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Altered Fluorescence of Buccal Cells as a Candidate Biomarker for Areca Nut Chewing

Laura A.F. Biggs^{*,a}, Adrian A. Franke^b, Christine E. Farrar^b

^aCollege of Applied and Natural Sciences, University of Guam, Mangilao, Guam

^bUniversity of Hawaii Cancer Center, University of Hawaii at Manoa, Honolulu, Hawaii, USA

Abstract

Background: Betel nut is used by an estimated 600 million people globally and is the 4th most widely used psychoactive substance in the world. Its use has been shown to cause oral and esophageal cancers. Therefore, cessation programs are needed in which an effective biomarker can be employed.

Objectives: Buccal cells are highly exposed to the betel nut during its use and are also easy to collect. However, it is unknown if there are significant changes to these cells upon exposure or how long any changes may last as the turnover of buccal cells is relatively fast. We sought to determine if optical changes could be detected on buccal cells after exposure to betel nut and if detected, how long these changes were sustained.

Methods: Flow cytometry was employed to determine whether fluorescence intensities differ between buccal cells exposed to betel nut and naïve cells. We further characterized the optical signature of buccal cells exposed to betel nut and other polyphenol-rich substances using lambda scans performed on a laser scanning confocal microscope.

Results: We demonstrate that the fluorescence of betel nut exposed cells is greater than that of cells exposed to other optically active compounds such as polyphenol-rich foods. We also demonstrate that the fluorescence spectra of betel nut quid exposed cells is distinct from that of cells exposed to other polyphenol-rich substances.

Conclusions: We conclude that detecting the altered fluorescence of buccal cells following exposure to betel nut quid may serve as a candidate biomarker for betel nut quid use.

Keywords

betel nut; areca nut; biomarkers; buccal cells; flow cytometry

Introduction

The nut from the palm species *Areca catechu* is commonly chewed in the Western Pacific, India, and Papua New Guinea among other locations, and is one of the most highly used

^{*}Address for correspondence and reprints: Laura A.F. Biggs, PhD., University of Guam, Mangilao, GU 96923, Tel: (671) 777-6609, drilaurabiggs@gmail.com.

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psychoactive substances in the world. Chewing this nut has cultural significance in many Asian and Oceanic countries and has been known to be used as early as 5 years of age in some communities (Mehrtash et al., 2017; Paulino et al., 2017). Its use among populations in the Western Pacific is on the rise in communities who are largely unaware of the cancer causing potential of its use (Hattori, 2018; Paulino et al., 2017). The Areca nut contains the alkaloid arecoline, that like nicotine, is an agonist for both muscarinic and nicotinic acetylcholine receptors and serves as the basis for its psychoactive properties. Areca nut has been designated as a Group 1 carcinogen by the International Agency for Research on Cancer (World Health Organization, 2003). Its long-term use has been associated with oral and oropharyngeal cancer, oral lesions, oral leukoplakia, submucosa fibrosis, gum disease, and cancer of the pharynx and esophagus, which has made its use one of the most neglected public health emergencies in the world (Garg & Chaturvedi, 2014; IARC, 2005; Javed et al., 2010; World Health Organization, 2003). Users of Areca nut may benefit from cessation programs and the University of Guam/University of Hawaii Cancer Center Partnership recently pilot tested a cessation program that showed great promise (Moss et al., 2015). As the presence of arecoline and other compounds from areca nut in the blood, saliva, and urine are relatively short lived and appear to return to baseline after 8 hours, biomarkers to validate qualitative reports of cessation are necessary, but currently unavailable (Franke et al., 2016; Wu et al., 2010; Lin et al., 2013; Mehrtash et al., 2017; Patrick et al., 1994).

