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Effects of areca nut extract on the apoptosis pathways in human neutrophils

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Background and Objective: Areca nut, a major component in area quid, possesses genotoxic and carcinogenic activities. Areca nut extract (ANE) may affect the defensive functions of neutrophils. Recent studies suggest that areca nut chewing is associated with a higher prevalence of periodontal disease as a result of the detrimental effects of ANE on the host defense system. This study examined the effects of ANE on the apoptosis pathways in human neutrophils.

Material and Methods: Apoptosis/necrosis of neutrophils was determined using flow cytometry. Proteins involved in the apoptosis pathway were determined using western blotting analysis.

Results: The results indicated that ANE reduced early apoptosis, but increased the primary necrosis of neutrophils. ANE may arrest neutrophils in the G0/G1 phase and reduce the apoptotic hypodiploid DNA contents. The levels of cleaved forms of poly(ADP-ribose) polymerase, and of caspase-3 and caspase-8 were decreased by treatment with ANE. Moreover, glycogen synthase kinase-3α/β may be involved in the ANE-modulated effects of neutrophils.

Conclusion: Areca nut may regulate death pathways in neutrophils. This may be one mechanism by which areca nut compromises the periodontal health of areca nut chewers.

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Areca (betel) quid chewing, an addictive habit, common in India and Southeast Asia, is associated with an increased risk of oral submucous fibrosis and oral squamous cell carcinoma (1). Areca quid chewing also enhances gingival bleeding and increases the prevalence of periodontal diseases (2,3). Areca quid generally consists of areca nut (*Areca catechu*), *Piper betle* leaf and slaked lime, with or without additives such as tobacco and *P. betle* inflorescence. Areca nut, the major component of areca quid, possesses cytotoxic, genotoxic and carcinogenic properties (1). Areca nut extract (ANE) impairs T-cell activation and induces the production of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in oral epithelial cells (4). Moreover, ANE enhances cytokine production in peripheral blood mononuclear cells (5,6). ANE down-regulates the production of alkaline phosphatase and up-regulates the production of RANKL in human osteoblasts (7), suggesting a possible mechanism by which areca nut is involved in the development of periodontal diseases associated with areca chewing. ANE also reduces the defensive functions of neutrophils (8).

Neutrophils, which represent the first line of the host defense mechanism, are recruited to sites of infection by sensing, and migrating towards, a gradient of chemotactic substances and they are capable of phagocytosis and killing a wide range of bacteria (9). Approximately 1 million neutrophils enter the oral cavity via the gingival crevicular fluid every minute (10). Neutrophils within the gingival crevice are able to promote periodontal health by decreasing the colonization and viability of periodontal pathogens (9). Altered numbers or function of circulating neutrophils may lead to severe periodontal diseases (11,12). Amplified activity of neutrophils may also be responsible for the destruction of periodontal tissues (12,13). At a nonlethal level, ANE inhibits the bactericidal activity of neutrophils and interferes with the release of superoxide

anion by neutrophils *in vitro* (8). The ability of cytochalasin B and fMet-Leu-Phe to trigger the production of intracellular reactive oxygen species and the extracellular release of lysosomal enzyme myeloperoxidase in human neutrophils is significantly suppressed by ANE (14). ANE also inhibits the phagocytosis of the oral pathogens, *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* and *Streptococcus mutans*, by neutrophils (15,16). Areca chewing is associated with a tendency for subgingival infection with the periodontal pathogens, *A. actinomycetemcomitans* and *Porphyromonas gingivalis* (2). The effects of ANE on the defensive functions of neutrophils may contribute to a less efficient elimination of bacteria from the periodontal environment.

Neutrophils survive in the circulation for approximately 24–36 h before undergoing apoptosis (17). Apoptotic neutrophils lose surface adhesion molecules and their ability to release granular contents, and thereby are phagocytosed by macrophages (18). Apoptosis, a mechanism essential for maintaining cellular homeostasis, is usually considered less inflammatory because the cellular membranes of apoptotic cells remain intact and cells are removed from the area of inflammation with minimal damage to the surrounding tissue (18). The main characteristics of apoptosis include plasma-membrane asymmetry, cell shrinkage, chromatin condensation and DNA fragmentation. Several caspases (cysteine aspartyl-specific proteases), including caspase-3 and caspase-8, are involved in the apoptosis of neutrophils (18). Caspase-8 may catalyze the proteolytic activation of caspase-3. Activated caspase-3 may further cleave poly(ADP-ribose) polymerase (PARP), which plays an essential role in DNA damage repair and cell death (19). The life span of neutrophils may be extended by the anti-apoptotic functions of a range of inflammatory mediators, including leukotriene B_4 (LTB_4) (20).

