Exfoliated Buccal Mucosa Cells as a Source of DNA to Study Oxidative Stress

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Abstract

The extent of oxidative DNA damage is considered a biomarker of carcinogenic process and could be investigated in population studies using easily obtained cells. The oxidized DNA base adduct 8-hydroxy-2-deoxyguanosine (8-OHdG) released by enzymatic hydrolysis of DNA is commonly assayed by high performance liquid chromatography with electrochemical detection. It is expressed as a ratio of 8-OHdG to unoxidized deoxyguanosine. We modified and improved this method, determined the optimal time for harvesting buccal mucosa cells (BMC), assessed whether they mirror peripheral circulating blood cell DNA damage, and compared the anticoagulants, heparin, and EDTA for consistency in measurement of leukocyte 8-OHdG. Thirty-one healthy participants, randomized into two groups, donated BMC and blood samples. Samples were collected at baseline and either 3 or 7 days after baseline. Results showed no correlation between 8-OHdG/deoxyguanosine ratios in BMC and peripheral blood leukocytes at any time point regardless of harvest time. BMC had much higher oxidative DNA damage, but displayed a 25.6% reduction in the oxidized DNA adduct level (P < 0.04) at 3 days after baseline. Leukocytes collected in heparin and EDTA had similar 8OHdG/deoxyguanosine ratios; however, EDTA was preferred, as it produced a clean nuclear pellet without hemoglobin contamination, and the results were less variable. This improved assay shows within subject stability over time in both leukocyte and BMC DNA damage, increasing the probability that small intervention differences can be detected in healthy subjects. Buccal cells provide an accessible pool of epithelial cells that represents higher levels of DNA damage than circulating leukocytes. (Cancer Epidemiol Biomarkers Prev 2008;17(1):212–9)

Introduction

The association between accumulated oxidative DNA damage and aging is well documented in literature. Oxidative stress has been shown to produce DNA damage (1-3), which suggests a strong association between oxidative stress and etiology of cancer, cardiovascular disease, cataracts, age-related maculopathy, and aging in general. Aged tissues are more susceptible to injury by free radicals and thereby more prone to oxidative damage. Reactive oxygen species can initiate cancer progression by causing structural alterations in DNA, such as base pair mutations, rearrangements, deletions, insertions, and sequence amplification (4). 8-Hydroxy-2-deoxyguanosine (8-OHdG) is the most common adduct formed in the reaction of free radicals with DNA. It is a sensitive marker of DNA damage in cells and tissues and more widely used than other methods, such as the Comet assay (5). 8-OHdG can be further oxidized to 2-deoxy-β-D-erythro-pentofuranosyl derivatives of oxaluric acid, oxazalone, and cyanuric acid that have been shown to be an order of magnitude more mutagenic (6). Because of their smaller molecular mass, they are unlikely to coelute with 8-OHdG with methods using high performance liquid chromatography (HPLC) separation. Numerous human studies have reported positive associations between environmental pollutants and increased levels of 8-OHdG in different tissues (7-10). Nutritional studies, however, report mixed results. For example, supplementing vitamin E, ascorbic acid, and coenzyme Q had little effect on levels of urinary 8-OHdG (11, 12), whereas increased consumption of green tea, Brussels sprouts, tomato sauce, red wine, and soy hypocotyl tea reduced levels of 8-OHdG in both urine and leukocyte DNA (13-17). These discrepancies are certainly due to differences in the methods and analytic conditions used by different laboratories. It has been reported that the important step of derivatization, before gas chromatography–mass spectrometry analysis, induces artifactual increase in the levels of 8-hydroxyguanine in DNA (18). Similarly, use of phenol and chloroform for DNA isolation is known to enhance the level of 8-OHdG (19), although it is recently claimed that use of phenol does not increase DNA base damage and variability in its reports by various laboratories are related to the quality of phenol used, the temperature of its storage during experiments, and any contamination with transition metal ions (20). Extensive reviews summarizing comparison and standardization of different approaches are discussed elsewhere (20-22).
The use of buccal mucosa cells (BMC) as study material offers a number of advantages, which include the following: (a) chemotherapeutic agents and radiation therapy limit the proliferative ability of the epithelium, producing thinning and ulceration manifested predominantly in gastrointestinal and oral mucosa (23); (b) the BMC lining serves as a mirror for health status, reflecting changes in the systemic conditions, such as oxidative stress (24), nutrient deficiency (25-27), or human exposure to genotoxins (28-31); (c) 90% of all cancers arise from epithelial tissue (32) and the use of oral exfoliated cells is therefore considered a powerful diagnostic tool for monitoring genetic damage in human populations (33); (d) BMCs are directly exposed to xenobiotics in food and the environment, making them the most interesting cell type to explore different aspects of oxidative stress and carcinogenesis. The ease of harvest and biochemical analysis (34) of good quantity and quality DNA (35) makes them an excellent indicator cell for the extent of DNA damage that the epithelium may be experiencing.

