Is the Blood Donated by Habitual Nut Quid Chewers Suitable for Use in Transfusion?

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Background/Purpose: Betel quid (BQ) chewing is a popular oral masticatory activity, and there are approximately 600 million BQ chewers worldwide. Although chewing BQ has been linked to the pathogenesis of oral cancer, leukoplakia, and oral submucous fibrosis. The question whether the mixed constituents present in areca nut, which may exert cytotoxic effects on red blood cells (RBCs), has never been addressed.

Methods: Heparinized blood specimens were obtained with informed consent from healthy laboratory personnel. RBCs were separated with the standard procedure and adjusted to 10% hematocrit with PBS. Various concentrations of areca nut extract (ANE; 100–800 µg/mL) were added to these RBC preparations and incubated at 37°C for 4 hours. Two portions (0.4 mL each) of the incubated RBCs were then used for measuring osmotic deformability index and for observing RBC morphology with scanning electron microscopy. The remaining RBCs were used for determining membrane sulfhydryl groups and protein profiles by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Results: Blood incubated with various concentrations of ANE showed concentration-dependent decreases in osmotic deformability index and membrane sulfhydryl groups. Membrane protein profiles revealed a significant loss of the band 3 fraction, with the concomitant appearance of several new protein bands in the electropheretogram. Finally, drastic morphological changes of ANE-treated RBCs were observed.

Conclusion: We suggest that to assure the quality of transfusion, the blood donated by a habitual BQ chewer should be used with caution because of its possible contamination with areca nut ingredients that may be cytotoxic to RBCs. [*J Formos Med Assoc* 2010;109(2):106–112]

Habitual betel quid (BQ) chewing, which is common in Taiwan, is associated causally with the pathogenesis of oral submucous fibrosis and an increased risk of oral cancer. It is estimated that more than 2 million people in Taiwan and approximately 600 million people worldwide are regular BQ chewers.[1,2] Furthermore, an average of 14–23 BQs are used per day by a Taiwanese chewer, which is relatively high compared with the amount consumed by chewers in other countries.[3,4]

A typical BQ chewer in Taiwan generally uses a mixture of areca nut, catechu, inflorescence, piper betel, and slaked lime; and a high proportion of chewers usually swallow the liquid mixture of these constituents. It has been documented that polyphenols are common in areca nut. The participation of these constituents in

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the generation of reactive oxygen species such as superoxide anion (O_2^-) and hydroxyl radical (OH•), which are involved in the formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), a marker for oxidative DNA damage, has been demonstrated.[5,6] For this reason, there is a concern as to whether the mixed constituents being swallowed and chronically circulated inside the body have an adverse effect on the red blood cells (RBCs).

Is the blood donated by a habitual BQ chewer, which may be contaminated with the mixed constituents of areca nut extract (ANE), still suitable for transfusion? To the best of our knowledge, this question has never been addressed. Thus, the specific aim of this study was to establish whether ANE ingredients can indeed exert adverse effects on the structural integrity of human RBCs.

Methods and Methods

Blood procurement

Heparinized blood specimens were obtained with informed consent from healthy college students and laboratory personnel. All blood samples were used within 24 hours of collection. Before any experimental manipulation, RBCs were washed three times in potassium-free PBS (1.9 mM NaH_2PO_4, 8.1 mM NaH_2PO_4 NaCl, pH 7.4), and the buffy coat was aspirated each time. Unless stated otherwise, all chemicals were reagent grade and were obtained from Sigma Chemical Company (St Louis, MO, USA).

Potassium measurement

The potassium content in the supernatant of each aliquot from the incubation mixture of the RBCs, with or without treatment with ANE, was determined with a Perkin–Elmer 413 flame photometer (Waltham, MA, USA). In brief, RBC suspensions of 10% hematocrit were incubated with various concentrations of ANE (0–800 μg/mL) at 37°C in an agitating water bath. At appropriate time interval (4 hours), aliquots were taken for potassium determination by flame photometry. Result represents an average of 10 specimens.