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is used by many cell types for the regulation of apoptosis and cell survival (21). Akt (also known as protein kinase B), is a serine–threonine kinase that has been implicated in the control of several cellular functions, including the promotion of cell survival and the blocking of apoptosis (22,23). Glycogen synthase kinase-3 (GSK-3) is constitutively active, but can be inactivated through phosphorylation by Akt (23,24). GSK-3, containing two isoforms (GSK-3α and GSK-3β), also plays roles in the apoptotic signaling pathway (24). ANE may activate the PI3K/Akt signaling in normal human oral keratinocytes (25). ANE induces apoptosis in cultured human keratinocytes (HaCaT cells) (26). However, ANE induces the cell cycle arrest, but not the apoptosis, of cultured oral KB epithelial cells (27). Whether ANE affects apoptosis in neutrophils has not yet been characterized. This study examined the effects of ANE on the apoptosis pathways in human neutrophils.

Material and methods

Preparation of ANE

ANE was extracted from dried ripe areca nuts without husk, as previously described (5). Briefly, dried nuts (10 g) were finely chopped and extracted with 250 mL of distilled water for 1 h. The filtrate was freeze-dried. After extraction the yield was approximately 12%. ANE was first dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA). Before use in experiments, the ANE stock solution was diluted, in DMSO, to different concentrations and then further diluted with Hank's balanced salt solution (HBSS) (Gibco BRL Laboratories, Grand Island, NY, USA) supplemented with 1.6 mM $CaCl_2$ and 10 mM HEPES (HBSS/Ca^{2+}). The final concentration of DMSO in each sample did not exceed 0.5%.

Preparation of neutrophils and incubation conditions

Neutrophils were freshly purified from human venous peripheral blood, obtained from systemically healthy and nonsmoking donors (age range: 23–30 years), by dextran sedimentation followed by Ficoll density-gradient centrifugation, as described previously (8). The time-course experiments were initially performed to determine the optimal experimental conditions. From these initial experiments, an 8-h incubation period showed more evident effects of ANE on apoptosis, and was therefore used in this study. Freshly isolated neutrophils (2×10^6 cells/mL) were incubated with various concentrations of ANE (0, 6.25, 12.5, or 25 μg/mL) in HBSS/Ca^{2+} for 8 h at 37°C.

For experiments studying the effects of inhibitors, the PI3K inhibitor, 2-(4-morpholinyl)-8-phenyl-4H-1-benzo pyran-4-one (LY294002; Calbiochem, EMD Biosciences, San Diego, CA, USA), the LTB_4 inhibitor, 3-[1-(p-chlorobenzyl)-5-(isopropyl)-3-t-butylthioindol-2-yl]-2,2-dimethyl popanoic acid,Na (MK886; Calbiochem), the NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI; Sigma) and the GSK-3 inhibitors, BIO-acetoxime (2′Z,3′E)-6-bromoindirubin-3′-acetoxime (GSK-3 inhibitor X; Calbiochem) and SB 216763 (Sigma), were first dissolved in DMSO as stock solutions and further diluted in HBSS/Ca^{2+}. Neutrophils were pretreated with HBSS/Ca^{2+} only or with HBSS/Ca^{2+} containing vehicle DMSO (0.5%), LY294002 (20 μM), MK886 (1 μM), DPI (10 μM), GSK-3 inhibitor X (20 μM) or SB 216763 (20 μM), for 30 min at 37°C. Neutrophils were further incubated with or without ANE for various periods of time. Each inhibitor was present throughout the incubation. Cell lysates were harvested and then analyzed by western blotting. The treated cells were also analyzed using flow cytometry.

Propidium iodide exclusion assay

The viability of the treated neutrophils was determined by analyzing the influx of propidium iodide (PI) into neutrophils, as described previously (14). Neutrophils fixed in 3% paraformaldehyde served as the controls for dead cells. Treated neutrophils were washed and incubated in HBSS alone, or in HBSS containing 4 μg/mL of PI

(Sigma), at 37°C for 15 min. After washing twice with HBSS, neutrophils were passed through a nylon filter (41 μm) (Spectrom®; Spectrum Laboratories, Inc., Laguna Hills, CA, USA) and analyzed using a flow cytometer (FACSort™; Becton Dickinson, San Jose, CA, USA) equipped with an argon laser operating at an excitation wavelength of 488 nm. Data were analyzed using the CellQuest® and WinMDI 2.8 software programs (Becton Dickinson). The light scatter profiles and fluorescence intensities of a total of 10,000 cells were measured. The ability of neutrophils to exclude PI in each sample was determined using the following formula: (number of unstained cells/total number of cells) × 100%.

Annexin V-fluorescein isothiocyanate/PI assay for apoptosis

The redistribution of phosphatidylserine to the outer leaflet of the plasma membrane, which indicates the early stage of apoptosis, was detected by incubating neutrophils with fluorescein isothiocyanate (FITC)-conjugated annexin V. Cells that had lost the integrity of their plasma membrane (i.e. necrotic and late apoptotic cells) were detected by PI staining. After 8 h of incubation with ANE at 37°C, cells were washed and resuspended in 100 μL of 1× binding buffer containing 5 μL of annexin V–FITC and 4 μg/mL of PI, then left to sit at room temperature (20–25°C) in the dark for 10 min according to the manufacturer's instructions (Annexin V–FITC kit; Clontech Laboratories, Palo Alto, CA, USA). The cells were washed and resuspended in PBS, then passed through a nylon filter (Spectrum®). Stained cells were kept on ice and subjected to flow cytometry analyses (FACSort™; Becton Dickinson). Green fluorescence (FITC) and red fluorescence (PI) were collected. The fluorescence intensities of a total of 10,000 cells were measured. Quadrant settings were based on the negative controls for each concentration of ANE examined. The lower left quadrant denotes cells that were negative for both PI (red fluorescence) and annexin V–FITC (green fluorescence) staining (viable cells). The lower right quadrant denotes cells stained mainly by annexin V–FITC (early apoptotic cells). The upper left quadrant represents cells stained mainly by PI (primary necrotic cells), while the upper right quadrant represents cells stained by both PI and annexin V–FITC (late apoptotic/necrotic cells). The percentage of cells in each quadrant was calculated.

