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Effects of sample collection and storage conditions on DNA damage in buccal cells from agricultural workers

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ABSTRACT

Buccal cells are becoming a widely used tissue source for monitoring human exposure to occupational and environmental genotoxicants. A variety of methods exist for collecting buccal cells from the oral cavity, including rinsing with saline, mouthwash, or scraping the oral cavity. Buccal cells are also routinely cryopreserved with dimethyl sulfoxide (DMSO), then examined later for DNA damage by the comet assay. The effects of these different sampling procedures on the integrity of buccal cells for measuring DNA damage are unknown. This study examined the influence of the collection and cryopreservation of buccal cells on cell survival and DNA integrity. In individuals who rinsed with Hank's balanced salt solution (HBSS), the viability of leukocytes (90%) was significantly ($p < 0.01$) greater than that of epithelial cells (12%). Similar survival rates were found for leukocytes (88%) and epithelial cells (10%) after rinsing with Listerine® mouthwash. However, the viability of leukocytes after cryopreservation varied significantly ($p < 0.01$) with DMSO concentration. Cell survival was greatest at 5% DMSO. Cryopreservation also influenced the integrity of DNA in the comet assay. Although tail length and tail moment were comparable in fresh or cryopreserved samples, the average head intensity for cryopreserved samples was ~6 units lower (95% CI: 0.8–12 units lower) than for fresh samples ($t_{25} = -2.36$, $p = 0.026$). These studies suggest that the collection and storage of buccal samples are critical factors for the assessment of DNA damage. Moreover, leukocytes appear to be a more reliable source of human tissue for assessing DNA damage and possibly other biochemical changes.

1. Introduction

Although peripheral blood lymphocytes (PBLs) are widely used in monitoring humans for exposure to occupational and environmental genotoxicants, buccal cells are becoming an increasingly popular tissue source, particularly because they can be obtained non-invasively [1–4]. There are several methods for collecting buccal cells from the oral cavity including rinsing the mouth with saline [3,5] or with commercial mouthwash [1,6–11], or scraping the buccal cavity with cytobrushes [8,11,12], tongue depressors [2], cotton swabs [13,14], or a soft-bristle toothbrush [4,15–19]. Commercial mouthwash products (*e.g.*, Listerine® or Scope®) and saline solution (*e.g.*, Hanks' balanced salt solution, HBSS) are the most common rinse agents [2,19,20]. Most studies describe the method used for buccal cell collection, but cell viability is rarely assessed. Regardless of collection method, the final cell suspension primarily consists of epithelial cells and leukocytes, the latter being more viable [3]. The recovery of viable cells is an important factor for *in vitro* studies with buccal cells [18,21].

Buccal cells are an excellent sample to monitor human exposure to occupational and environmental genotoxicants, because are in direct contact with ingested or inhaled pollutants [22]. Buccal cells have been used widely to assess DNA damage by the comet assay. Rojas et al. [23] isolated buccal cells from smokers and non-smokers and found that DNA damage is greater in smokers. Buccal cells collected from individuals who were exposed to air pollution [24] or ionizing radiation [16] have also been examined for DNA damage. More recently, McCauley et al. [25] and Kisby et al. [26] examined oral leukocytes of agricultural workers by the comet assay and demonstrated that DNA damage is greater in farmworkers who were exposed to pesticides. Buccal cells are reportedly more sensitive than PBLs to the cytogenetic damage induced by cigarette smoke [22] or other environmental mutagens [27–32]. These studies demonstrate that buccal cells are an important source of tissue for assessing the effects of environmental or occupational genotoxicants on human health.