Analyzing 8-OHdG by HPLC with electrochemical detection is a highly sensitive method (36). Measurement is usually expressed as proportion of 8-OHdG to deoxyguanosine, which attenuates incomplete DNA extraction or hydrolysis. In current study, the method developed by Chen et al. (37) was modified and optimized, including the consideration of optimal anticoagulant for leukocyte harvesting.

Heparinized blood is often used to obtain leukocytes for 8-OHdG analyses. Recently, EDTA was reported to be a better anticoagulant than heparin or citrate for delayed plasma processing to study cell-free circulating DNA (38). In our laboratory, we observed that leukocyte DNA isolated from blood with heparin as anticoagulant is frequently contaminated with hemoglobin, whereas DNA isolated from EDTA-treated blood was free from hemoglobin contamination. Preliminary analysis showed a higher ratio of 8-OHdG to deoxyguanosine in DNA from heparinized blood compared with blood treated with EDTA. Based on these observations, the present study was undertaken to examine (a) the effect of cell aging on DNA damage in BMCs, (b) feasibility of using buccal cells in place of leukocytes to determine 8-OHdG as a possible surrogate end-point biomarker for cancer risk in population studies, and (c) the effect of heparin and EDTA on 8-OHdG levels in leukocytes.

Materials and Methods

Selection of Subjects. Thirty-two volunteers, both male and female at ages 18 to 65 years, were recruited from among the staff and students of the Human Nutrition Department at University of Illinois at Chicago. Inclusion criteria were nonsmoking, no heavy alcohol consumption, and absence of chronic or acute diseases, as assessed by self-reports. The Institutional Review Board of the Office of Protection from Research Risks at University of Illinois at Chicago approved the study. Volunteers were recruited by campus advertisement, and informed consent was obtained before study participation. The sample collection procedure was done in the morning to avoid any diurnal variation in the assessed variables.

Study Design. The subjects were randomly divided into two groups: the 3-day group and 7-day group. Each group donated blood and buccal cell samples on the first day of participation (day 0), and they returned for the second sample collection after 3 or 7 days, based upon their group assignment. The sample collection procedure was done in the morning to avoid any diurnal variation in the assessed variables.

Analytic Methods

Chemicals. All chemicals were of highest quality and obtained from Sigma Chemical Co., except if otherwise stated.

Water. The distilled water was further polished by filtering through a C-18 SEP-PAK cartridge (Waters) under hydraulic vacuum pressure. Polishing removes any ionic compounds from the distilled water that may interfere in the electrochemical detection.

Leukocyte Samples: Extraction of Nuclei. In the non-fasting state, 6 mL of venous blood was collected into both heparin and EDTA-coated tubes by a trained phlebotomist. The tubes were gently tilted up and down to mix the blood with the anticoagulants uniformly. The samples were stored on ice until they were processed. Blood samples were centrifuged at 4°C for 15 min at 800 × g. Plasma was carefully separated, and the packed red cells with the buffy coat interface containing the WBC layer was transferred completely into a 50-mL Falcon tube for lysis. For most human studies, plasma is collected for other assays and theuffy coat and red cells remain and are often discarded. Using the whole centrifugate for nucleated cell collection, we minimize any artifactual DNA damage because separation of different cell types may enhance damage to DNA during processing as reported by Rehman et al. (20) and processing and aliquoting of various assays is simplified. Lysis buffer (0.32 mol/L sucrose, 0.05 mol/L mannitol, 0.005 mol/L magnesium chloride, 0.16 mmol/L Triton X-100, and 1 mol/L Tris at pH 7.4) was used to wash the vccutatons clean, and the contents of Falcon tubes were made up with the lysis buffer to 30 mL for extraction of nuclei. Falcon tube contents were vortexed at high speed for 60 s and then centrifuged for 15 min at 800 × g at 4°C. The supernatant was discarded, and the pellet was washed again with 25 mL of lysis buffer. The process was repeated for a third time with 10 mL of lysis buffer or until the nuclei pellet appeared clear of hemoglobin. Heparinized blood samples often required overnight freezing and thawing between washings to eliminate hemoglobin contamination. Leukocytes, isolated from the buffy coat, represent a variety of cell types with highly variable biological half-lives and functions and likely to reflect a range of 8-OHdG/deoxyguanosine ratios. The extracted nuclei were stored at −80°C until DNA isolation.