DNA content analysis

For determination of late stages of apoptotic cell death, apoptotic hypodiploid nuclei were detected using the flow cytometry analysis (28). Neutrophils were treated with various concentrations of ANE for 8 h. After washing once with HBSS, neutrophils were fixed with 1 mL of 70% ethanol precooled to −20°C and were then stored at −20°C until required for further analyses. Fixed neutrophils were washed with PBS and incubated in PBS containing 0.1% Triton X-100, 0.2 mg/mL of RNase A and 20 μg/mL of PI for 15 min at room temperature (20–25°C) in the dark. Neutrophils were washed and resuspended in 1 mL of PBS and analyzed using a flow cytometer. According to the DNA contents, cell cycle distribution was divided into four phases, sub-G1, G0/G1, S and G2/M phases.

Western blotting analysis

Neutrophils were incubated with ANE (0, 6.25, 12.5 or 25 μg/mL) for various periods of time at 37°C. Treated cells were lysed with the lysis buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris–HCl (pH 7.5), 100 mM NaCl and 1× protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)]. For detection of phosphorylated proteins, the lysis buffer also contained 100 mM NaF and 100 mM Na_3VO_4 as phosphatase inhibitors. Cell lysates were analyzed by electrophoresis on a 10% or 12% sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred onto a polyvinylidene difluoride membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA) and the membrane was immunoblotted with polyclonal antibody against cleaved PARP (Asp214) (1:1000 dilution) (Cell Signaling Technology, Inc., Danvers, MA, USA), caspase-3 (1:1000 dilution) (Cell Signaling Technology), caspase-8 (1:3000 dilution) (BD Pharmingen, San Diego, CA, USA) and the phosphorylated GSK-3α/β (Ser21/9) (1:1000 dilution) (Cell Signaling Technology) or with monoclonal antibody against both phosphorylated and nonphosphorylated GSK-3α/β (0.2 μg/mL) (sc-7291) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or against β-actin (1:2500 dilution) (Chemicon Inc., Temecula, CA, USA) at room temperature (20–25°C) for 1 h. Each membrane was then incubated with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (1:2000 dilution) (Cell Signaling Technology) or with goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (1:1000 dilution) (Chemicon Inc.). Between each step, the membranes were washed with the blocking buffer [20 mM Tris-HCl (pH7.6), 137 mM NaCl, 0.1% Tween-20 and 5% dry milk]. Proteins were revealed using the enhanced chemiluminescence detection system (Super signal®; West Pico Chemiluminescent substrate; Pierce Biotechnology Inc., Rockford, IL, USA). Band intensities were quantified using a densitometer. The signals of cleaved PARP, and of the proform and cleaved forms of caspase-3 and caspase-8, were normalized to those of β-actin. The signals of phosphorylated GSK-3α and GSK-3β were normalized to those of total GSK-3α and GSK-3β, respectively. The expression from the control cells was designated as 1. The relative expression was then calculated.

Statistical analysis

All values were expressed as mean ± standard error of the mean. Differences between the experimental sample and buffer-treated neutrophils in the absence or presence of inhibitors were analyzed using the paired *t*-test. Each experiment was repeated independently, at least three times, using neutrophils from different blood donors

to ensure reproducibility. A p-value of < 0.05 was considered to represent a significant difference.

Results

Effects of ANE on size, granularity and viability of neutrophils

The effects of ANE on the size, granularity and viability of neutrophils were analyzed using flow cytometry. Changes in light scatter profiles were observed when neutrophils were treated with ANE (Fig. 1A). The size and granularity of ANE-treated neutrophils increased slightly when compared with the control. Viable cells exhibited low background fluorescence (Fig. 1B, a). The mean background fluorescence increased when neutrophils were treated with 25 μg/mL of ANE for 8 h (Fig. 1A, d). The treatment of neutrophils with ANE impaired the ability of neutrophils to exclude PI in a dose-dependent manner (Fig. 1B): the percentage was reduced from 98.38 ± 0.84% to 85.94 ± 3.34% ($p = 0.04$) and to 73.94 ± 2.74% ($p = 0.007$) when 12.5 and 25 μg/mL of ANE were used, respectively (Fig. 1B, e). However, there was no difference in PI exclusion between control cells and neutrophils exposed to 6.25 μg/mL of ANE.