Sulfhydryl group determination of RBC ghosts

To examine the effects of ANE on RBC membrane proteins, ghost controls and ANE-treated RBCs were prepared by the method of Dumaswala et al.[6] The sulfhydryl groups of RBC membranes were then quantified spectrophotometrically at 412 nm with Ellman's reagent (5,5′-dithiobis-2-nitrobenzoate), as described by Beutler et al,[7] with minor modifications.[8]

RBC membrane protein assessment

The isolated ghost preparations were subjected to total membrane protein determination by the method of Smith et al[10] and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using the method of Laemmli with minor modifications by Dumaswala et al.[9]

Measurement of RBC deformability

The osmotic deformability profile of intact RBCs was determined by continuously monitoring the deformability index as a function of the medium osmolality (50–500 mOsm/kg) at a constant shear stress of 160 dynes/cm^2.[11,12] In brief, RBC suspensions of 10% hematocrit were incubated at 37°C with or without various concentrations of ANE. At the end of 4 hours incubation, aliquots were taken to determine the osmotic deformability profile using a custom-built laser viscodiffractometer (FAK Scientific Instruments, CA, USA). In a typical run, 100 μL whole blood was mixed with 4 mL sample buffer, which was a mixture of 37.5 mL low polyvinyl-pyrrolidone buffer and 12.5 mL high-polyvinyl-pyrrolidone buffer, as described elsewhere.[13]

Scanning electron microscopy

RBC suspensions of 10% hematocrit were incubated at 37°C with or without added ANE (0, 200, 400 or 800 μg/mL) for 4 hours. Upon completion of incubation, the blood cells in the PBS solution were fixed by adding equal amounts of

2.0% glutaraldehyde solution in 300 mOsm/kg cacodylate buffer. The extracellular fluids were replaced by freshly prepared 2.0% glutaraldehyde solution for fixation for 2 hours. The cells were washed in 0.1M cacodylate buffer solution and post-fixed for 1 hour with 1.0% O_sO_4 in cacodylate-buffered solution at the same osmolality as the glutaraldehyde solution. The post-fixed cells were washed in water and rinsed in ethanol (50%, 70%, 80%, 90%, and 95%, and three times in 100%). Some specimens were transferred into containers of filter paper and dried in a critical point dryer (HCP-2; Hitachi®, Tokyo, Japan). The filter paper with dried cells was glued to a metal stub, coated with gold using an EiKo IB-2 ion coater (EiKo Co., Tokyo, Japan), and examined and photographed in a Hitachi S-2300 scanning electron microscope (Schaumburg, IL, USA) at 20 KV.[14,15]

Statistical analysis

The data were expressed as mean ± standard deviation. Statistical analysis was carried out by one-way analysis of variance, and the *t* test was performed to make comparisons among the different groups. Differences among the groups were considered to be significant at $p < 0.05$.

Results

RBCs exposed to ANE showed a concentration-dependent passive potassium leak (Figure 1). However, it was observed that the passive potassium leak was less pronounced when the concentration of ANE was < 400 μg/mL. Conversely, higher concentrations of ANE (> 400 μg/mL) caused permanent damage that led to major passive potassium leak, which was accompanied by the leakage of macromolecules such as hemoglobin (Figure 2). Some target-cell-like transformation of RBCs also occurred when exposed to a higher ANE concentration (Figure 3).

To assess further the possible adverse effects of ANE on RBCs, deformability of control RBCs and those treated with various concentrations of ANE
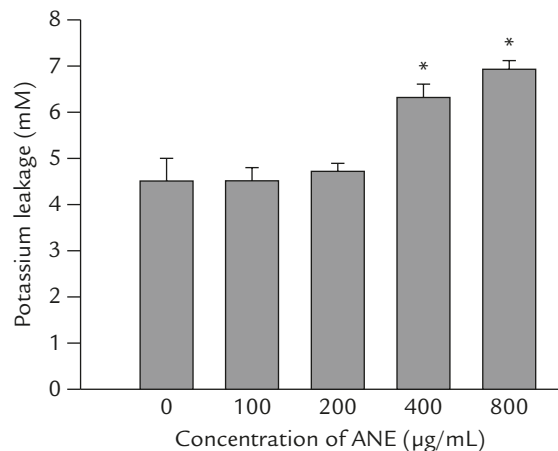

Figure 1. Effect of areca nut extract on potassium permeability of human red blood cells. *Significant difference between the mean of ANE-treated and the respective control group. ANE = areca nut extract.
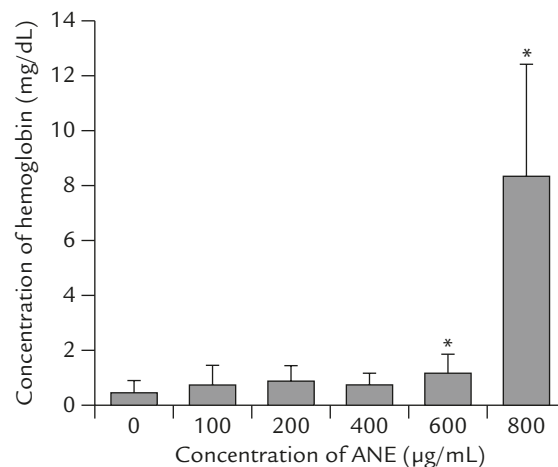

Figure 2. Effect of areca nut extract on the release of hemoglobin from red blood cells. Each result represents an average of 10 specimens. *Significant difference between the mean of areca nut extract-treated and the respective control group. ANE = areca nut extract.