Buccal Cell Collection Technique. The participants rinsed their mouth thoroughly with distilled water before collection of the buccal cells, and this preliminary rinse was discarded. A soft bristle toothbrush was then used to collect the cells from inside the cheeks. A gentle scraping of the inside cheek was done by moving the brush up and down ~20 times on each side of the cheeks. The mouth was then rinsed twice with 20 mL of distilled water, and the rinse was collected into a 50-mL Falcon tube. The brush was agitated in this rinse to loosen the cells that had adhered to the bristles. This buccal cell suspension was centrifuged at 800 × g at 4°C for 10 min. The supernatant was decanted, and lysing solution was added to lyse the cells.
Buccal Cell Lysis. The cell pellet was resuspended in 15 mL lysing solution and centrifuged for 15 min at 800 \( \times g \) at 4°C. The supernatant was discarded, and the pellet was washed twice with 10 mL of lysis buffer. The remaining pellet was covered with a small amount of lysis buffer and stored at -80°C until it was analyzed.

Isolation and Hydrolysis of Leukocyte and Buccal Cell DNA. Frozen cell nuclei were defrosted gradually on ice, and the lysis buffer was discarded. Then 900 \( \mu L \) of 1% w/v sodium dodecyl sulfate-EDEA, 90 \( \mu L \) of 5 mol/L NaCl, 200 \( \mu L \) RNase A, and 10 \( \mu L \) RNase T1 (7,500 units/mL) were added. The samples were vortexed well and incubated at 37°C for 30 min to hydrolyze any RNA present in the nuclei. Next, protease K (12 units/mg; 40 mg/mL) was added, followed by incubation at 60°C for 30 min to hydrolyze protein contaminants. The sample was then transferred to a round-bottomed tube, and 180 \( \mu L \) of 5 mol/L NaCl and 10 \( \mu L \) of 1 mol/L Tris (pH 8.0) were added. The sample was then mixed by repeated inversion. n-Butanol (1.5 mL) was added, and the samples were mixed for 2 min. The tubes were centrifuged at 2,000 \( \times g \) for 15 min at 4°C to separate the top butanol layer that was discarded. Ice-cold absolute ethanol (100%; 3 mL) was added to the remaining bottom layer and vortex-mixed until DNA precipitation. The tubes were centrifuged at 14,000 \( \times g \) for 15 min at 4°C to pellet the DNA. After discarding the supernatant, the DNA pellet was washed with 70% ethanol and the tubes were centrifuged again at 14,000 \( \times g \) for 15 min. The supernatant was removed, and the tubes were drained by invasion on absorbent paper and tapped to remove small droplets, taking care that the pellet was not dislodged. C-18 purified water and tapped to remove small droplets, taking care that the pellet was not dislodged. C-18 purified water (100 \( \mu L \)) and 50 mmol/L ammonium acetate (100 \( \mu L \)), containing 0.2 mmol/L ZnCl\(_2\) at pH 5.2, were added to the DNA pellet with adequate mixing to dislodge the pellet from the wall of the tube. Buccal cell DNA was mixed with 50 \( \mu L \) C-18 purified water and 50 \( \mu L \) of 50 mmol/L ammonium acetate buffer. The original method used 10 \( \mu L \) of deferoxamine mesylate (48 \( \mu mol/L \)) with ammonium acetate buffer to bind free iron present in the sample, which would prevent artificial oxidation. However, we observed that deferoxamine produced a peak on the HPLC with electrochemical detection, which interfered with the accurate identification of the 8-OHdG peak, and it also caused distortion of the baseline. Similar results were reported by Huang et al (39) and Helbock et al. (40). The omission of deferoxamine did not produce increased 8-OHdG values in equivalent samples, so it was eliminated from the assay. The samples were incubated at 37°C for 1 h with gentle shaking every 15 min to dissolve DNA completely. For quantification and purity assessment, the absorbance of each DNA sample [5 \( \mu L \) sample in 450 \( \mu L \) of bis-Tris, i.e., 2-[bis[2-hydroxyethyl]-iminol]-2-hydroxy-methyl]-1,3-propane-diol; Kodak; 5 mmol/L; pH 5.2 with 1 mmol/L EDTA] was assessed in a spectrophotometer at 260 and 280 nm. Purity of DNA is considered high when the ratio of A\(_{260}/A_{280}\) is between 1.7 and 2.0. The ratios for all samples were between 1.7 and 1.9.