The comet assay (single cell gel electrophoresis or SCGE) is widely used by epidemiologists to monitor the extent of DNA damage in human cells, including buccal cells [4,33,34]. However, most

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comet studies use either buccal epithelial cells or exfoliated buccal cells. Few studies have used the comet assay to assess DNA damage in different types of buccal cells. A prerequisite for using any cell in the comet assay is that it must be viable. However, ≥90% of the cells in a buccal sample are epithelial cells, a cell type with low viability (10%) [3,18]. In epidemiological studies, buccal cells are routinely collected from subjects in the field, the cells cryopreserved and then immediately stored in a laboratory freezer until analysis. We report here that the concentration of the cryoprotectant DMSO is a significant factor that influences both the survival and the amount of DNA in oral leukocytes. Such factors are likely to be important confounders in epidemiological studies that use the comet assay to assess genotoxicity.

2. Materials and methods

2.1. Study population

The study population for the comet studies was recruited from Oregon farmworkers who worked in the berry crops during the summer of 2005. A total of 58 study participants signed a consent form prior to their participation in the study. The consent forms and study protocols were approved by the Institutional Review Boards of the University of Pennsylvania and Oregon Health & Science University. Twenty-six out of the 58 buccal mucosal samples were examined for DNA damage both before and after cryopreservation while the remaining 32 samples were examined for cell viability.

2.2. Collection of buccal cells

Participants ($n = 58$) were given two labeled sterile 50 ml conical polypropylene centrifuge tubes, each containing 20 ml sterile HBSS (Gibco-Invitrogen, Grand Island, NY, USA). Each participant was instructed to rinse their mouth for 60 s with the first tube of HBSS and then spit out the mouth rinse into a sterile 50 ml conical centrifuge tube. This procedure was repeated with the second tube of HBSS. Both rinses were combined in the sterile 50 ml centrifuge tube, the samples were transported on ice to the laboratory, and the cells were processed within 4 h of collection. To compare the effect of different rinse solutions on cell viability, a subset of participants ($n = 20$) used Listerine® mouthwash instead of HBSS to rinse their mouth. Another subset of participants ($n = 5$) rinsed their mouth with Listerine® and then HBSS. First they rinsed their mouth twice with 20 ml Listerine® mouthwash and cells were collected. Then, after 1 h, they repeated the procedure by rinsing their mouth twice with 20 ml HBSS; each time, cells were collected.

2.3. Isolation of leukocytes and epithelial cells

The oral rinses from each participant were centrifuged for 15 min at 1100 × *g*. The supernatant was carefully discarded; the cell pellets were re-suspended in 15 ml HBSS and the samples were centrifuged for 15 min at 1100 × *g*. The supernatant was discarded and the cell pellet re-suspended in RPMI 1640 cell culture medium. Leukocytes and epithelial cells were isolated from the cell suspension by density gradient centrifugation using Histopaque® 1077 (Sigma–Aldrich, St. Louis, MO, USA), as previously described by Obwald et al. [3] with minor modifications. Briefly, buccal cell suspension (4.0 ml) was carefully layered over 5.0 ml Histopaque®, and centrifuged for 30 min at 400 × *g*. The upper layer was gently withdrawn, leaving the interface (leukocytes) and cell pellet (epithelial cells) undisturbed. The interface was removed with a sterile Pasteur pipette, the cells were mixed with 5.0 ml PBS, and the cell suspension was centrifuged for 15 min at 1100 × *g*. The pellet was re-suspended in 1.0 ml RPMI 1640 culture medium and leukocytes were examined for viability. The pellet containing the epithelial cells was re-suspended in 5.0 ml PBS; the cell suspension was processed in a similar manner as the leukocyte fraction and the cells were examined for viability.

2.4. Viability

The leukocyte and epithelial cell fractions from each participant were examined for viability by trypan blue exclusion. Briefly, an aliquot (20 μl) of each fraction was mixed with trypan blue solution (20 μl) (Cellgro™, Manassas, VA), and the cells were counted using a hemocytometer (Hauser Scientific, Horsham, PA). The numbers of live (translucent) and dead (stained) cells were determined with a bright field microscope (200×) (Fig. 1). Cell viability was calculated as the ratio of live to total counted cells.

