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Areca nut extracts modulated expression of alkaline phosphatase and receptor activator of nuclear factor κB ligand in osteoblasts

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Abstract

Objectives: Areca chewers have a higher prevalence of periodontal diseases than non-chewers. This study was to determine the possible effects of ripe areca nut extracts (rANE) on viability and gene expression of alkaline phosphatase (ALP), receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) in human osteoblasts.

Methods: The effects of rANE on cell viability of osteoblast-like MG63 cells were determined using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) that measures metabolic activity. Gene expression of ALP, RANKL and OPG was examined using reverse transcription-polymerase chain reaction. ALP activity and RANKL protein were further examined using substrate assay and confocal laser scanning microscopy, respectively.

Results: Relative viability was reduced to approximately 50% when 25 μg/ml of rANE was used. The expression of OPG mRNA in MG63 cells was not altered by rANE. However, decreased levels of mRNA and enzyme activity of ALP were observed. Moreover, the expressions of mRNA and protein of RANKL were stimulated by rANE in a dose-dependent manner.

Conclusions: The rANE affected morphology and viability of osteoblasts. We also present novel evidence demonstrating that areca nut may compromise the periodontal health of areca chewers by suppression of ALP gene expression and elevation of RANKL gene expression in osteoblasts.

Key words: alkaline phosphatase; areca nut; cytotoxicity; osteoblasts; osteoprotegerin; receptor activator of nuclear factor-κB ligand

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Areca (betel) chewing is associated with an increased risk of oral squamous cell carcinoma and oral submucous fibrosis (Jeng et al. 2001). Studies have also shown a higher prevalence of periodontal disease among areca chewers than non-areca chewers (Mehta et al. 1955, Waerhaug 1967). Poorer plaque control might explain the higher prevalence of periodontal disease in areca chewers (Mehta et al. 1955). However, at the same plaque level, areca chewers have a higher periodontal index than non-areca chewers (Waerhaug 1967). This result suggests a direct influence of areca chewing on periodontal health, irrespective of plaque infection (Waerhaug 1967). Areca chewing is associated with a higher prevalence of bleeding on probing where higher clinical levels of disease existed, and with a likelihood of subgingival infection with periodontal pathogens, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* (Ling et al. 2001). The overall periodontitis levels are significantly higher in areca chewers and smokers than in non-tobacco users studied in Sri Lanka (Amarasena et al. 2002). Areca chewing also significantly enhances gingival bleeding in the population studied in Sri Lanka (Amarasena et al. 2003). In addition, areca quid-

chewers (current and former) have a significantly higher risk of chronic periodontitis compared with control subjects who never chew areca quid (Teng et al. 2003).

Epidemiological and experimental studies have revealed the genotoxic and carcinogenic potencies of areca nut, a major ingredient in areca quid (Jeng et al. 2001). Areca nut extracts inhibit growth, attachment and matrix protein synthesis of cultured gingival fibroblasts (Chang et al. 1998). Areca nut extracts enhance the production of prostaglandin and cyclooxygenase-2 of human gingival keratinocytes (Jeng et al. 2000) and may be associated with tissue inflammatory responses. Areca nut extracts also impair T cell activation and induce tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production in gingival keratinocytes (Jeng et al. 2003). Areca nuts may be responsible to inflammatory damage to oral tissues in areca chewers. Areca nut extracts also reduce the defensive functions of neutrophils (Hung et al. 2000), suggesting that areca nuts might promote bacterial colonization and periodontal infection. This effect may contribute to a less efficient elimination of bacteria from the periodontal environment.

Little is known about the possible influence of areca nuts on osteoblasts. Alkaline phosphatase (ALP) is one of the markers of osteoblastic differentiation. Receptor activator of nuclear factor-κB ligand (RANKL), produced by osteoblasts, is a key factor regulating osteoclast formation (Lacey et al. 1998, Yasuda et al. 1998). Osteoprotegerin (OPG), a naturally occurring inhibitor of osteoclast differentiation, binds to RANKL and blocks RANKL from interacting with RANK (Lacey et al. 1998, Yasuda et al. 1998). The purpose of this study was to determine whether ripe areca nut extract (rANE) could influence the functions of osteoblasts. The effects of rANE on human osteoblast-like MG63 cells were determined through measuring cell viability and gene expression of ALP, RANKL and OPG.

