J Periodont Res 2009; 44: 175–183

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01104.x

Areca nut extracts increased expression of inflammatory cytokines, tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8, in peripheral blood mononuclear cells

L-Y. Chang[1,2,3], H-C. Wan[4], Y-L. Lai[1,2], Y-F. Kuo[4], T-Y. Liu[5], Y-T. Chen[1,4], S-L. Hung[3,4]

[1]Department of Dentistry, National Yang-Ming University, Taipei, Taiwan, [2]Dental Department, Taipei Veterans General Hospital, Taipei, Taiwan, [3]Department of Education and Research, Taipei City Hospital, Taipei, Taiwan, [4]Institute of Oral Biology, National Yang-Ming University, Taipei, Taiwan and [5]Institute of Environmental and Occupational Health Sciences, National Yang-Ming University, Taipei, Taiwan

Chang L-Y, Wan H-C, Lai Y-L, Kuo Y-F, Liu T-Y, Chen Y-T, Hung S-L. Areca nut extracts increased expression of inflammatory cytokines, tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8, in peripheral blood mononuclear cells. J Periodont Res 2009; 44: 175–183. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: Cytokines represent a central role in inflammatory tissue destruction and regulate the immune responses that may govern the progression of periodontal diseases. This study investigated the effects of areca nut extracts on the expression of inflammatory cytokines, tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8 in peripheral blood mononuclear cells. The role of oxidative stress of areca nut extracts was also examined using curcumin.

Material and Methods: The expression of cytokines in peripheral blood mononuclear cells treated with extracts of ripe areca nut or extracts of tender areca nut was analyzed using enzyme-linked immunosorbent assay and reverse transcription–polymerase chain reaction.

Results: Both extracts of ripe areca nut (≤ 40 μg/mL) and extracts of tender areca nut significantly enhanced the production of tumor necrosis factor-α and interleukin-1β in peripheral blood mononuclear cells in a dose-dependent and time-dependent manner. The kinetics of mRNA expression of both cytokines was also enhanced by areca nut extracts. The stimulatory effects of areca nut extracts on the secretion of tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8 and on the mRNA expression of tumor necrosis factor-α, interleukin-1β and interleukin-6 at 4 h of incubation were reduced by curcumin (20–50 μM). However, the level of interleukin-8 transcripts was not affected by curcumin. Moreover, interleukin-1β induction by extracts of tender areca nut, but not by extracts of ripe areca nut, was weakened by 10 μM curcumin. The inhibitory effects of curcumin may vary with different cytokines and with different areca nut extract treatments.

Conclusion: The complex cytokine profile induced by areca nut extracts-treated peripheral blood mononuclear cells implied the possibility of enhanced local

Dr Shan-Ling Hung, Institute of Oral Biology, National Yang-Ming University, No. 155, Sec. 2, Li-Nong St., Pei-Tou, Taipei 11221, Taiwan
Tel: +886 2 2826 7224
Fax: +886 2 2826 4053
e-mail: slhung@ym.edu.tw

Key words: areca nut; peripheral blood mononuclear cells; cytokine; curcumin

Accepted for publication March 18, 2008

inflammation and altered immune functions by the areca chewing habit. The inhibitory effects of curcumin on cytokine expression suggested that oxidative stress might be involved in areca nut extracts-associated immune alteration.

Areca quid chewing is a popular habit in countries of south and southeast Asia and the Asian Pacific region (1). The habit is strongly associated with oral carcinogenesis, like oral submucous fibrosis and oral cancer (2,3), and is also closely related to periodontal disease (4–8). Periodontal disease is characterized by a progressive and destructive inflammatory process of the supporting tissues of teeth, which results from the interaction of bacteria in dental plaque biofilm and host immune responses (9,10). Constant chewing of areca quid may provide access of areca ingredients for stimulation of oral tissue and cause mucosal microtrauma, hence increasing the inflammatory reaction and disease progression.

Histologically, the inflammatory infiltrate in the periodontal lesion consists of lymphocytes and macrophages (11). T cells predominate in the early lesion, whereas the amounts of B cells and plasma cells are increased in the established lesion (11). Studies have demonstrated that a number of inflammatory cytokines are released by resident cells and inflammatory/immune cells in response to periodontopathogens (9–11). Cytokines, which exert biological effects on both host inflammatory and immune responses, are important in the linkage of inflammation/immune response and disease status (12,13). Excessive and/or continuous production of inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8, is believed to play a role in leukocyte recruitment and periodontal tissue destruction (10). Among the myriad of cytokines, tumor necrosis factor-α and interleukin-1β are potent and crucial in stimulating bone resorption in periodontitis (10). They are often present simultaneously in chronic inflammatory diseases and are known to induce expression of a variety of inflammatory mediators, including themselves (10,14–16). Therefore, the inflammatory reaction may be amplified and prolonged, leading to more tissue damage (14).