Areca nut is typically consumed in varying combinations with betel leaf, tobacco, and slaked lime, together known as betel quid (BQ). Epithelial cells of the oral mucosa encounter high amounts BQ during chewing. The turnover rate of all layers of epithelial cells in the buccal region typically ranges from 5 to 6 days, but the rate at which the outer layer of buccal cells is shed is approximately every 2–4 hours (Borthakur et al., 2008; Sequier CA, 2001). One function of the buccal cells is to provide a protective barrier to the underlying tissue, however the permeability is affected by the polarity, pH, and concentration of the compounds to which it is exposed; therefore, some compounds are able to penetrate and become adsorbed into the intercellular compartment of the barrier layer and diffuse into the deeper layers of the tissue (Borthakur et al., 2008). We suspect that, following exposure to BQ, compounds from the quid penetrate several layers deep and could persist in the buccal epithelium for some time after exposure; perhaps similarly to how the red coloring from the BQ can persist on the teeth and gums of those who use BQ regularly. Therefore, quantitative analysis of buccal cell optical properties could serve as a viable biomarker for exposure to BQ. Because of the nature of the BQ pigmentation, we sought to determine if there is a difference in the optical properties of BQ-exposed buccal cells compared to unexposed cells, utilizing flow cytometry, due to its ability to quantify differences in optical intensities. Furthermore, we hypothesized that the optical signature of the BQ-exposed cells would differ from that of unexposed cells and from cells exposed to other polyphenol-rich substances.

Materials and Methods

Buccal Cell Collection for Preliminary In Vivo Experiment and Fluorescence Analysis:

Betel nut quid was prepared using one (1) white areca nut (husk included), one (1) betel leaf, and approximately a 1/2 teaspoon of slaked lime (all locally sourced in Honolulu, Hawaii). Buccal cells from healthy male volunteers were collected prior to the exposure to BQ or other substances. For nine minutes, a volunteer chewed either BQ or a 72% cacao chocolate bar as a control for an optically active compound. Buccal cells were also collected 24 hours following exposure to all substances. Cells were collected by gently scraping a soft-bristle toothbrush up and down five times on the buccal cell region. The brushes were inserted into 15 ml of PBS inside a 50 ml conical tube, covered with parafilm, and vortexed for 15 seconds to loosen the cells adhered to the bristles and suspend them in the PBS. The tubes were then centrifuged at $1000 \times g$ for 5 min. The cells were then fixed and frozen according to a modified protocol from the BD Biosciences website (www.bdbiosciences.com/us/resources/s/cytokinesfca). Briefly, cells were mixed with 1X Formal-Fixx (Thermo Fisher Scientific, Waltham, MA) for 20 mins at 4°C then centrifuged at $500 \times g$ for 5 mins, resuspended with cryoprotectant mixture (500 μ l 90% FBS/10% DMSO), and stored at -80°C until analysis by flow cytometry and microscopy.

Preparation of Betel Quid, Betel Quid Components, and Polyphenol Rich Extracts for Ex Vivo Experiments:

BQ extract was prepared using 16.3 g white areca nut (husk included), 2.0 g betel leaf, 0.3 g tobacco (Largo brand), and 0.2 g slaked lime, all obtained locally in Honolulu, Hawaii. Extracts of individual and paired components of BQ were made using the same weights of each component as indicated in the above BQ extract preparation. In each case, the nut and leaf were chopped into smaller pieces using a razor blade prior to grinding with a mortar and pestle with 20 mL deionized water (diH_2O) for 30 mins. The ground BQ was transferred to a 50 mL conical tube and the mortar and pestle were rinsed with 5 mL diH_2O , which was added to the BQ contents. After vortex-mixing for 1 min, the ground BQ was filtered using a 0.22 μM vacuum filter unit for 2 hrs. The final volume (12–14 mL) was pipetted into 1 mL aliquots then stored at -80°C until analysis.

Other polyphenol rich extracts were prepared using: 36 g soy beans + 20 mL diH_2O ; 73 g apples; 18 g spinach; and 50 g black berries. These substances were ground separately with mortar and pestle and filtered for 2 hours through a 0.45 μM filter. Cocoa extract was prepared by dissolving 5.0 g Hershey's unsweetened cocoa powder in 20 ml with hot diH_2O and vacuum filtering through 0.2 μM filter for 2 hr. Green tea extract was prepared by steeping 5.0 g dried green tea leaves (Bigelow) in 40 ml hot diH_2O and vacuum filtering through 0.2 μM filter for 2 hr. All extracts were aliquoted into 1 ml portions and stored at -80°C .