The effects of ANE on apoptosis and necrosis of neutrophils were further determined using double-staining with annexin V-FITC and PI followed by flow cytometry analysis. A higher percentage of nonstimulated neutrophils became apoptotic, reaching 35.66 ± 5.97% (Fig. 1C, b). The percentage of apoptotic cells was reduced to 13.97 ± 3.54% ($p = 0.023$), 8.69 ±

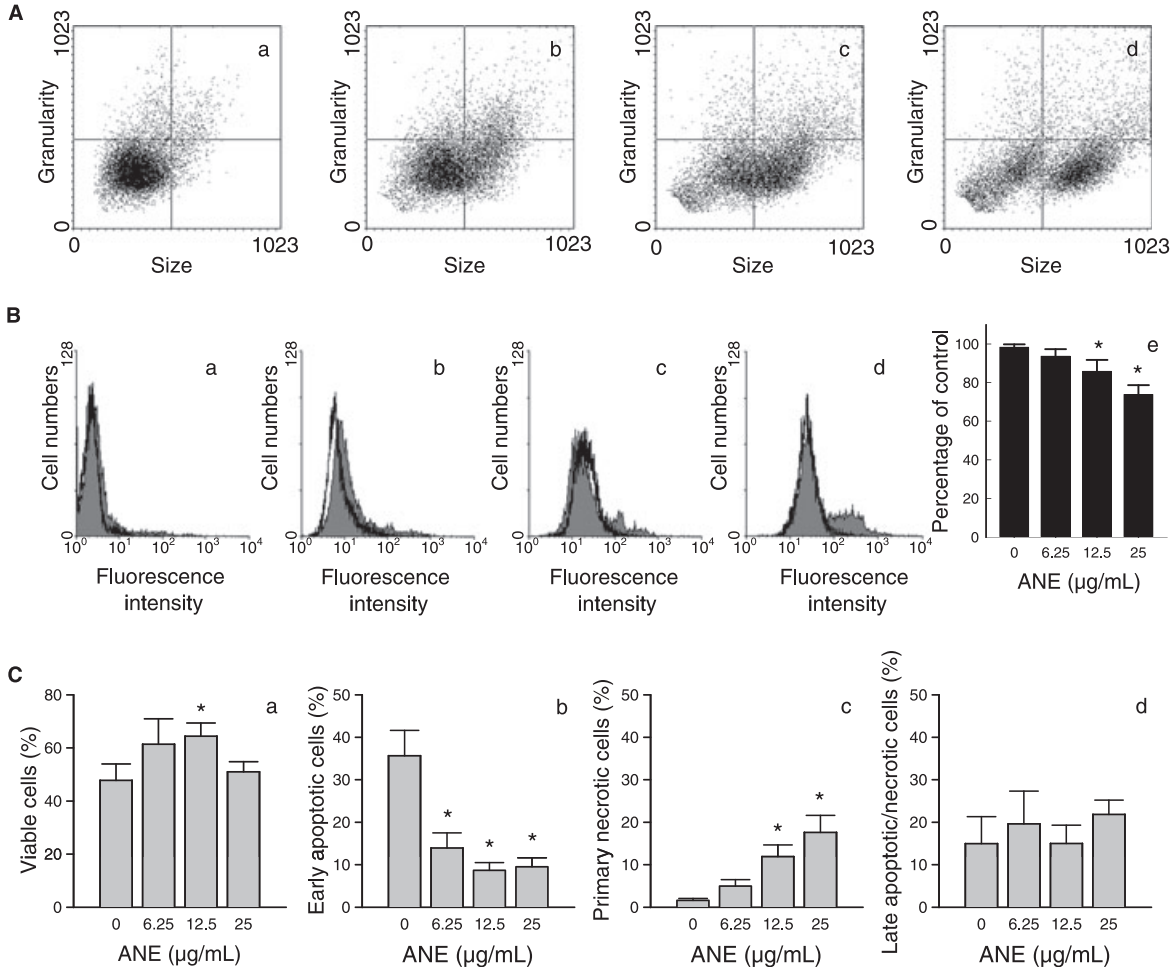

Fig. 1. Effects of areca nut extract (ANE) on the size, granularity and viability of neutrophils. (A) The forward scatter (size), sideward scatter (granularity) signals and (B) the propidium iodide (PI) staining of neutrophils treated with (a) 0, (b) 6.25, (c) 12.5 or (d) 25 μg/mL of ANE for 8 h are the representative results of three independent experiments. (B) In the illustrated histograms, black lines represent the fluorescence intensity of neutrophils incubated without PI, whereas grey areas represent the fluorescence intensity of neutrophils incubated with PI. (e) The abilities (expressed as percentage of control) of neutrophils to exclude PI, determined from the results of three independent experiments, are shown as mean ± standard error of the mean (SEM). A significant difference ($p < 0.05$) compared with control neutrophils is indicated with an asterisk. (C) Treated neutrophils were also analyzed by annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining and flow cytometry. The percentages of (a) viable, (b) early apoptotic, (c) primary necrotic and (d) late apoptotic/necrotic cells from five independent experiments are shown as mean ± SEM. A significant difference ($p < 0.05$) compared with control neutrophils in the same quadrant is indicated with an asterisk.