Areca nut, the fruit of the tropical palm tree *Areca catechu*, is the main component of areca quid. The areca nut used in Taiwan is fresh, tender and with husk, whereas the ripe and fully grown areca nut, without fibrous husk, is used in India and some other countries. Extracts of areca nut can stimulate the production of inflammatory molecules, such as prostaglandins, tumor necrosis factor-α and interleukin-6, in keratinocytes (17). Several *in vitro* studies have pointed out that areca nut extracts and other areca quid ingredients can generate reactive oxygen species and induce oxidative damage (18–23). Reactive oxygen species are proposed to be a major cause of the cell and tissue damage associated with many chronic inflammatory diseases via several pathways, including stimulation of the release of a variety of inflammatory cytokines (24,25). Compared with the aqueous extracts of tender areca nut, extracts of ripe areca nut are more cytotoxic to CHO-K1 cells and generate a higher level of oxidative DNA damage (18). The different cellular effects between extracts of ripe areca nut and extracts of tender areca nut may be related to the greater oxidative stress generated from the higher phenolic amount in ripe areca nut (26–28). To understand in more detail the role of areca quid in oral diseases, the effects of two types of areca nuts on cells and the role of areca-associated oxidative stress are worthy of more investigation.

Immunolocalization of greater levels of cytokines in oral tissue from patients with oral submucous fibrosis (29) suggests that inflammatory/immune cells, as well as structural cells, could be stimulated by areca quid chewing. Local immunocompetent cells derived from peripheral blood are important sources of inflammatory cytokines (12). Recent *in vitro* studies demonstrate that areca nut extracts can affect the cytokine production function of immunocompetent cells (23,30). However, little is known about the effects of areca on the profile of the crucial cytokines tumor necrosis factor-α and interleukin-1β that are produced by immune cells. The purpose of this study was to investigate the effects of extracts of ripe areca nut and extracts of tender areca nut on expression of the inflammatory cytokines tumor necrosis factor-α and interleukin-1β in peripheral blood mononuclear cells (the immunocompetent cells). The possible role of oxidative stress of areca nut extracts on the production of tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8 on peripheral blood mononuclear cells was also examined.

Material and methods

Preparation of areca nut extracts

Extracts of ripe areca nut were prepared from dried ripe areca nuts without husk and extracts of tender areca nut were prepared from fresh tender areca nuts with husk, as previously reported (18,19). The yield, after extraction, was approximately 12 and 26% for extracts of ripe areca nut and extracts of tender areca nut, respectively. The extracts of tender areca nut were dissolved in calcium-free and magnesium-free Hanks' balanced salt solution (Gibco BRL Laboratories, Grand Island, NY, USA). The extracts of ripe areca nut were dissolved in dimethylsulfoxide (Sigma Chemical Co., St Louis, MO, USA) to help dissolution before being diluted in Hanks' balanced salt solution. The final concentration of dimethylsulfoxide in each extracts of ripe areca nut-treated sample was less than 0.4%.

Preparation of peripheral blood mononuclear cells and culture conditions

Twenty-one healthy human volunteers (11 men and 10 women; age range: 20–31 years) were recruited in the present study. Peripheral blood mononuclear cells were freshly prepared from venous peripheral blood of volunteers using dextran sedimentation followed by Ficoll (Ficoll–Paque PLUS; Amersham Pharmacia Biotech, Bucks., UK) density-gradient centrifugation, as described previously (30). The buffy coats were harvested, washed twice, then the purified peripheral blood mononuclear cells were resuspended in RPMI-1640 medium (Gibco BRL Laboratories) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin G sodium, 100 μg/mL of streptomycin sulfate and 0.25 μg/mL of amphotericin B (Gibco BRL Laboratories). Peripheral blood mononuclear cells (1×10^6 cells/mL) were treated with different concentrations (0, 10, 20, 40, 80 and 160 μg/mL) of either extracts of ripe areca nut or extracts of tender areca nut in 14-mL polypropylene tubes at 37°C for 4 or 24 h. Cells treated with 0.025, 0.05, 0.1, 0.2, or 0.4% dimethylsulfoxide under similar conditions served as the vehicle controls. For curcumin experiments, peripheral blood mononuclear cells were pre-incubated with curcumin (0, 2.5 or 10 μM) (Sigma Chemical Co.) for 1 h and then incubated with media only, with 20 μg/mL of extracts of ripe areca nut, or with 80 μg/mL of extracts of tender areca nut for 24 h. Preliminary tests revealed the toxic effects of 20 μM curcumin on extracts of ripe areca nut-treated cells for 24 h, and thus subtoxic concentrations of curcumin ($\leq$ 10 μM) were used for the 24-h treatments. At the end of each incubation period, the viability of treated cells was examined using the Trypan Blue dye-exclusion assay (31). The culture supernatants were collected, aliquoted and stored at −80°C until future analysis of cytokine levels using enzyme-linked immunosorbent assay (ELISA). For experiments of the kinetics of cytokine mRNA expression and 4-h curcumin effects on treated cells (media only, 20 μg/mL of extracts of ripe areca nut or 80 μg/mL of extracts of tender areca nut), the cell concentration used was about $3–4 \times 10^6$ cells/mL. At the end of each period of incubation, the viability of treated cells was examined and the supernatants were collected and stored at −80°C until further analysis using ELISA, then the pellets of harvested cells were prepared for RNA purification and the reverse transcription–polymerase chain reaction (RT-PCR) assay. All measurements were carried out in at least three independent experiments.