Ex Vivo Exposure of Buccal Cells to Polyphenol Rich Extracts:

Buccal cells from three healthy volunteers were collected as described above. The buccal cell suspensions were then centrifuged at $1000 \times g$ for 5 min. The supernatant was decanted and the pelleted buccal cells were resuspended in 500 μL PBS and aliquoted into 100 μL

portions. Each tube was mixed with 900 µl of the above described extracts for 60 min at 37°C on a rotator. All samples were then fixed, suspended in cryoprotectant as described above. The cells were stored at -80°C until analysis by flow cytometer or microscopy.

Collection of Buccal Cells from Betel Chewers in Pilot Participant Study:

The IRB (CHRS-121SU) for this participant study was reviewed by the institutional review board from the University of Guam (UOG) and approved on June 13, 2017. Informed consent was reviewed during in-person interviews and eligible participants signed an informed consent prior to sample collection. Betel chewing participants were self-reported occasional BQ chewing adult males (>18 yrs) recruited in Guam in 2018 and 2019. Interested participants were first screened in person to determine study eligibility. Information was obtained regarding age, ethnicity, health condition, and typical BQ chewing habits. Exclusion criteria included: active cough, fever, self-reported diagnosis of HIV, Hepatitis B, Hepatitis C, tuberculosis, cough that had lasted two weeks or more within the two previous weeks, or if dataset was missing more than one collection timepoint. Participants were asked to abstain from chewing BQ and any BQ related material, including tobacco, for at least five days prior to the study commencement and up to 120 hrs post-BQ chewing. After the screening, a total of nineteen (19) participants were included in the study (13 BQ chewers and 6 non-BQ chewing controls (including 2 non-user controls, 2 smokers and 2 chocolate chewing individuals). Three participants were excluded based on the exclusion criteria. On the day of the study, participants refrained from eating or drinking for 30 mins prior to BQ chewing. If food was consumed, participants brushed their teeth no less than 10 mins before donating baseline and longitudinal samples. Buccal cells were collected by gently scraping a soft-bristle toothbrush up and down five times on the buccal cell region. The brushes were inserted into 15 ml of PBS inside a 50 ml conical tube, covered with parafilm, and vortexed for 15 seconds to loosen the cells adhered to the bristles and suspend them in the PBS. The tubes were then centrifuged at $1000 \times g$ for 5 min. The cells were then fixed and frozen as described above. Buccal cells were collected prior to the start of the study (baseline). Following the baseline buccal cell collection, the participants were provided with BQ components (sourced in Guam; young white and young red *Areca catechu* nuts, betel leaf, and slaked lime) to assemble and chew for the time period typical to their chewing habit. Samples from participants chewing quid composed of white *Areca catechu* nuts, betel leaf, and slaked lime with a chewing time of ~6–16 min were included in the flow cytometry portion of this IRB study (this included 10 of the 13 BQ chewing participants, 3 participants were excluded). Buccal cells were collected 30 min, 24-hrs, 48-hrs, and 72-hrs post chewing. Five-hundred ml aliquots of the buccal cell suspensions from each timed collection were fixed and frozen as described above. After all collections from the participants were completed, samples were shipped on dry ice to the UH Cancer Center for analysis.

Flow Cytometry:

Samples for the preliminary fluorescence analysis were analyzed on a LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA) using 14 emission channels and 4 excitation lasers (405 nm, 488 nm, 561 nm, and 640 nm). All subsequent samples were analyzed on an Accuri C6 flow cytometer using a 488 nm laser for excitation and a 670 nm long pass filter

(FL3) for emission detection. As many of the buccal cells are collected in sheets or aggregate during processing, cell suspensions were filtered through 30- μ m nylon mesh cell strainers prior to flow analysis on a BD LSRFortessa or BD Accuri C6 (BD Biosciences, San Jose, CA). Both instruments were run using medium fluidic settings. All flow cytometry data was analyzed by FlowJo (FlowJo Inc., Ashland, OR) or Accuri C6 software (BD Biosciences) to obtain median fluorescence intensity statistics. Gating strategies were the same for all samples within compared groups. Significance for group comparisons was calculated using the Student's t-test.