Ten microliters of nuclease P1 [prepared by resuspending the contents of one vial of nuclease P1 in 1 mL of 20 mmol/L sodium acetate (pH 5.2) buffer] were added to 100 \( \mu L \) of DNA (0.5-1 \( \mu g/\mu L \)). The final concentration of nuclease P1 in the sample was 280 units/mL or 0.1 mg/mL. The samples were incubated at 37°C for 60 min to hydrolyze DNA. Next, 5 \( \mu L \) of alkaline phosphatase (Boehringer Mannheim; 1 units/\( \mu L \)) were added to each sample. Tris hydroxymethyl aminoethane was evaluated at several concentrations, and 15 \( \mu L \) of 0.15 mol/L Tris hydroxymethyl aminoethane at pH 10.4 was found to be adequate to maintain the sample pH at 8.5. This pH adjustment was found to be critical for complete hydrolysis of the DNA sample. The sample was incubated at 37°C for 30 min to remove the phosphate groups from the nucleotides. Incomplete hydrolysis of DNA is usually responsible for underestimated values for 8-OHdG. The hydrolysate was filtered through a 10K microcentrifuge filter (Microcone, Millipore) at 4°C for 30 min. The type of filter and the prolonged centrifugation are required to produce a clear chromatogram with a smooth baseline. The sample was then transferred to auto injector vials with low volume inserts for HPLC with electrochemical analysis. The quantity of free deoxyguanosine base liberated from known concentrations of salmon sperm DNA was used as a control in each assay to determine the completeness of hydrolysis. The average efficiency of DNA hydrolysis was calculated to be 75% to 90%. This is an improvement over our original method (37) using DNase and two phosphodiesterases, which reported only 62% hydrolysis. The enzymatic hydrolysis method used in the original method was reported to be lengthy and inefficient (41) because a long incubation period at high temperature enhances increased DNA damage (20).

HPLC with Electrochemical Detection Analysis of 8-OHdG. The hydrolyzed samples were subjected to HPLC separation on the day of hydrolysis. However, there was no increase in the values of 8-OHdG in equivalent samples after they were stored at -80°C for 2 months. The HPLC with electrochemical detection system consisted of an ESA refrigerated autoinjector model 542, ESA model 580 solvent delivery module with ESA model 5020 guard cell, ESA Coulomex II detector equipped with an ESA model 5011 analytic cell, and a model 490 Waters UV detector. The column used was a SUPELCO (LC-18, 15 cm x 4.6 mm; Supelco Co.), operating at a flow rate of 1.0 mL/min. The column temperature was maintained at 20°C by using a column cooler jacket with temperature controller (CERA Inc., Series-250). Stock solutions of