Material and Methods

Cell culture

The human osteoblast-like cell line (MG63) (Franceschi et al. 1985) retains markers of osteoblastic differentiation, such as activity of ALP and production of osteocalcin (Lincks et al. 1998, Pan et al. 1998). MG63 cells were propagated in minimum essential medium (MEM) supplemented with 10% foetal calf serum and antibiotic–antimycotic solution containing penicillin, streptomycin and amphotericin B (Gibco BRL, Rockville, MD, USA).

Cell viability experiments

The rANE was extracted from dried ripe areca nuts without husk as previously described (Nair et al. 1987, Liu et al. 1996). The rANE was first dissolved in dimethyl sulphoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA) before being diluted in MEM. The final concentration of DMSO in each sample was less than 0.5%. MG63 cells (1.25×10^4/100 µl) were inoculated into each well of 96-well tissue culture plates and cultured for 20 h at 37°C. Cells were then treated with various concentrations of rANE (12.5, 25, 50, 100 µg/ml) at 37°C. Cells treated with media only under similar conditions served as the controls. After 24 h of incubation, cellular morphology was examined under the inverted microscope. Unattached cells were then removed by phosphate-buffered saline (PBS) washes. Viability of attached MG63 cells after rANE treatment was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (Mossman 1983, Pellen-Mussi et al. 1997) to determine the activity of mitochondrial dehydrogenase. The background signal inherent to the plates when no cells were present was subtracted from the absorbance obtained from viable cells. The optical density (OD) of control cells was considered to be 100%. The relative viability of MG63 cells was calculated by the formula: (OD of experimental sample/OD of control cells) × 100%. Experiments were performed in duplicate, and the relative viability was averaged from three independent experiments.

RNA purification and reverse transcription-polymerase chain reaction (RT-PCR)

MG63 cells (8×10^5) were treated with various concentrations of rANE (12.5, 25, 50 µg/ml) at 37°C. Cells treated with media only under similar conditions served as the controls. After 24 h of incubation, unattached cells were removed by PBS washes. Total cellular RNA was extracted using the Trireagent-RNA® isolation reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) as instructed by the manufacturer. Expression of mRNA was analysed by RT-PCR using the Superscript™ one-step™ RT-PCR system, which consists of Superscript II RT/Platinum® Taq DNA polymerase (Gibco BRL). Each RT-PCR reaction (50 µl) contained 50 ng of RNA sample, 10 pmol each of two primers (Table 1), 1 µl Superscript II RT/Taq DNA polymerase and 1 × reaction mixture containing 0.2 mM of each dNTP and 1.2 mM $MgSO_4$ (for ALP, RANKL, β-actin) or 1.5 mM $MgSO_4$ (for OPG, GAPDH, 28S rRNA). Samples were initially incubated at 50°C for 30 min. to generate cDNA followed by one cycle of denaturing at 95°C for 5 min. To quantify various mRNA present in MG63 cells treated with or without rANE, the kinetics of accumulation of PCR products was initially analysed to determine the optimal condition for each gene. The amplification was per-

Table 1. Oligonucleotide primers used in RT-PCR

Target gene	Nucleotide sequence	Size in bp	References
ALP	5′–ACGTGGCTAAGAATGTCATC–3′ 5′–CTGGTAGGCGATGTCCTTA–3′	475	Rickard et al. (1996)
RANKL	5′–GCCAGTGGGAGATGTTAG–3′ 5′–TTAGCTGCAAGTTTTCCC–3′	486	Granchi et al. (2002)
OPG	5′–GCTAACCTCACCTTCGAG–3′ 5′–TGATTGGACCTGGTTACC–3′	324	Granchi et al. (2002)
β-actin	5′–ACACTGTGCCCATCTACGAGG–3′ 5′–AGGGGCCGGACTCGTCATACT–3′	621	Chang et al. (2000)
GAPDH	5′–TGGTATCGTGGAAGGACTCATGAC–3′ 5′–ATGCCAGTGAGCTTCCCGTTCAGC–3′	190	Nobori et al. (1994)
28S rRNA	5′–GTTCACCCACTAATAGGGAACGTGA–3′ 5′–GGATTCTGACTTAGAGGCGTTCAGT–3′	212	Lambert et al. (2001)