1.80% (p = 0.008) or 9.50 ± 2.15% (p = 0.023) when 6.25, 12.5 or 25 μg/mL of ANE was used, respectively. In parallel to the reduction of neutrophil apoptosis, exposing neutrophils to ANE produced a significant increase in the percentage of cells undergoing primary necrosis without first initiating apoptosis or expressing phosphatidylserine. The percentages of primary necrotic cells were significantly increased from 1.59 ± 0.49% to 4.97 ± 1.54%, 11.89 ± 2.81% (p = 0.021) and 17.63 ± 3.99% (p = 0.017) (Fig. 1C, c). The results indicated that ANE reduced the percentage of cells that underwent apoptosis, but increased those that underwent primary necrosis.

Effects of ANE on cell cycle distribution of neutrophils

The apoptosis-suppressing effect of ANE was further confirmed when the later event of apoptosis induction was analyzed. Cell cycle distribution was determined using PI staining and flow cytometry. Exposure of neutrophils to ANE led to an increased number of cells being arrested in the G0/G1 phase, but fewer cells in the sub-G1 phase (Fig. 2). When 25 μg/mL of ANE was used, the percentage of cells in the sub-G1 phase was reduced from 28.30 ± 5.23% to 8.43 ± 0.68%, whereas that in the G0/G1 phase was increased from 59.58 ± 6.29% to 83.84 ± 2.14% (Fig. 2B). Thus, the results indicate that ANE may arrest cells in the G0/G1 phase and reduce the apoptotic hypodiploid DNA contents in neutrophils.

Effects of ANE on PARP, caspases and GSK-3

The amounts of the activated (cleaved) forms of PARP, caspase-3 and caspase-8 were decreased after treatment with ANE for 8 h (Fig. 3A). The cleaved forms of caspase-3 and caspase-8 were barely detectable when 25 μg/mL of ANE was used. The levels of cleaved forms of PARP (Fig. 3A, a), caspase-3 (Fig. 3A, c) and caspase-8 (Fig. 3A, e) were reduced significantly (p < 0.05) to 0.33-, 0.01- and 0.44-fold when 25 μg/mL of ANE was used, respectively. In addition, the proforms of caspase-3 (Fig. 3A, b) and caspase-8 (Fig. 3A, d) increased significantly (p < 0.05) when 25 μg/mL of ANE was used.

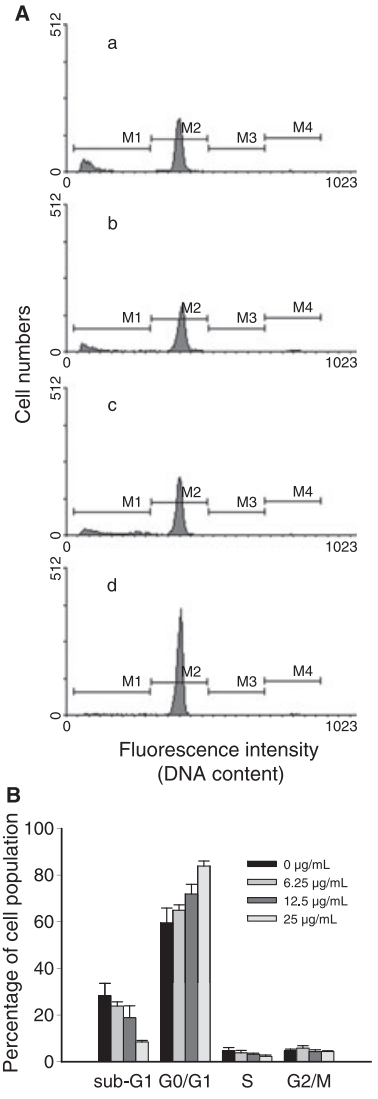

Fig. 2. Effects of areca nut extract (ANE) on the cell cycle phase distribution in neutrophils. Neutrophils were exposed to (a) 0, (b) 6.25, (c) 12.5 or (d) 25 μg/mL of ANE for 8 h followed by propidium iodide (PI) staining and cell cycle analysis. (A) The histograms illustrated are the representative results of three independent experiments. M1, M2, M3 and M4 indicate sub-G1, G0/G1, S and G2/M phases, respectively. (B) The percentages of the cell population in different phases of the cell cycle are presented as mean ± SEM. The results were averaged from three independent experiments.

To determine the possible mechanisms involved in the effects of ANE, several inhibitors were used to analyze whether cleavage of caspase-3 reduced by ANE could be reversed. The PI3K inhibitor (LY294002) (Fig. 3B), the LTB_4 inhibitor (MK886) and the NADPH oxidase inhibitor (DPI) (data not shown) did not affect the reducing effects of ANE on the cleavage of caspase-3 in neutrophils.

The effects of ANE on the phosphorylation of GSK-3α and GSK-3β were also determined. The total amounts of GSK-3α and GSK-3β were not altered when incubated with buffer only for 15 or 30 min. However, phosphorylation of GSK-3α and GSK-3β was activated by ANE (Fig. 3C). The relative intensity of phosphorylated GSK-3α (Fig. 3C, a) and GSK-3β (Fig. 3C, b) increased when compared with that of control neutrophils.