### Table 1. Characteristics of the subjects (\( n = 31 \))

<table>
<thead>
<tr>
<th></th>
<th>3-d group</th>
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<td>No. subjects</td>
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<td>15</td>
</tr>
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<td>Age (range)</td>
<td>40 (22-62)</td>
<td>35 (21-62)</td>
</tr>
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<tr>
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<tr>
<td></td>
<td>Ever smoked (%)</td>
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<tr>
<td>Alcohol intake</td>
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<td>1-2 servings/wk (%)</td>
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<td></td>
<td>&gt;3 servings/wk (%)</td>
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</tr>
<tr>
<td>Race/ethnicity</td>
<td>Euro-American (%)</td>
<td>56</td>
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<tr>
<td></td>
<td>Afro-American (%)</td>
<td>6</td>
</tr>
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<td>Asian (%)</td>
<td>19</td>
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<td></td>
<td>Others (%)</td>
<td>19</td>
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*Smokeless for at least 6 mo was one of the entry criteria.
2'-deoxyguanosine and 8-OHdG were prepared and stored at -20°C. Fresh working standards were made from the stock solutions on the day of the assay. The eluting solvent was 5% methanol (EM Science) and 95% of 100 mmol/L sodium acetate buffer (pH 5.2), adjusted with glacial acetic acid from Baker Chemical Company. The buffer was prepared with C-18 purified water, filtered, and degassed through 0.22-μm Millipore filters under hydraulic vacuum. The mobile phase is preferably made at least 1 day before and stored at 4°C. It is important that the mobile phase should come to room temperature (24°C or 75°F) before use. The room temperature was maintained by using an air conditioner. At higher room temperatures, the separation of the peaks became suboptimal as the run time becomes shorter due to warming of the mobile phase. If the temperature of the mobile phase is maintained at 20°C, which is the column temperature, the run time becomes too long (~45 min per run). Running the sample at column temperatures of 20°C with the mobile phase at room temperature (24°C) produced optimal separation of peaks with a run time of 32 min per sample. For the HPLC with electrochemical detection, the peak potential for 8-OHdG was set at +350 mV after running a voltogram, 150 mV for cell 1 (conditioning cell) and 400 mV for cell 2 (analytic cell). The approximate limit of detection was 0.1 pg. Unoxidized 2-deoxyguanosine was analyzed simultaneously by UV detector at 260 nm. The peak heights were measured for both 8-OHdG and 2-deoxyguanosine peaks. The concentrations of 8-OHdG was calculated and expressed as a ratio of oxidized species to 10^6 unoxidized 2-deoxyguanosine. The coefficient of variation for intra-assay and interassay were 4% (n = 26) and 7.7% (n = 26), respectively, based on pooled heparinized human leukocyte DNA samples.

**Statistical Analyses.** Values were tested for normal distribution, and Student's t tests were used on log-transformed values to compare the baseline and the endpoint values of 8-OHdG/2-deoxyguanosine ratios in each time period and to find differences in leukocyte DNA 8-OHdG/2-deoxyguanosine ratios between different groups. Correlations coefficients were calculated using simple Pearson product-moment equations. The P value of <0.05 was considered statistically significant.

Results

The purity of the chemicals used in the assay was critical, so it is recommended that chemicals be obtained only from the companies mentioned in Materials and Methods. The most crucial improvement to the method was the purification of the HPLC grade distilled water using a simple one-step polishing method that resulted in a smoother baseline of the chromatograms obtained. Another significant improvement of the method was the adjustment of the pH in the hydrolyzing buffer using 0.15 mol/L Tris hydroxymethyl aminoethane, which optimized the reaction of the alkaline phosphatase enzyme. One of the major limitations of the traditional method was the use of deferoxamine as a redox-inactive metal chelator to limit oxidation of DNA ex vivo. Deferoxamine interferes with the electrochemical cells and produces peaks, which are close to the peak of 8-OHdG in the chromatogram. We have found that there was no additional increase in the levels of 8-OHdG in the samples when this compound was not used. It was therefore omitted from this method.

Of the 32 subjects recruited, 31 completed the study (Table 1). The subjects were mainly young healthy adults from diverse ethnic origins. The two groups were roughly equivalent in characteristics, except that the 7-day group was composed of more Asians, had a higher proportion of never smokers, and consumed less alcohol per week. However, there was no significant difference among the groups based on age, sex, smoking and alcoholic status, and ethnicity.

Figure 1 presents the DNA content of leukocytes collected with two different anticoagulants and buccal cells collected at three different time points. Bars, SE.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Amount of DNA obtained from BMC and from leukocytes collected in heparin and EDTA. Statistical significance was determined between the groups versus baseline values. Bars, SE.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** DNA damage in leukocytes collected with two different anticoagulants and buccal cell samples collected at three different time points. Bars, SE.

**Figure 2.** DNA damage in leukocytes collected with two different anticoagulants and buccal cell samples collected at three different time points. Bars, SE.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** DNA damage in leukocytes collected with two different anticoagulants and buccal cell samples collected at three different time points. Bars, SE.