RT-PCR, reverse transcription-polymerase chain reaction; ALP, alkaline phosphatase; RANKL, receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin.

formed using the following profile for each gene: ALP (32 cycles of 94°C for 30 s, 55°C for 2 min. and 72°C for 2 min.); RANKL (42 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min.); OPG (35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min.); β-actin (20 cycles of 95°C for 1 min., 54°C for 1 min. and 72°C for 1 min.); GAPDH (29 cycles of 95°C for 1 min., 54°C for 1 min., and 72°C for 1 min.); and 28S rRNA (15 cycles of 94°C for 1 min., 66°C for 1 min. and 72°C for 1 min.). A final extension was performed at 72°C for 10 min. Reaction without RNA was served as a negative control. The PCR amplified fragments were visualized in a 2% agarose gel stained with ethidium bromide. The intensity of each band was quantified by densitometer. The expression of each gene was normalized by its GAPDH expression. The expression of each gene in control cells without rANE treatment was considered to be 100%. The relative expression was calculated.

ALP activity

The effects of rANE on the ALP activity, an osteogenic property, of MG63 cells were determined (Kuru et al. 1998, Kuru et al. 1999). MG63 cells were seeded into each well of 96-well tissue culture plates in full culture medium. The ALP activity was determined colorimetrically using an ALP kit 104-LL as described by the manufactures (Sigma Chemical Co.) (Kuru et al. 1998, Kuru et al. 1999). The ALP activity was compared with the standard curve derived from *p*-nitrophenol solution (Sigma Chemical Co.). The OD of control cells was considered to be 100%. The relative ALP activity of MG63 cells was calculated by the formula:(OD of experimental sample/ OD of control cells) $\times$ 100%. Experiments were performed in duplicate, and the relative viability was averaged from three independent experiments.

Confocal laser scanning microscopy

MG63 cells on the chamber slide (Lab-Tek® II, Nalge Nunc International, Rochester, NY, USA) were incubated with 0, 12.5, 25 or 50 µg/ml of rANE for 24 h at 37°C. Cells were fixed with 4% paraformaldehyde (15 min.); permeabilized with 0.5% Triton X-100 in 4% paraformaldehyde (15 min.), washed twice in PBS; and blocked with blocking reagent (Dako A/S, Glostrup, Denmark) for 30 min. at 37°C. Rabbit polyclonal antibody against RANKL (Chemicon International, Inc., Temecula, CA, USA) was diluted 1:100 in blocking reagent and mouse monoclonal antibody against GAPDH (Chemicon) was diluted in 1:250. Cells were incubated with primary antibodies at 37°C for 30 min., followed by washing twice in PBS. Cells were further incubated at 37°C for 30 min. with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate isomer (GAR-FITC) (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or goat anti-mouse IgG-FITC (GAM-FITC) (1:400) (Sigma Chemical Co.). Cells were viewed using a Leica TCS NT confocal laser-scanning microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with an argon–krypton mixed gas laser. As control experiment, immunostaining with secondary antibody only was also performed.

Statistical analysis

All values were expressed as mean $\pm$ standard deviation (SD). Quantitative analysis was carried out using Student's *t*-test.

Results

Effects of rANE on morphology and viability of MG63 cells were determined. Microscopic evaluation of MG63 cells exposed to rANE showed morphologic characteristics that did not appear normal (Fig. 1). Numerous intra-cellular vacuoles appeared when cells were exposed to rANE for 24 h. Pyknosis, rounded, swelled, irregular cell membrane borders and loss of attachment to the dish were evident (Fig. 1). Addition of rANE at concentration of 25, 50 and 100 µg/ml significantly decreased the cellular viability in a dose-dependent manner ($p < 0.01$) (Fig. 2). Approximately 50% of the cellular viability was affected when 25 µg/ml of rANE was used (Fig. 2).