Effects of ANE on neutrophils in the presence of GSK-3 inhibitor X

The GSK-3 inhibitors, GSK-3 inhibitor X and SB 216763, were further used to determine whether GSK-3 is involved in the modulation of apoptosis in ANE-treated neutrophils. The apoptosis-suppressing effects of ANE, with or without pretreatment of the GSK-3 inhibitors, were determined using annexin V–FITC and PI staining methods. In the absence of the GSK-3 inhibitor X, the percentage of primary necrotic cells increased significantly from 1.63 ± 0.38% to 10.11 ± 2.03% (p = 0.014) or to 27.02 ± 8.28% (p = 0.049) when 12.5 or 25 μg/mL of ANE was used, respectively (Fig. 4A, c). In the presence of the GSK-3 inhibitor X, the percentage of primary necrotic cells was lower by comparison with neutrophils in the absence of the inhibitor when 25 μg/mL of ANE was used (Fig. 4A, c). Similar results were observed for another GSK-3 inhibitor, SB 216763 (Fig. 4B, c). The GSK-3 inhibitors partially reduced the modulating effects of ANE on neutrophils. Thus, phosphorylation of GSK-3 may be involved partly in the ANE-modulated effects on apoptosis and necrosis of neutrophils.

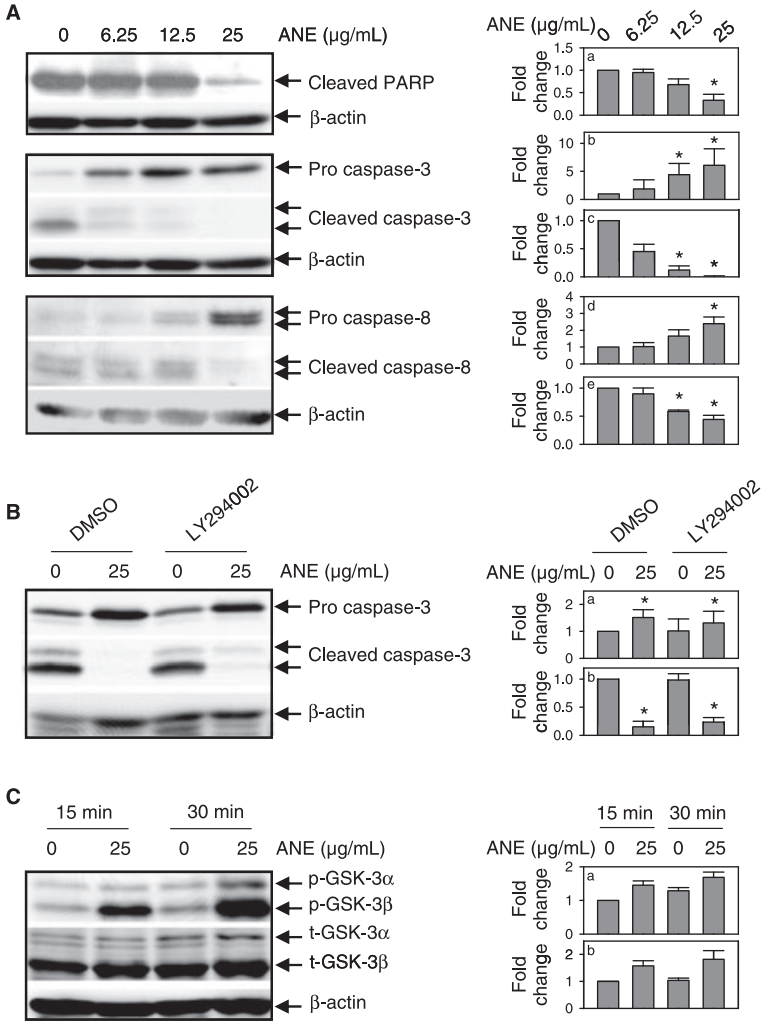

Fig. 3. Effects of areca nut extract (ANE) on enzymes involved in apoptosis. Neutrophils were incubated with (A) various concentrations of ANE (0, 6.25, 12.5, or 25 μg/mL) for 8 h at 37°C, with (B) ANE (0 or 25 μg/mL) in the presence of the phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002, 20 μM) or equivalent volumes of the solvent [dimethylsulfoxide (DMSO)] for 8 h, or with (C) ANE (0 or 25 μg/mL) for 15 or 30 min at 37°C. Cell lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis using antibodies against cleaved poly(ADP-ribose) polymerase (PARP), the proform or the cleaved form of caspase-3 and caspase-8, phosphorylated glycogen synthase kinase (GSK)-3α/β or total GSK-3α/β. Beta-actin was the internal control. The images shown are the representative results of three independent experiments. Band densities were quantified using a densitometer. The expression relative to that found in control neutrophils, from three independent experiments, is shown for (A) (a, cleaved PARP; b, pro caspase-3; c, cleaved caspase-3; d, pro caspase-8; e, cleaved caspase-8); (B) (a, pro caspase-3; b, cleaved caspase-3); and (C) (a, p-GSK-3α; b, p-GSK-3β). Significant differences ($p < 0.05$) compared with control neutrophils are indicated with an asterisk.