(100–800 μg/mL) were determined by laser viscodiffractometer. Figure 4 shows typical osmotic deformability profiles of RBCs before and immediately after addition of various concentrations of ANE. At these concentrations, the deformability at isotonicity (290 mOsm/kg) decreased. There was also a concentration-dependent decline in DI.

An additional study indicated that ANE also caused a concentration-dependent reduction in membrane sulfhydryl groups (Figure 5). Moreover, SDS-PAGE of the membrane protein profile from ANE-treated RBCs (800 μg/mL) revealed
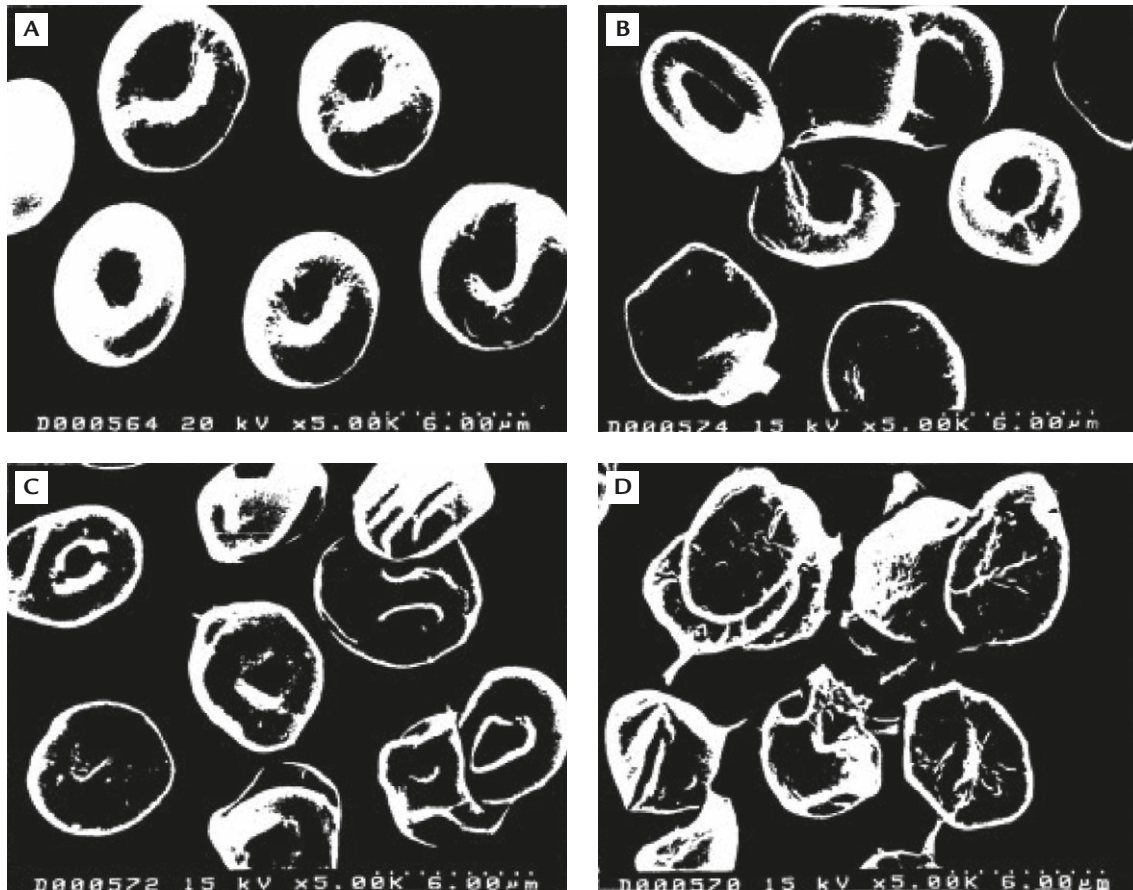
Figure 3. Effect of areca nut extract on the morphology of intact red blood cells. (A) Control red blood cells (RBCs); (B) RBCs + 200 μg/mL ANE; (C) RBCs + 400 μg/mL ANE; and (D) RBCs + 800 μg/mL ANE. ANE = areca nut extract.