Effects of rANE on the expression of ALP, RANKL, OPG, β-actin, GAPDH and 28S rRNA were determined by RT-PCR of RNA extracted from MG63 cells with specific primers. No PCR product was detected for the negative control (data not shown). OPG, β-actin, GAPDH and 28S rRNA were amplified at similar levels in MG63 cells after treated with various concentrations of rANE (Fig. 3). The expression of ALP mRNA was inhibited by rANE; whereas the expression of RANKL mRNA was enhanced by rANE in a dose-dependent manner. Messenger RNA expression levels of each gene relative to mRNA expression levels of GAPDH were shown in Fig. 4. The relative expression of ALP mRNA reduced to 39 $\pm$ 14% when 50 µg/ml of rANE was used ($p = 0.002$) (Fig. 4a). However, the relative expression of RANKL was enhanced to 291 $\pm$ 94% ($p = 0.025$) and 423 $\pm$ 197% ($p = 0.047$) when 25 and 50 µg/ml of rANE was used, respectively

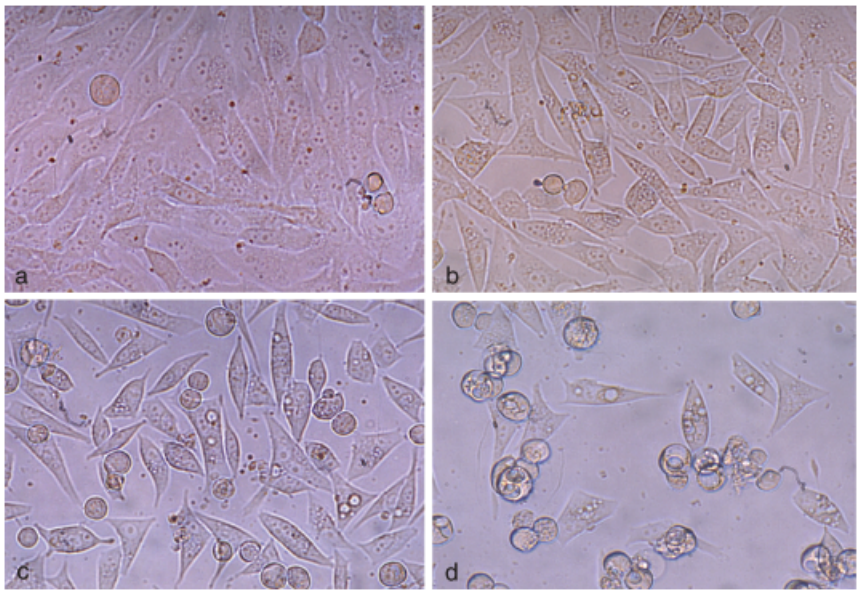

Fig. 1. Effects of ripe areca nut extracts (rANE) on cellular morphology. The results shown are MG63 cells incubated with (a) 0, (b) 12.5, (c) 25 or (d) 50 µg/ml of rANE for 24 h.

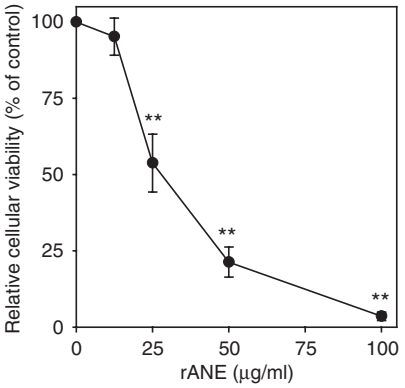

Fig. 2. Effects of ripe areca nut extracts (rANE) on the relative viability of MG63 cells as determined by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide assay. The percentages of cellular viability in the presence of rANE relative to that in the control are shown. The results were averaged from three independent experiments. Standard deviation (SD) is shown as an error bar (**$p < 0.01$).

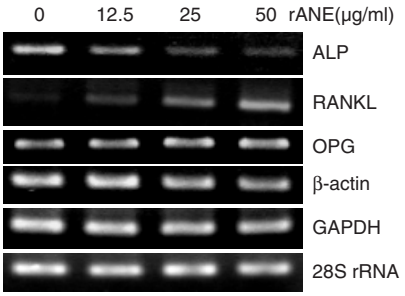

Fig. 3. Detection of mRNAs by reverse transcription-polymerase chain reaction (RT-PCR) assay. RT-PCR products were amplified from cDNAs of the MG63 cells treated with various concentrations of rANE. Agarose gel electrophoresis of RT-PCR-amplified sequences specific for alkaline phosphatase (ALP), receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), β-actin, GAPDH, and 28S rRNA, is shown.