Discussion

Neutrophils play an important role in the initial host immune system. Spontaneous apoptosis of neutrophils has been observed both *in vitro* and *in vivo* (18,29). With a high percentage of cells undergoing spontaneous apoptosis, neutrophils have a rapid turnover rate in the circulation. Apoptotic neutrophils can be phagocytosed by macrophages, leading to a shift in macrophage phenotype from activated to reparative (30). By contrast, necrosis of neutrophils represents a more pathological form of cell death, characterized by loss of membrane integrity and leakage of cellular contents (31,32). This *in vitro* study investigated the possible effects of ANE on the apoptosis of neutrophils. The results indicated that ANE may reduce the spontaneous apoptosis of neutrophils, but increase the proportion of necrotic cells concomitantly. ANE might cause tissue damage by releasing higher levels of protease and reactive oxygen species during necrosis. As a more vigorous inflammatory response may be generated in tissue during the process of necrosis, these results suggested unfavorable effects of ANE in areca nut chewers.

The effects of ANE on cellular apoptosis have been examined in several studies. Treatment with ANE for 24 h may induce apoptosis in HaCaT cells (26) and in KB epithelial cells (4). In epithelial cells, treatment with ANE leads to arrest in the G2/M phase (27). However, the results of the present study showed that the number of apoptotic neutrophils was decreased by treatment with ANE. In addition, ANE reduced the percentage of sub-G1 neutrophils and induced arrest in the G0/G1 phase. Neutrophils in the sub-G1 phase are cells with hypodiploid DNA, which represents cells undergoing apoptosis. Obvious, different effects of ANE were observed with regard to regulation of the cell cycle. These discrepancies may be a result of differences in cell types examined, incubation time, culture conditions, or preparation protocols for ANE. Nevertheless, the physiologic regulations in the cell cycle are highly variable among different cell types. In the absence of noxious stimuli, neutrophils are committed to undergo apoptosis in normal physiologic condition. *In vitro* studies have demonstrated that apoptosis of neutrophils is inhibited by a wide range of inflammatory stimuli (33,34). Delayed apoptosis of neutrophils may also promote inflammation (35). Thus, reduced apoptosis of neutrophils by treatment with ANE might suggest the presence of a proinflammatory signal.

Two main pathways are involved in apoptotic cell death: one is known as extrinsic, which is initiated through the

