proposed to increase the sensitivity of the comet assay for human biomonitoring studies (25,32). Crebelli *et al.* (32) used cytosine arabinoside as a repair inhibitor to study the genotoxic effects of occupational exposure to polycyclic aromatic hydrocarbons (PAHs) and other agents among primary aluminium industry workers.

In the present study, we used this approach to develop a NER cellular phenotype assay to investigate repair capacity by measuring the influence of APC on BPDE-induced DNA damage. We defined the individual repair capacity as the amount of DNA strand breaks damage induced by BPDE in presence of APC, diminished with the damage induced by BPDE and damage induced by APC alone, which corresponds to the incision capacity of the NER enzymes. There are some advantages to this newly developed assay. First, this new approach assesses repair capacity on the basis of the enzymatic mechanisms responsible for DNA repair and replication. Second, experimental biases were minimized since time between blood sampling and processing was kept to a minimum. Moreover, the repair phenotype was evaluated on fresh cells without any freezing step. Lymphocytes are also easy to isolate and the fact that they are circulating cells means that their metabolic state (including the state of their DNA)

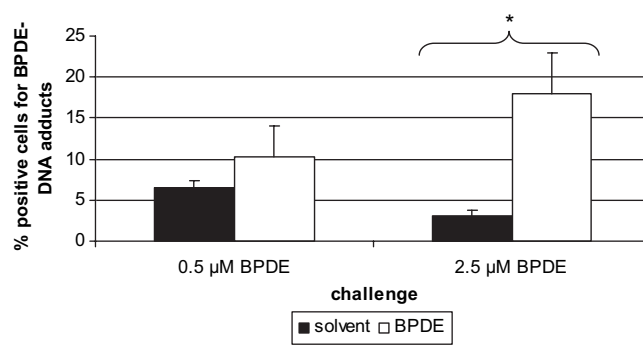


Fig. 7. Comparison of BPDE-induced DNA adducts in donors challenged to low ($N = 10$) and high ($N = 12$) BPDE concentration. Per individual and per treatment, two parallel cultures were processed. * $P < 0.001$ (Mann–Whitney U) for BPDE compared to solvent (DMSO).

reasonably reflects the overall body environment to which they are exposed. To exclude inter-electrophoresis differences, an erythroleukemia K562 cell line was used as internal standard (24). Using this standard, all different electrophoresis sessions could be compared. Inter-experimental variation was minimized because experiments were processed and measured by one researcher. The choice of the BPDE concentrations was based on a dose-range experiment showing a positive increase in BPDE-induced DNA damage. To verify whether a saturation effect could be detected and influence the results, we decided to continue further the experiments with both a high (2.5 μM) and a low (0.5 μM) BPDE concentration.

The use of DNA repair inhibitors such as APC increases the sensitivity of the assay. However, this kind of compounds can also interfere with DNA replication and may disturb the cell cycle of proliferating cells. To determine whether APC affected cell proliferation in our test system, we performed the *in vitro* CBMN assay on PBMCs. This method allowed us to investigate both fixed DNA damage and disturbance of cell proliferation by calculating the CBPI (26). The results indicated no modification of cell proliferation or MN induction. Therefore, it can be considered that APC did not induce any secondary effects in the comet assay. Speit *et al.* (33) also investigated the effect of APC in order to better understand the effect of inhibited replication and cell cycle disturbance. Using a similar APC concentration used in the present study, an increase of the chromosomal aberration frequency was observed, whereas no significant effects in the *in vitro* comet

assay were detected (33). These results indicate minor disturbances in DNA replication. Similar to our results with the comet assay, highly variable effects between independently repeated tests were detected. This observation can be explained by the indirect nature of the genotoxic effect of APC.

To validate this new methodology, the assay was applied to a NER-deficient human fibroblast cell line (XPA^{-/-}) in comparison to wild-type human fibroblasts. During NER, two mechanisms can be described, DNA excision and gap filling–ligation. Both mechanisms will have opposite effects on the formation of DNA breaks. The XPA protein is involved in the cooperative binding of RPA, XPA and XPC-THFII and plays a central role in DNA damage recognition and the positioning of the repair machinery around the lesions (34–37). Exposure to APC inhibits the gap filling–ligation phase. As expected, XPA-deficient cells showed a lower DNA repair capacity and higher amount of BPDE-induced DNA adducts as compared to the wild-type fibroblasts. These findings are in agreement with those of Langie *et al.* (17).