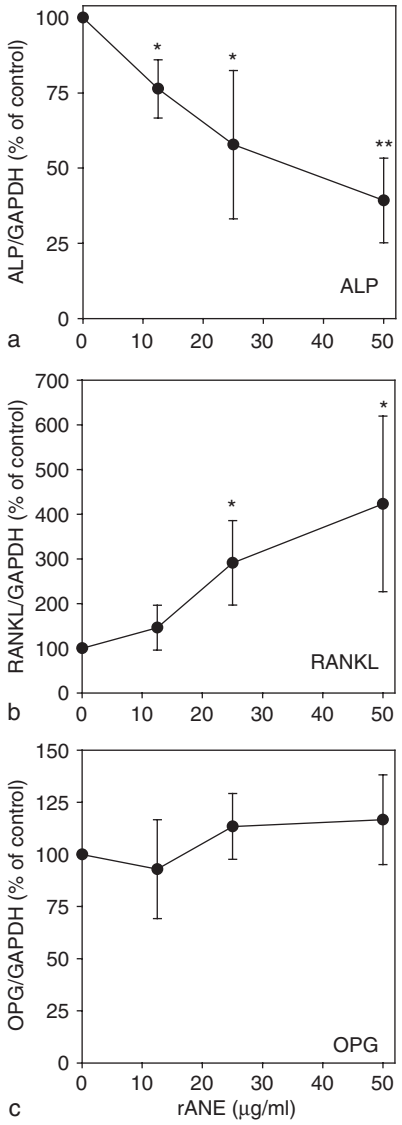

Fig. 4. Effects of ripe areca nut extracts (rANE) on the relative expression of mRNA. The percentages of mRNA expression relative to that in the control are shown for (a) alkaline phosphatase (ALP), (b) receptor activator of nuclear factor-κB ligand (RANKL) and (c) osteoprotegerin (OPG). The results were averaged from three independent experiments (*$p < 0.05$; **$p < 0.01$)

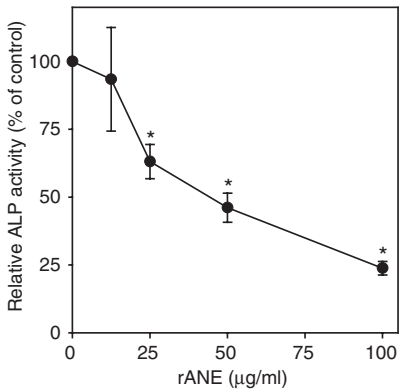

Fig. 5. Effects of ripe areca nut extracts (rANE) on the relative alkaline phosphatase (ALP) activity. The percentages of ALP activity in the presence of rANE relative to that in the control are shown. The results were averaged from three independent experiments (*$p < 0.05$).

(Fig. 4b). The relative expression of OPG was not altered (Fig. 4c).

The effects of rANE on the ALP activity were determined. Similar to the mRNA results, the ALP activity decreased significantly after rANE treatment in a dose-dependent manner ($p < 0.05$) (Fig. 5). The relative ALP activity reduced to $46 \pm 5\%$ when 50 μg/ml of rANE was used ($p < 0.05$). Expression of GAPDH and RANKL proteins in osteoblasts after rANE treatment was further determined through immunofluorescence staining and confocal laser scanning microscopy. In the absence of the primary antibodies (either anti-RANKL or anti-GAPDH), cells remained unstained. Similar level of GAPDH proteins was observed in MG63 cells after 24 h of incubation with 0, 12.5, 25 or 50 μg/ml of rANE (Figs 6a–d). For RANKL antibody staining, the untreated cells show low level of protein expression (Fig. 6e). The fluorescent intensity of MG63 cells increased apparently after rANE treatment (Figs 6f–h).