Imaging and Lambda Scans of Buccal Cells by Confocal Microscopy:

In addition to ex vivo samples, two volunteer participants were used to identify fluorescence spectral signatures (one BQ chewer and one who chewed a 72% dark chocolate bar). Cells were collected and processed as described above. Ten microliters of the exposed cells in suspension were spotted onto a glass slide and covered using a #1.5 coverslip. Fluorescence and transmitted light images of the buccal cells were obtained using a Leica SP5 inverted laser scanning confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL). Confocal microscopy settings for three color channels were as follows: PMT1 - ex 405 nm, em 410–475 nm; PMT2 - ex 488 nm, em 520–620 nm; PMT 3 - ex 633 nm, em 650–750 nm. The images were acquired using a PL APO 63 $\times$ /1.4 oil immersion Leica objective with 2.5X zoom. For lambda scans, the pinhole was set to 2 AU. The entire field of view from the 63X/1.4 with 2.5X zoom was excited with the 488 nm laser and spectral emission was collected every 5 nm using a 5 nm band width from 500–700 nm. All images were recorded and processed with LAS AF software (Leica Microsystems).

Results

Preliminary Fluorescence Analysis of Buccal Cells Before and 24 Hours After In Vivo Exposure to BQ

In order to determine if chewing BQ caused a change in the fluorescence of buccal cells, confocal microscopy was used to image before and 24 hours after exposure to BQ (Fig. 1). This analysis showed very little fluorescence was visible in these cells prior to BQ exposure. However, 24 hours after exposure, approximately 80% of the cells exposed to BQ showed high levels of fluorescence across the visible spectrum (Fig. 1A). The high level of fluorescence from the buccal cells following exposure demonstrated that fluorescence intensity may serve as a candidate biomarker for BQ exposure.

It is important to note that there was also a distinct difference in the appearance of many of the BQ exposed cells in the transmitted light images (Fig. 1). The pre-exposed cells generally appeared large and smooth while many of the post-exposed cells appeared somewhat shriveled and wrinkled. It is possible that this is due to damage caused by the slaked lime in the quid as this component is quite caustic and capable of causing acute cellular damage.

In order to determine the best parameters for using flow cytometry to analyze buccal cells following BQ exposure, we used an LSR Fortessa flow cytometer with 4 lasers and 14

channels. An increase in fluorescence was seen in all 14 channels when pre-exposed cells were compared to post exposed cells. The channel showing the greatest percent increase from pre-exposure was the 675–715 nm channel using 488 nm laser excitation (Fig. 1B, left histogram). Knowing the ideal excitation and emission for BQ exposure allowed us to perform future analysis with a less expensive and more accessible instrument, the Accuri C6 flow cytometer, using 488 nm laser excitation and a 670 long pass filtered channel (FL3). Reanalysis of these samples using this instrument confirmed that the FL3 channel also gave the greatest difference between pre- and post-exposed cells (Fig. 1B, right histogram). In order to demonstrate the achievable separation between BQ exposed and unexposed cells when analyzed on the two flow cytometers, the histograms presented in Fig. 1B represent samples containing a mixture of cells, half exposed to BQ (right peak) and half naïve or unexposed buccal cells (left peak).

Analysis of Ex Vivo Collected Buccal Cells Exposed to Polyphenol-Rich Extracts

One reason that buccal cells may fluoresce following exposure to BQ is the presence of photoactive catechins and other phenols or polyphenols in the quid. It was unknown, however, if other foods rich in polyphenols would also cause the same or similar changes in the fluorescence of buccal cells. Therefore, in order to determine if other foods might also cause changes in fluorescence to these cells, we conducted a preliminary ex vivo study to analyze buccal cells exposed to various polyphenol-rich extracts. In addition, each individual component of the BQ was examined to determine their contribution to the fluorescence of the BQ. Buccal cells collected from three healthy volunteers were exposed ex vivo to various food extracts rich in polyphenols or BQ component extracts and analyzed on an Accuri C6 flow cytometer using an excitation wavelength of 488 nm and a 670 long-pass emission filter (Fig. 2A). These ex vivo studies demonstrate that BQ causes a greater increase in fluorescence, by 3–20 fold, when compared to other polyphenol-rich sources. BQ component analysis revealed that the component contributing overwhelmingly to the level of fluorescence was the areca nut, but only when combined with either slaked lime or betel leaf or both leaf and lime. In addition, the extracts (as prepared) showed that the areca nut in combination with either lime or leaf had the highest red coloring when compared to the other component extracts (Figure 2B). The inclusion of slaked lime increased the pH of each extract to which it was added, however, the inclusion of the areca nut in these extracts containing slaked lime increased the pH to a lesser degree (Fig. 2B). For example, the pH of extracts with lime were approximately 11–11.5, while those containing both lime and areca nut were approximately 8–8.5.