When developing a new phenotypic assay to assess NER capacity, it is important to take into account intra- and inter-individual variations. A reliable methodology should be able to detect differences between individuals, resulting in significant inter-individual variation, but should show a minimal intra-individual, intra-experimental and inter-assay variation.

In the literature, intra- and inter-individual variation in comet assay has been addressed using the CV. Several studies observed relatively high intra-individual variation (18,19,38–40) for comet assay results. As far as our study is concerned, we evaluated the inter- and intra-individual variation both by statistical analysis (Kruskal–Wallis) and by calculating the CV, the latter in order to be able to compare our results with data from the literature.

Statistical analysis of the data for BPDE- and APC + BPDE-induced DNA damage measured with the comet assay revealed a low intra-individual, inter-assay and intra-experimental variation, indicating the reliability of our methodology. In addition, our CV values were comparable with those reported by others (18,19,38–40). Although no statistically significant differences were observed between experiments from the same donor, i.e. intra-individual, inter-assay and intra-experimental variation, relatively high CV values were sometimes observed. This may reflect the high sensitivity of the assay variation in the experimental conditions (conditions of the different electrophoresis runs and hydration of agarose layers) or physiological variations of the donors between different blood samplings (e.g. diet or infections). Nevertheless, we observed a low intra-individual variation for repair capacity, especially for the low BPDE concentration.

Looking at the inter-individual variation, a statistically significant difference in BPDE-induced DNA damage and DNA damage induced by APC + BPDE was observed between high and low concentrations. A higher CV after challenging with the high BPDE concentration was detected as compared to the low concentration, indicating that the low BPDE concentration is more suitable to assess NER capacity.

Besides genotype, a possible confounding factor in the assessment of inter-individual differences in *in vitro* repair capacity performed on lymphocytes is the effect of PHA, which is known to induce mainly the proliferation of T lymphocytes in PBMC cultures. DNA repair is tightly regulated within the cell cycle and thus the number of proliferating cells, and the subpopulation of lymphocytes

involved, could affect the repair capacity as measured with this procedure. Although few studies have been published on this question, Mayer *et al.* (41) could not detect any difference in repair capacity of γ -radiation-induced DNA damage between non- and PHA-stimulated PBMCs measured with the comet assay (41). Using CA and MN as end points, the same conclusion was drawn by Durinec *et al.* (42). By comparing the CBPI and the repair capacity, we found no statistically significant correlation between both in PHA-stimulated PBMCs from adult women (data not shown). However, because no discrimination between T-lymphocyte subpopulations was made and because of the small population size of the study population, this point deserves further attention.

Different points can be addressed that support the recommendation of using the low BPDE concentration for further implementation of the APC NER assay. First, the repair capacity of XPA-deficient cells was only significantly lower than that of normal human fibroblasts with the low concentration. Second, although the repeated experiments in four donors showed no difference in intra-individual, inter-assay and intra-experimental variation for both concentrations, lower CV values for repair capacity were observed with 0.5 μ M than with 2.5 μ M BPDE. Third, in the extended population ($N = 22$), similar results for the repair capacity were obtained for both concentrations. Although no statistically significant values could be found for inter-individual variation, differences in repair capacity between individuals were more pronounced after exposure to the low BPDE concentration. The CV values calculated for the repair capacity showed higher levels with inter-individual experiments than intra-individual experiments and with high BPDE concentration than low BPDE concentration. Moreover, in contrast with the high BPDE concentration, no saturation effect of BPDE was observed with the low concentration. All this indicates the adequacy of the method and supports the recommendation of using the low BPDE concentration for further implementation of the APC NER assay.

In conclusion, the APC-block repair assay is a promising methodology to assess NER phenotype since it allows assessment of DNA repair incisions and therefore provides a molecular-based interpretation. Further improvement of the method by applying use of the low BPDE concentration to a larger population and taking into account the relevant genotypes for NER should be recommended and could allow predictions on individual level for genetic disturbances or cancer.

Supplementary material

Supplementary data are available at *Mutagenesis* Online.

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