ELISA

The culture supernatants were assayed in duplicate to determine the concentrations of tumor necrosis factor-α, interleukin-6, interleukin-8 (EH3TN-FA, EH2IL6, EH2IL8, respectively; Pierce Biotechnology, Inc., Rockford, IL, USA) and interleukin-1β (Hu IL-1β kit; BioSource International, Inc., Camarillo, CA, USA) using solid-phase sandwich ELISA kits according to the manufacturer's recommendation. The sensitivities (or lower limit of detection) of the ELISA kits were 2, 1, 2 and 1 pg/mL for tumor necrosis factor-α, interleukin-6, interleukin-8 and interleukin-1β, respectively. In brief, 50 μL of sample was added to each well in duplicate and incubated with biotinylated antibody against tumor necrosis factor-α, interleukin-6, interleukin-8 or interleukin-1β, followed by incubation with streptavidin-horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. The plates were washed three to four times between each incubation step. Absorbance was measured at 450 nm using an ELISA reader (EL 312e Microplate Bio Kinetics Reader; BIO-TEK Instruments, Winooski, VT, USA). The amount of cytokine in each well was calculated using the standard curve generated from the purified cytokines. The cytokine level of each sample was the mean of duplicate values.

RT-PCR

The cell pellets of treated cells were collected and the total RNA of each sample was isolated by a single-step method using Trireagent-RNA® isolation reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Gene expression was examined using RT-PCR. RT was performed as follows: 16 μL of DEPC (diethyl pyrocarbonate)-water containing 2 μg of total RNA and 10 pmoles of oligo $(dT)_{18}$ primer (Protech Technology Enterprise, Co., Ltd, Taipei, Taiwan) was incubated at 70°C for 10 min, chilled on ice for 5 min and then mixed with 2 μL of 10× StrataScript buffer (Stratagene, La Jolla, CA, USA), 1 μL of 10 mM dNTP and 1 μL of StrataScript reverse transcriptase (50 U) (Stratagene). After incubation at 42°C for 50 min, then at 70°C for 15 min, the final cDNA products were harvested and stored at −20°C until further analyzed. cDNA products from 80 ng of total RNA were used for PCR amplification in a total volume of 20 μL containing 4 mM dNTP, 10 pmoles of each specific primer (Clontech Laboratories, Inc., Palo Alto, CA, USA), 1 unit of Taq DNA polymerase and 1× reaction buffer (MoBiTec GmbH, Göttingen, Germany). The primer pairs and their annealing temperatures used in this study were as follows: tumor necrosis factor-α, 5′-GAGTGACAAGCCTG TAGCCCATGTTGTAGCA-3′ and 5′-GCAATGATCCCAAAGTAGAC CTGCCCAGACT-3′ (annealing temperature = 57°C) (32); interleukin-1β, 5′-ATGGCAGAAGTACCTAAGCT-CGC-3′ and 5′-ACACAAATTGCAT-GGTGAAGTCAGTT-3′ (annealing temperature = 62°C) (33); interleukin-6, 5′-ATGAACTCCTTCTCCACAAG-CGC-3′ and 5′-GAAGAGCCCT-CAGGCTGGACTG-3′ (annealing temperature = 55°C) (34); interleukin-8, 5′-ATGACTTCCAAGCTGGCCGT-GGCT-3′ and 5′-TCTCAGCCCT-CTTCAAAAACTTCTC-3′ (annealing temperature = 60°C) (35); and glyceraldehyde-3-phosphate dehydrogenase, 5′-TGGTATCGTGGAAGGACTCA-TGAC-3′ and 5′-ATGCCAGTGA-GCTTCCCGTTCAGC-3′ (annealing temperature = 55°C) (36). Reactions using buffer only or an RNA sample from treated cells served as negative controls. The reaction mixture was heated to 95°C for 5 min and

amplification was performed for 24–31 cycles of 95°C for 1 min, annealing temperature for 1 min and 72°C for 1 min. A final extension was performed at 72°C for 10 min. The PCR products were loaded onto a 2% agarose gel for electrophoresis. Gels were stained with ethidium bromide and pictures were taken. The amplified products were 444, 802, 628, 289 and 190 base pairs for tumor necrosis factor-α, interleukin-1β, interleukin-6, interleukin-8 and glyceraldehyde-3 phosphate dehydrogenase, respectively.