Signature Fluorescence Spectra of BQ exposed Buccal Cells

While the results from the previous ex vivo experiment suggest that a component or a combination of components in BQ is causing the buccal cells to significantly increase in fluorescence intensity, they do not exclude the possibility that other foods or substances not tested could cause a similar increase in fluorescence intensity. Therefore, we sought to determine if the fluorescence of the BQ exposed cells had a distinct spectral signature when compared to some of the more fluorescent phenol-rich extracts. In order to determine the fluorescence emission spectra of buccal cells exposed to BQ or other phenol-rich extracts, we conducted lambda scans (three buccal cells for each category) of cells exposed to BQ,

cocoa, or green tea extracts (data not shown) using PBS as a negative control. Using 488 nm excitation, the emission values from 500 – 700 nm were averaged and compared (Fig. 3; left panel). These data show that the fluorescence spectra from BQ-exposed cells, whether in vivo or ex vivo, are consistent and have maximum emission at 585 nm. We also used these scans to compare the spectra of cells after ex vivo or in vivo exposure to cocoa, another polyphenol rich substance (Fig. 3; right panel). These results demonstrate that the spectra of the in vivo samples, whether betel quid or chocolate, are comparable to the lambda scan spectra of their respective ex vivo extract exposed buccal cells. This data also implies that fluorescence from BQ-exposed cells (with white areca nut, betel leaf, and slaked lime) has a specific spectral signature that can be distinguished from the spectra of other polyphenol-rich sources.

Analysis of Buccal Cells from Individuals Chewing BQ

In order to determine the practicality of using optical analysis as a biomarker for BQ use, a pilot participant study was conducted as described above. Buccal cells were collected from individuals prior to chewing BQ and 30 min, 24, 48, and 72 hours after chewing. The samples were then analyzed for fluorescence intensity on the Accuri C6 flow cytometer. Increased fluorescence could be seen for participants at 30 min and at 24 hours after exposure (Fig. 4 – left panel). Control non-BQ exposed buccal cells (including 2 non-substance users, 2 smokers, and 2 chocolate chewers) showed no significant increase in fluorescence (Fig. 4 – right panel) at timepoints when compared to baseline levels.

Discussion

In this study we found a change in fluorescence intensity in buccal cells exposed to BQ. As these cells are relatively easy to collect, this difference could potentially serve as a useful and practical biomarker in cessation trials. The increase in fluorescence emitted from BQ exposed buccal cells was seen across the visible spectrum, but the greatest difference from baseline using flow cytometry was seen using 488 nm excitation and 670 nm emission. In addition, when BQ is composed of white areca nut, betel leaf, and slaked lime, there is a consistent and distinct spectral signature detectable for BQ-exposed cells when excited at 488 nm using lambda scans by laser scanning confocal microscopy. It is interesting that the lambda scans revealed that the wavelength giving the highest intensity fluorescence for betel quid exposed cells compared to controls (when examined between 500 nm and 700 nm and excited at 488 nm) was at 585 nm, while the flow cytometry results indicated that the highest emission intensity using 488 nm excitation was greater than or equal to 670 nm. This is likely due to the limited range chosen for spectral analysis by confocal microscopy. For future lambda scans or other microscope-based spectral analysis, we will examine a range above 700 nm for betel quid exposed cells.

Our results also indicated that the fluorescence of the white areca nut extract alone was not fluorescent (or red in color) without the inclusion of either slaked lime and/or betel leaf (Fig. 2). We hypothesize that the red coloring is caused by accelerated oxidation of areca nut catechins or other polyphenols caused by the slaked lime (calcium hydroxide) or by some component of the betel leaf. Further analysis is needed to determine if this is the cause and,

if so, what specific components of the areca nut and the betel leaf may contribute to this. In addition, we will investigate whether the red areca nut produces the same increase in fluorescence, as the current analysis did not include participants using this variety of areca nut.