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ratios between the samples of leukocyte DNA collected with EDTA versus heparin. Similarly, the DNA damage in BMC collected 7 days after baseline was not significantly different from the baseline. In contrast, cells harvested 3 days after baseline collection had 25.6% lower 8-OHdG/deoxyguanosine ratios ($P < 0.04$). This may indicate that younger buccal cells, represented by the measurement at day 3, have less oxidative DNA damage. The overall 8-OHdG/deoxyguanosine ratios were roughly 3-fold to 4-fold higher in BMCs compared with leukocytes.

To find whether the leukocyte 8-OHdG/deoxyguanosine ratio is a stable characteristic for an individual subject over time, correlation coefficient analysis was done along with the line-fit plot. Correlations between leukocyte DNA damage at baseline and those collected at 3 and 7 days after baseline are presented in Fig. 3. Leukocyte 8-OHdG/deoxyguanosine ratios from both heparin and EDTA groups were significantly correlated with baseline ratio, indicating fair within-person stability of the measurement, at least over these short time periods. There was less scatter and stronger linear correlations in the samples collected in EDTA for both time periods. Therefore, EDTA is the preferred anticoagulant for leukocyte 8-OHdG measurement.

Figure 4 presents the relationship of 8-OHdG/deoxyguanosine ratios in BMC harvested at baseline versus those collected at 3 and 7 days later. The samples collected after 3 days are more scattered, whereas samples collected after 7 days are better correlated ($P < 0.00001$) with baseline values. These comparisons add further support to the idea that cells collected on day 3 after baseline are younger than those collected on day 7, which are more similar to the old cells collected at baseline. The younger cells are more likely to reflect oxidative status, whether it is from oxidative assault or antioxidant protection.

There was no correlation between BMC and leukocyte DNA damage for individuals at baseline and 7 days. However, the 3-day samples of BMC showed a marginal relationship with the leukocyte samples (with heparin, $r = 0.38 \ P < 0.17$; with EDTA, $r = 0.45 \ P < 0.11$).

Discussion

The described method of 8-OHdG assessments as a biomarker of DNA damage represents a simpler and more robust assay. Recently, an improved method for the analysis of 8-OHdG in DNA by using the purified base-excision repair enzyme formamidopyrimidine glycosylase

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Between-day correlations in leukocyte 8-OHdG/2-deoxyguanosine (2-dG) $\times 10^6$ collected in heparin or EDTA from same individuals.
The leukocyte samples (6.94 study showed low ratios of 8-OHdG/deoxyguanosine in there is still a need for an easier method of analyzing F to 9.22 anticoagulants for quantitative leukocyte DNA analysis therefore acceptable to use either EDTA or heparin as the was similar to the damage in heparin-collected cells. It is showed that DNA damage in EDTA-collected leukocytes according to DNA and therefore prevents artificial oxidation difference between the two. Our assumption was based heparin-treated blood, but we found no significant ratios in EDTA-treated blood would be lower than in decreasing artifactual 8-OHdG. The strong correlation between desirable because they indicate a lower generation of normal individuals in Germany (43). Lower ratios are reported (42). However, purification of formamidopyrimidine glycosylase and its reaction with isolated DNA requires many elaborate steps, which makes it difficult for routine analysis of DNA damage in vivo. Therefore, there is still a need for an easier method of analyzing 8-OHdG in a large number of biological samples. Our study showed low ratios of 8-OHdG/deoxyguanosine in the leukocyte samples (6.94 ± 0.85 / 10^6 deoxyguanosine to 9.22 ± 1.21 / 10^6 deoxyguanosine) compared with higher ratios reported (14.5 ± 3.6 / 10^6 deoxyguanosine to 17.5 ± 4.0 / 10^6 deoxyguanosine) in a similar group of normal individuals in Germany (43). Lower ratios are desirable because they indicate a lower generation of artifactual 8-OHdG. The strong correlation between collection times reflects both the reproducibility of the method and also the stability of leukocyte 8-OHdG/deoxyguanosine ratios as a steady-state biomarker of DNA damage.