Discussion

This study clearly demonstrated that the expression of ALP in human osteoblast-like MG63 cells was inhibited by rANE. In addition, the expression of RANKL was stimulated by rANE, whereas the expression of OPG was not altered by rANE. RANKL is a member of the tumour necrosis factor superfamily that plays an essential role in osteoclastogenesis, as well as the activation and survival of mature osteoclasts. RANKL is expressed on osteoblasts as a transmembrane ligand and it also exists in a biologically active soluble form (Lacey et al. 1998, Yasuda et al. 1998, Manabe et al. 2001). RANKL acts through its receptor RANK, which is expressed on osteoclast precursors, mature osteoclasts, as well as dendritic cells (Hofbauer 1999). RANKL promotes osteoclast survival (Komarova et al. 2003). The interaction of RANKL and RANK is important in terminal differentiation and activation of osteoclasts. OPG, a soluble decoy receptor, binds RANKL and blocks its interaction with RANK (Hofbauer 1999). In binding to RANKL, OPG blocks differentiation and activation of osteoclasts. Expression of RANKL in osteoblasts induced by rANE may promote osteoclastogenesis. Further analysis is required to determine whether rANE-treated MG63 cells may be able to stimulate differentiation of osteoclasts.

Treatment of mice in vivo with RANKL activates osteoclasts, promotes

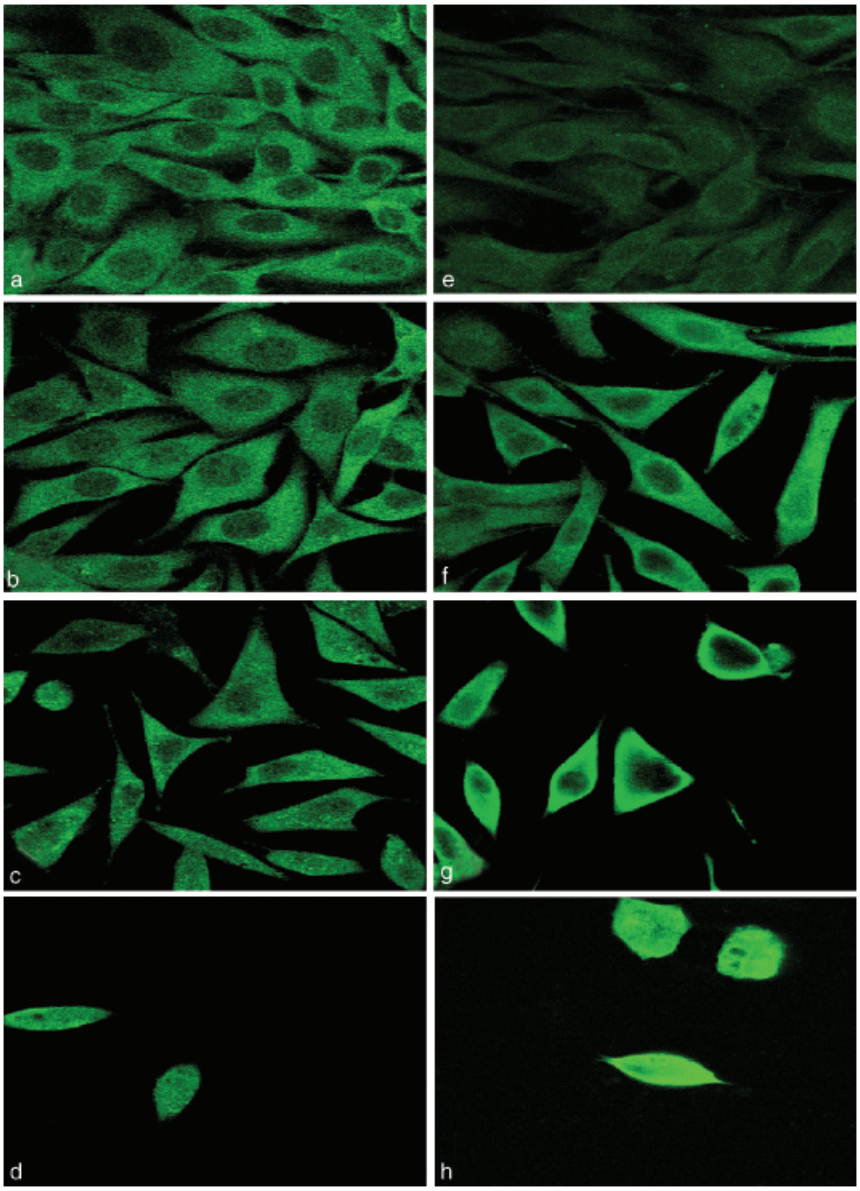

Fig. 6. Effects of ripe areca nut extracts (rANE) on GAPDH and receptor activator of nuclear factor-κB ligand (RANKL) protein expression. The results shown are MG63 cells incubated with 0 (a and e), 12.5 (b and f), 25 (c and g) or 50 μg/ml (d and h) of rANE for 24 h followed by immunofluorescence staining for GAPDH protein (a–d) or RANKL protein (e–h) and confocal laser scanning microscopy.

bone loss and causes severe hypercalcemia (Lacey et al. 1998). An animal study supports that RANKL is the key molecule regulating bone destruction in periodontitis (Teng et al. 2000). Higher levels of RANKL protein and lower levels of OPG protein have been found in the tissue and gingival crevicular fluid of patients suffering periodontitis (Crotti et al. 2003, Mogi et al. 2004), suggesting that these key regulators of osteoclast differentiation may play a major role in pathogenesis of periodontitis. Areca chewers have a higher prevalence of periodontal diseases than non-chewers. However, it is not known whether an association exists between the expression of RANKL/OPG and areca chewing in vivo.