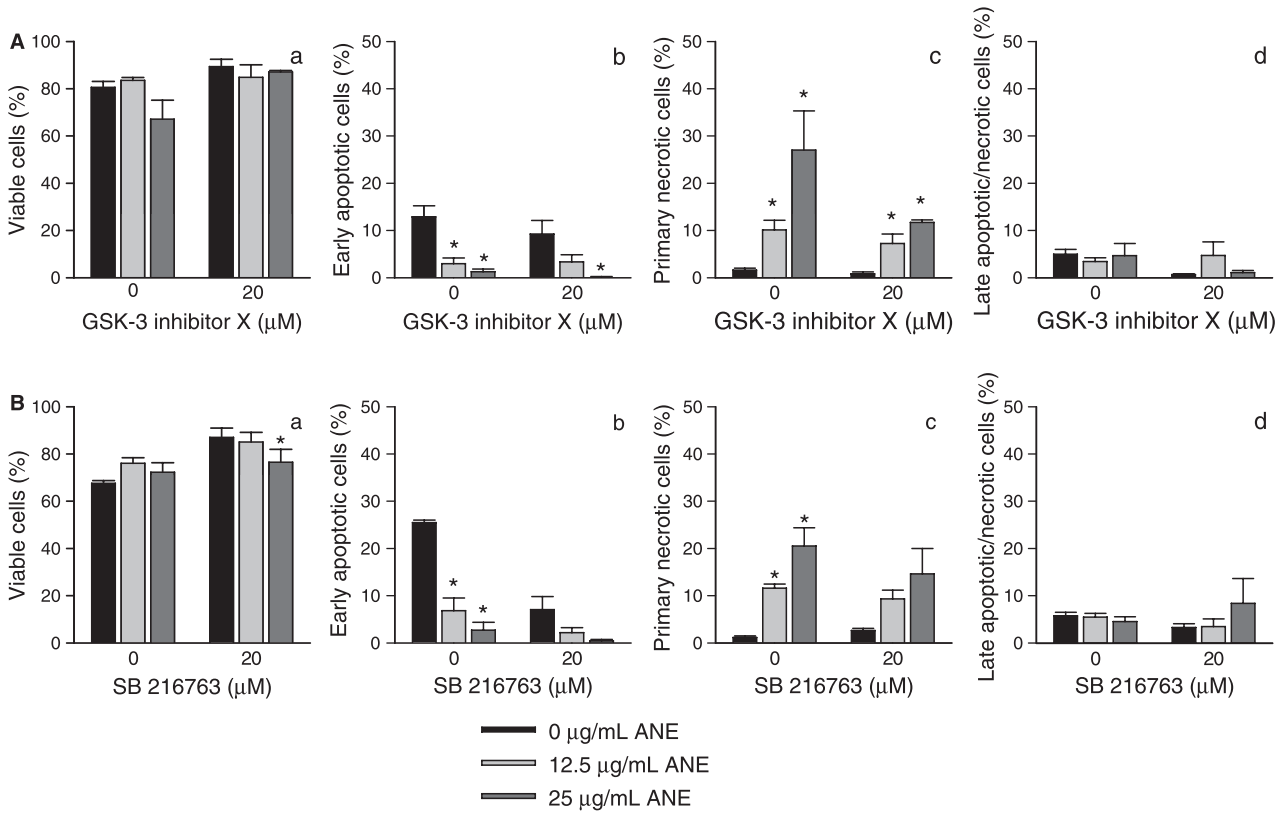

Fig. 4. Effects of the glycogen synthase kinase (GSK-3) inhibitors and areca nut extract (ANE) on the apoptosis and necrosis of neutrophils. Neutrophils were exposed to ANE in the presence or absence of (A) the GSK-3 inhibitor X or (B) SB 216763 for 8 h followed by annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining and flow cytometry. The percentages of (a) viable, (b) early apoptotic, (c) primary necrotic and (d) late apoptotic/necrotic cells, obtained from at least three independent experiments, are shown as mean ± standard error of the mean. For each figure, a significant difference ($p < 0.05$) compared to control neutrophils treated with the same concentration of inhibitor is indicated by insertion of an asterisk.

interaction of death receptors, such as Fas or TNF receptors with their ligands; the other pathway is known as the intrinsic pathway and requires the participation of mitochondria (36). It has been reported that proinflammatory cytokines such as IL-1β, TNF-α and IL-6 are able to modulate the survival of neutrophils (37). In addition, IL-8 has been shown to delay neutrophil apoptosis through the extrinsic pathway (38,39). The results of the present study showed that constitutive neutrophil apoptosis is impacted by ANE. ANE has been demonstrated to induce the expression of the inflammatory cytokines, TNF-α and IL-6, in both oral epithelial cells and peripheral blood mononuclear cells (4,6). Further studies are needed to confirm whether cytokine signals are involved in the reduction of neutrophil apoptosis induced by ANE.

Caspases are proteases that participate in both pathways as essential regulatory factors. It has been shown that inhibition of caspase activity could lead to the reduction of apoptosis, but increase primary necrosis (40,41). Caspase-8 is considered as the key initiator of death-receptor-mediated apoptosis, while caspase-3 is an important downstream effector caspase that cleaves major cellular substrates in apoptotic cells (42). Both caspase-3 and caspase-8 play critical roles in neutrophil apoptosis, and activation of these caspases is observed in freshly isolated neutrophils (43). In this study, exposure of neutrophils to ANE suppressed the activation of caspase-3 and caspase-8. However, the PI3K inhibitor (LY294002), LTB_4 inhibitor (MK886) and NADPH oxidase inhibitor (DPI) failed to reverse the suppression of caspase-3 activity regulated by ANE. These results indicate that ANE may reduce neutrophil apoptosis through mechanisms other than the PI3K signaling pathway.

It has been suggested that phosphorylation cascades, including phosphorylation on tyrosine, serine and threonine residues, may be important in the intracellular signaling control of neutrophil apoptosis (44). GSK-3 is a constitutively active serine–threonine kinase that participates in several cellular processes, including cell membrane-to-nucleus signaling, gene transcription and cell survival (23,24). In addition, GSK-3 plays roles in the apoptotic signaling pathway (24). It has been reported that active GSK-3 induces apoptosis by activating the mitochondrial death pathway and inducing cleavage of caspases (45,46). Moreover, active GSK-3 phosphorylates various molecules, including glycogen synthase,

β-catenin, c-Jun, c-Myc, cAMP response element binding protein and Tau (microtubule binding protein) (24). The results of the present study showed that GSK-3 phosphorylation was increased after treatment with ANE. Phosphorylation of GSK-3 may reduce apoptosis through the anti-apoptotic proteins MCL-1 and Bcl-2 (47,48). This study also suggested that phosphorylation of GSK-3 may play a part in the ANE-modulated effects of neutrophils. However, because the inhibitors used in this study did not completely abolish the effects of ANE, the definite mechanisms involved remain to be elucidated.

The alteration of neutrophil apoptosis is associated with inflammation in systemic diseases (49). To the best of our knowledge, this is the first report to demonstrate that exposure to ANE activates the anti-apoptotic signaling pathway and reduces spontaneous apoptosis in neutrophils. These findings are in line with previous reports showing that ANE may enhance local inflammation and induce the production of proinflammatory cytokines (5,6). The concentration of arecoline, the major component in areca nut, in saliva during areca chewing is about 140 μg/mL (50). Thus, the concentrations of ANE used in this study would be present in the gingival tissues and crevicular fluid of areca chewers. Taken together, the results suggest that ANE may alter the functions of immune cells. This could be one of the possible mechanisms by which ANE compromises the defense system of areca nut chewers.

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