several discrete new protein bands. The most noticeable had a molecular weight smaller than that of bands 5 and 7. Also, there was a marked reduction in the molecular weight of bands 3 and 6, and the effect was dose-dependent. We also noted that when RBCs were exposed to a higher concentration of ANE (800 μg/mL), a sizeable hemoglobin band was seen in the electrophoretogram, which indicated that hemolytic damage had occurred (Figure 6). These data were in accordance with the results of direct measurement of hemoglobin release (Figure 2).

Discussion

Notwithstanding extensive studies that have dealt with the etiological roles of betel nut constituents with oral cancer, the potential adverse effects of these constituents on the structural integrity of human circulating RBCs of habitual chewers seldom have been investigated. For that reason, we set out to study the potential adverse effects of ANE on the structural integrity of human RBCs.

It has been well documented that ANE contains a large concentration of polyphenols, and incubation of herring sperm DNA with ANE generates 8-OH-dG in a dose-dependent fashion.[5] The formation of 8-OH-dG is attributed to attack by OH•, which can be produced by the iron-catalyzed redox-cycling (Fenton) reaction. This originates from the conversion of O_2^- to hydrogen peroxide by superoxide dismutase followed by interaction with ferrous iron.[16] In the same way, ANE, being rich in polyphenols, can generate phenolic radicals via reaction with an oxidant. The resulting phenolic radicals are capable of abstracting
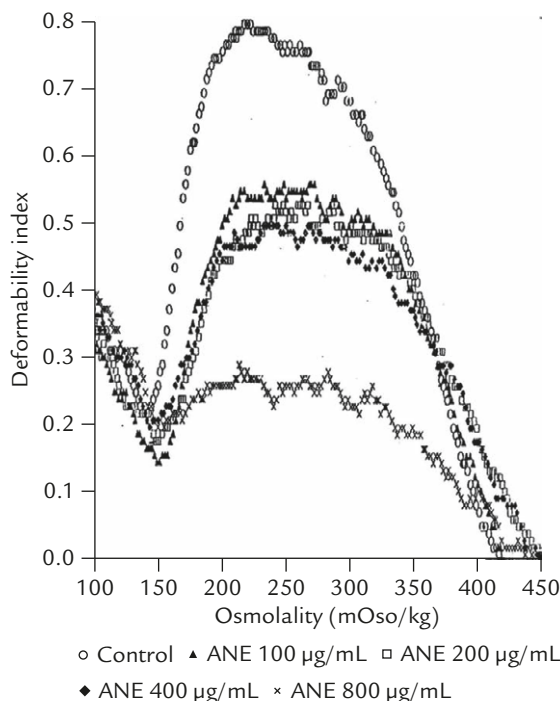
Figure 4. Effect of areca nut extract on membrane deformability. ANE = areca nut extract.

Figure 6. Effect of treatment with various concentrations of areca nut extract on red blood cell membrane protein profiles. Extra protein bands formed as a result of areca nut extract treatment (arrows). Lane 1 = molecular markers; lane 2 = control red blood cells ghosts; lane 3 = 200 μg/mL ANE; lane 4 = 400 μg/mL ANE; lane 5 = 800 μg/mL ANE. ANE = areca nut extract; Hb = hemoglobin.
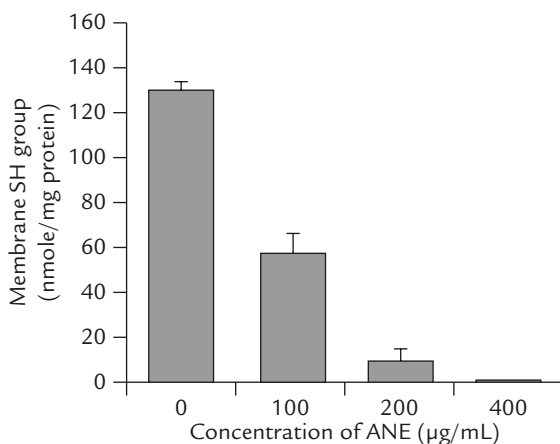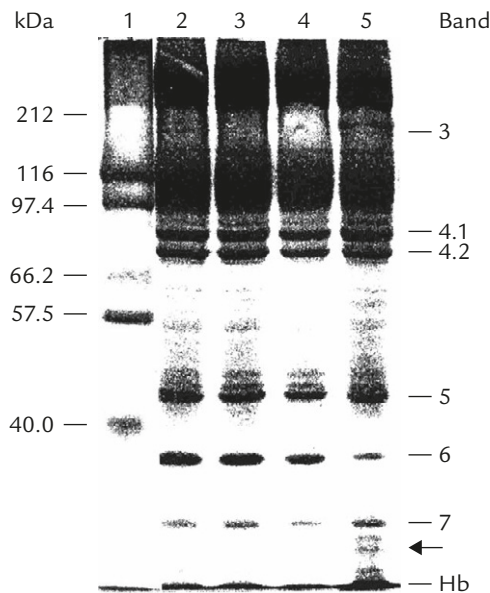