Ultimately, the process of collecting, preserving, storing, and shipping cells for analysis by flow cytometry and microscopy may not be as practical as a biomarker test that could be employed in the field. Therefore, we plan to test a simple cell counter (such as the Countess II FL; Thermo Fisher Scientific, Waltham, MA) that can detect cells in bright field in addition to multiple fluorescence channels. Applying this methodology in the field in which samples could be collected and analyzed immediately would save both time and money. In addition, testing fresh samples may demonstrate a more significant difference in fluorescence than what can be distinguished in fixed and frozen samples.

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Figure 1. Betel quid exposed buccal cells show increased fluorescence.

A) Confocal laser scanning microscopy was used to analyze buccal cells before in vivo exposure to BQ (left 2 panels) and after exposure to BQ (right 4 panels). The top panels are scans of representative cells with fluorescence from three overlaid channels. Fluorescence emission (top panels) was collected at 410 – 475 nm off the 405 nm laser (blue); 520–620 nm off the 488 nm laser (green); and 650–750 nm off the 633 nm (red). All scans were taken with the same excitation laser power and the same voltages applied to the detectors. The bottom panels represent transmitted light images of the same cells. Scale bar = 10 μ m. n = 2.

B) Representative flow cytometry histograms from the Fortessa (Ex. 488 nm and Em. 695/40 nm) compared to the Accuri C6 (Ex. 488 nm and Em. >670 nm or FL3). For each panel, the sample is a mixture of buccal cells, half exposed to BQ and half in PBS. The left peak in each histogram represents cells only exposed to PBS and the right peak represents cells exposed to BQ. Gating to eliminate debris was applied.

Figure 2. Buccal cells show increased fluorescence when exposed to specific components of betel quid, especially areca nut when combined with either betel leaf and/or slaked lime.

A) Buccal cells show greater fluorescence after exposure to betel quid components compared to other polyphenol-rich extracts in ex vivo samples. Box plot representing the median fluorescence intensity (MFI) of buccal cells exposed to various phenol rich extracts or components of betel quid as measured by flow cytometry on the Accuri C6 using the 488 nm laser for excitation and FL3 (670LP filter) for emission detection ($n = 3$, $** p < 0.01$). **B)** Extracts of betel quid components show an increase in red color intensity when combined together, specifically when calcium carbonate (slaked lime) or betel leaf is combined with white areca nut. The pH of the extract is indicated below the names of the components included in each extract.

Figure 3. Betel quid-exposed cells have consistent emission spectra.

In the left panel, cells exposed to BQ extract ex vivo (open circles), BQ in vivo (30 minute timepoint; closed circles), or PBS alone (horizontal line) were used to generate emission spectra by performing lambda scans with emission intensity measured every 5 nm using on a laser scanning confocal microscope with excitation at 488 nm (n=3). For comparison, the right panel shows lambda scans of cells from two individuals exposed to either cocoa extract ex vivo (open circles), chocolate bar in vivo (30-minute timepoint; closed circles), or PBS alone (horizontal line). At 488 nm excitation, the emission maximum for the BQ-exposed cells was 585 nm and the emission maximum for cocoa or chocolate-exposed cells was 545 nm.

Figure 4. Pilot study shows in vivo betel quid exposed buccal cells have altered fluorescence after exposure.

Box plots representing the median fluorescence intensity of buccal cells for progressive timepoints from the participant pilot study as measured by flow cytometry using the FL3 channel. The left graph includes measurements of cells from ten individuals that chewed BQ (white areca nut, betel leaf, and slaked lime for an average of 10 min); and the right graph includes measurements of cells from six individuals that did not chew BQ (2 non-user controls, 2 smokers, and 2 chocolate chewers). The left graph of BQ chewers shows a significant increase in fluorescence intensity at the 30 minute and 24 hour timepoints when compared to buccal cells at baseline (prior to BQ exposure). MFI = Median Fluorescence Intensity. Note: y-axis is in log scale. (* $p < 0.05$).