We hypothesized that 8-OHdG/deoxyguanosine ratios in EDTA-treated blood would be lower than in heparin-treated blood, but we found no significant difference between the two. Our assumption was based on the fact that EDTA can prevent free iron from attaching to DNA and therefore prevents artificial oxidation of extracted DNA. Laulhere and Briat (44) also reported that EDTA strongly inhibits iron release (induced by ascorbate) and uptake by ferritin. However, our results showed that DNA damage in EDTA-collected leukocytes was similar to the damage in heparin-collected cells. It is therefore acceptable to use either EDTA or heparin as the anticoagulants for quantitative leukocyte DNA analysis and oxidative DNA damage studies. However, EDTA blood is preferred for ease of handling, especially in obtaining a clean nuclear pellet without hemoglobin contamination. Yokota and his group (45) obtained similar results while extracting DNA from surplus blood samples remaining after routine clinical tests.

Our results also showed that DNA damage in BMC was not correlated with DNA damage in leukocytes. This is supported by earlier reports showing no correlation between oxidative stress biomarkers, such as TBARS in BMC and plasma (46). However, there is evidence showing significant correlation between DNA adduct levels in BMC and oral biopsies (47) and total DNA methylation (a biomarker of neoplasia) in BMC and malignant lung tissue (48). Our results, therefore, suggest that BMC might be a very good noninvasive target to assess human exposures to environmental genotoxins or oxidative stress and may correlate better with different target tissues than peripheral blood cells. The lack of association of BMC with plasma or leukocytes is vital to consider while determining the variables to measure in BMC. The simple technique of BMC collection provides a mixed sample that consists of epithelial cells from oral cheek mucosa, buccal leukocytes that migrate from blood through the gingival crevice into the oral cavity, erythrocytes, and fibroblasts (49-52). The relative proportions of different cells in the mixed cell suspension depend primarily on the aging of the buccal cells, in addition to the isolation procedure and physiologic state of the donor. In contrast to areas of the oral cavity, such as the hard palate, which is subject to mechanical forces associated with mastication and covered by keratinized epithelium, the lining of the mouth floor, cheek region, and esophagus, which require flexibility to accommodate chewing, speech, or swallowing of a bolus, are covered with a nonkeratinizing epithelium supported by elastic and flexible connective tissue (23). Moreover, unlike the simple lining of the stomach and small and large intestines, all of which consist of a single layer of cells, oral mucosa is covered by a stratified epithelium composed of multiple layers of cells that show different stages of differentiation (maturation) between the deepest cell layer and the most superficial layer. To maintain epithelial homeostasis, cell production in the deeper oral mucosa layers is balanced by loss of cells in the surface layer. The complete turnover rate of these epithelial cells in the buccal region is quite rapid, ranging from 5 to 6 days (53), but the mature and nonviable cells remain on the surface. Simple brushing at the 7-day collection likely removes a larger proportion of these more oxidized cells compared with the 3-day collection, wherein the number of newly generated and less oxidized cells would be proportionately higher. These facts may explain our results, showing similarity in the higher observed DNA damage at baseline and after 7 days, due to increased numbers of nonviable epithelial cells on the surface after differentiation. On the other hand, the reduced DNA damage after 3 days may have another explanation. It is possible that there was a lower ratio of buccal epithelial cells to buccal leukocytes in the 3-day collection. The alternate explanation is supported by Osswald et al. (49) who reported the presence of equal parts of leukocytes and epithelial cells in a cell collection from the oral cavity, although our method of cell collection probably removed a major portion of the leukocytes. We discarded
the first mouth rinse before collection of buccal cells with toothbrush. The assumption is that epithelial leukocytes would reflect the lower levels of DNA damage as was found in the circulating leukocytes. This explanation could also be supported by our result of a relatively higher correlation between BMC and the circulating leukocytes DNA damage in the 3-day group (r = 0.38 for heparin and r = 0.45 for EDTA samples), both representing whole-body oxidative stress. Therefore, further research may be undertaken to confirm the proportion of leukocytes that may be left in a collection of BMCs after discarding the first mouth wash and separating the leukocytes, if any, from the nonviable BMC to differentiate the responses of these two different types of cells. Because it is not always feasible in large-scale epidemiologic studies to collect BMC on two occasions, it is suggested to lightly scrape the inner cheek linings to remove old, well-differentiated BMC from the surface before final collection of newer viable BMC for biomonitoring studies.

In conclusion, the easy access and noninvasive collection make oral mucosa cells a suitable cell type to investigate surrogate biomarkers to detect exposure to genotoxic agents or in intervention studies. The described assay for 8-OHdG in leukocytes and buccal cells is sufficiently robust and stable over time to be used as a marker of oxidative damage to DNA in population studies.

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