2.5. Cryopreservation

The leukocyte and epithelial cell fractions were mixed with freezing medium [50% RPMI 1640 cell culture medium, 45% fetal bovine serum (FBS), and 5% dimethyl sulfoxide (DMSO)], and the cells aliquoted into cryogenic vials. The cells were cryopreserved slowly, at a rate of 1 °C/min by placing the vials in a cold ethanol bath, and the vials were stored at −80 °C. For the survival studies, leukocytes were mixed with freezing medium (50% RPMI 1640, 40–48% FBS and 2–10% DMSO) and the cells were cryopreserved and stored as described above.

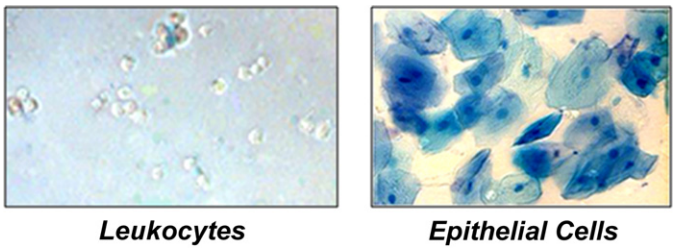

Fig. 1. Viability of cells in oral rinses from a non-agricultural control. Photomicrographs of leukocytes and epithelial cells that were stained with trypan blue. Note that most epithelial cells are dead (stained) while the smaller leukocytes are viable (unstained). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.6. Comet assay

Cryopreserved leukocytes were thawed quickly by submerging in a 37 °C water bath and washing the cells with ice-cold RPMI 1640 medium. Cryopreserved and fresh leukocytes were analyzed by the alkaline comet assay according to the methods of Singh et al. [35] with minor modifications [36]. Briefly, leukocytes (1×10^4) were embedded between a layer of 1% normal melting point agarose and a layer of 0.7% low melting point agarose. After solidification, the slides were immersed in lysing solution (2.5 M NaCl, 200 mM Na_2EDTA, 10 mM Tris–HCl, 10% DMSO, and 1% Triton X-100, pH 10) for 1 h at 4 °C to allow the DNA to unwind and the slides placed in alkaline buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 12) for 30 min at 4 °C. The slides were electrophoresed (0.8 V/cm) for 30 min in freshly chilled alkaline buffer, neutralized with Tris–HCl buffer (400 mM, pH 7.4), and stained with a fluorophore (20 μg/ml propidium iodide). DNA damage was determined by measuring the tail length, tail moment, and head intensity of 10–50 cells/sample, using a fluorescence microscope equipped with an automated digital imaging system running COMET Assay III™ software (Perceptive Instruments, UK).

2.7. Data analysis

Comet assay parameters were determined by measuring the tail length, tail moment, and head intensity of at least ten cells in each of the 26 samples. Mixed-effect models were used to compare fresh and cryopreserved samples for each comet parameter, with fresh/cryopreserved serving as a fixed effect and the subject serving as a random factor. Tail length and tail moment were transformed (fourth-root) to improve symmetry and stabilize variation prior to analysis; head intensity was analyzed without transformation. Within-subject variation by sample type was analyzed using a robust version of Levene's test using absolute deviations from the median. The test was conducted on untransformed data for all comet parameters. Influence of oral rinse and effect of cryoprotectant concentration on buccal cell viability was assessed with non-parametric procedures (signed rank and Kruskal–Wallis tests). Reported *p*-values are two-sided with significance level = 0.05 for all comparisons.

3. Results

3.1. Study sample

For the DNA damage study (comet assay) we recruited 16 Hispanic farmworkers (81.3% male) and ten Hispanic non-agricultural workers (50% male). The primary job activity of the farmworkers was picking berries. However, 2% of the farmworkers reported that they also handled pesticides during the 6 months prior to their participation in this study. Table 1 shows the characteristics of the participants in the study.

3.2. Influence of oral rinse solutions on buccal cell viability

While the methods for collecting buccal samples have been previously described, a detailed analysis of the effects of these methods on the yield and survival of the two major cell types in buccal samples (epithelial cells and leukocytes) has not been presented. Our first objective was to determine the viability of