Figure 5. Effect of areca nut extract on the contents of red blood cell membrane sulfhydryl groups. The sulfhydryl groups were undetectable when the cells were exposed to areca nut extract at concentration > 400 μg/mL, which indicated that membrane thiols had been oxidized completely. Each result represents an average of 10 specimens. ANE = areca nut extract; SH = sulfhydryl.

hydrogen atoms from glutathione (GSH) to allow its return to the original configuration, with commensurate formation of thiyl radical. The thiyl radical can generate O_2^- after molecular oxygen receives an electron. Also, arecoline and eugenol,

two of the major constituents of ANE, have been shown to be capable of forming a conjugate with reduced GSH, thereby diminishing cellular GSH antioxidant reserves.[17-19] These pathways could help to explain why exposure of human KB cells to ANE can result in GSH depletion, as reported elsewhere in the literature.[2,20]

As a result of the above-mentioned characteristics, it can be inferred that, if the constituents of ANE can enter into the circulation by swallowing the liquid mixture of betel nut chewing, an oxidative-stress-evoked effect on RBC membrane integrity can ensue. Using a simulated *in vitro* system, we were able to show that ANE could indeed exert alterations in RBC morphology, deformability, membrane permeability and membrane protein profiles.

ANE has a threshold concentration of 400 μg/mL and caused a concentration-dependent passive potassium leak (Figure 1), and such damage was accompanied by the leakage of macromolecules such as hemoglobin (Figure 2). These phenomena were accompanied by a morphological alteration

that resembled target-cell-like transformation. Maridonnean et al[21] reported that damage to membrane thiol groups can induce blockage of the essential ionic pump, which results in disruption of ionic movement with consequent leakage of intracellular contents. Studies by Deuticks[22] demonstrated clearly that membrane sulfhydryl groups play a pivotal role in modulating membrane permeability. It is therefore conceivable that the observed effect of ANE on RBC permeability might proceed through a similar type of mechanism, namely modification of membrane sulfhydryl contents. Indeed, we did observe that exposure of RBCs to ANE did result in a concentration-dependent loss of membrane sulfhydryl groups (Figure 5). There are two pathways that could help to interpret the possible mechanism(s) associated with the ANE-induced RBC membrane sulfhydryl depletion. First, the depletion of membrane sulfhydryl groups can proceed via ANE-provoked reactive oxygen species production that is preceded by the oxidation reaction of membrane thiols. Alternatively, the constituents in ANE such as arecoline and eugenol could also form conjugates with the membrane thiol groups. The decrease in membrane deformability by ANE can also be explained by the effect of ANE on membrane thiols. It has been documented that alkylation of sulfhydryl groups by N-ethylmaleimide can alter spectrin self-association, which leads to instability of the membrane skeleton.[23] Oxidation of sulfhydryl groups involved in ankyrin-spectrin or the ankyrin-band[3] binding site might also weaken the membrane.[24] Finally, SDS-PAGE clearly demonstrated that ANE could give rise to an abnormal membrane protein pattern, with the appearance of discrete new bands (Figure 6). We speculate that these new bands are proteolytic fragments of ANE-modified membrane proteins or could be complexes between fragments of protein with denatured hemoglobin. These possibilities await further clarification.

In conclusion, we demonstrated that human RBCs exposed to ANE can directly alter membrane structural integrity, including morphology, deformability, membrane permeability, and protein profiles. It is therefore possible that blood donated by a habitual betel quid chewer might be contaminated with areca nut ingredients that can have cytotoxic effects on the RBCs. This phenomenon could lead to unsatisfactory post-transfusion survival of RBCs, and its significance should not be overlooked.

Acknowledgments

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