TNF-α, interleukin-1β (IL-1β) and prostaglandin E_2 (PGE_2), are reported to stimulate production of RANKL (Hofbauer et al. 1999, Nakashima et al. 2000). OPG is also modulated by inflammatory cytokines, such as TNF-α and IL-1β, present in periodontitis (Hofbauer et al. 1998, Brandstrom et al. 1998a, Brandstrom et al. 1998b, Nakashima et al. 2000). Areca nut extracts induce TNF-α production and prostaglandin in gingival keratinocytes (Jeng et al. 2000, 2003). Whether production of TNF-α, IL-1β and PGE_2 in osteoblasts is affected by rANE is not known. It is possible that rANE stimulates the production of these inflammatory mediators and then leads to the induction of RANKL. The detail mechanisms involved in the stimulation of RANKL in osteoblasts remain to be defined.

This study showed that viability of MG63 cells was affected by rANE. Areca nut extracts were cytotoxic to gingival keratinocytes (Panigrahi & Rao 1984). Areca nut extracts also inhibit growth of human buccal mucosa fibroblasts at concentrations of 50–150 μg/ml (corresponding to 3.5–10 μg/ml of arecoline) (van Wyk et al. 1994). Arecoline is a main alkaloid in the areca nut, and catechin is a phenolic component in areca nut. The concentrations of arecoline and catechin in rANE were 9.1 and 16.6 μg/mg, respectively (Liu et al., unpublished data). Whether the components of areca nuts, such as arecoline and catechine, contribute to the effects of rANE on modulation of gene expression observed in this study remains to be examined.

Areca chewers may also smoke or chew areca quid containing tobacco. The results in this study indicated that rANE affect expression and activity of ALP. Nicotine also influences ALP activity (Fang et al. 1991, Ramp et al. 1991), the synthesis of collagen (Ramp et al. 1991) and bone matrix protein, osteopontin (Walker et al. 2001). In an animal model, nicotine also negatively impacts on bone healing (Hollinger et al. 1999). People who combine habits of areca chewing with cigarette smoking could be more susceptible to periodontium damage than areca chewing alone. Factors that are able to inhibit the functions of osteoblasts may also impair the reparative and regenerative potential of periodontal tissues of those who chew areca quid. Whether there is an additive effect of nicotine and areca nut on osteoblasts remains to be elucidated.

To conclude, our results suggest that rANE affect cellular morphology and viability of osteoblasts. We also present novel evidence demonstrating that rANE has an effect on human bone cells in down-regulation of ALP and in upregulation of RANKL. This study suggests a possible mechanism by which areca nut can be involved in the development of periodontal diseases associated with the areca chewers.

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