Statistical analysis

The Mann–Whitney test was performed using the analytical software (SPSS 10.0; SPSS Inc., Chicago, IL, USA). Differences were considered as statistically significant for a p-value of < 0.05.

Results

Production of tumor necrosis factor-α and interleukin-1β in peripheral blood mononuclear cells after treatment with extracts of ripe areca nut or extracts of tender areca nut

The levels of tumor necrosis factor-α and interleukin-1β in the supernatants of control cells treated with medium only were consistently low after incubation for 4 and 24 h. There was no significant difference in the secretion of tumor necrosis factor-α and interleukin-1β between control cells treated with medium only, with or without dimethylsulfoxide ($\leq$ 0.4%) (data not shown). Both extracts of ripe areca nut ($\leq$ 40 μg/mL) and extracts of tender areca nut showed dose-dependent enhancing effects on cytokine production in peripheral blood mononuclear cells at 4 and 24 h of incubation (Fig. 1). Concentrations of extracts of ripe areca nut and extracts of tender areca nut as low as 20 and 80 μg/mL, respectively, significantly induced the production of cytokines. Compared with the control cells treated with medium only, 20 μg/mL of extracts of ripe areca nut increased the secretion of tumor necrosis factor-α by 3.7-fold and 53.4-fold, at 4 and 24 h of incubation, respectively (Fig. 1A and 1B), while 80 μg/mL of extracts of tender areca nut increased the secretion of tumor necrosis factor-α by 3.1-fold and 6-fold, at 4 and 24 h of incubation, respectively. In addition, 20 μg/mL of extracts of ripe areca nut increased the secretion of interleukin-1β by 5.8-fold and 155-fold (Fig. 1C and 1D), while 80 μg/mL of extracts of tender areca nut increased the secretion of interleukin-1β by 2.5-fold and 17.1-fold, at 4 and 24 h of incubation, respectively. However, the level of tumor necrosis factor-α in cells treated with 160 μg/mL of extracts of ripe areca nut was as low as that in cells treated with medium only, at both 4 and 24 h.

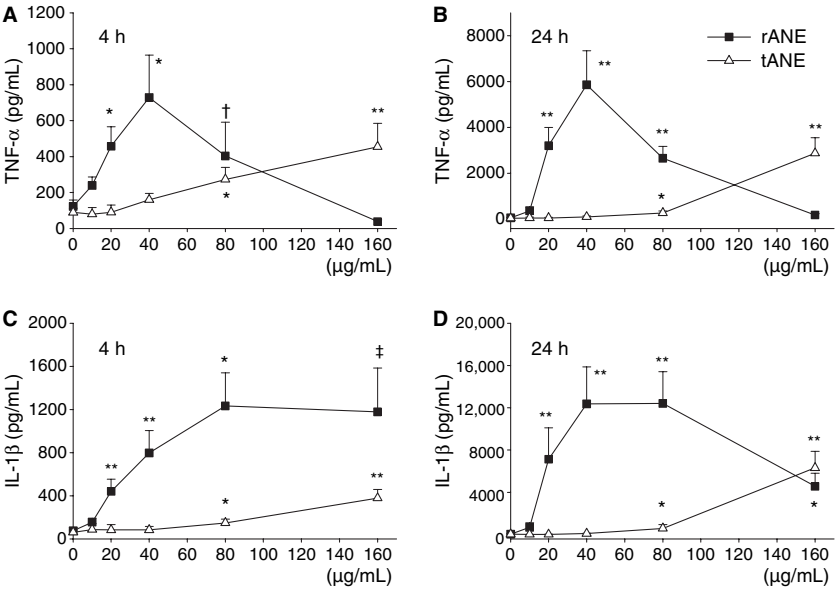

Fig. 1. Effects of areca nut extracts on the cytokine production in peripheral blood mononuclear cells were determined using enzyme-linked immunosorbent assay. The levels of tumor necrosis factor-α (A, B) and interleukin-1β (C, D) in the supernatants of peripheral blood mononuclear cells treated with indicated concentrations of extracts of ripe areca nut or extracts of tender areca nut for 4 h (A, C) or 24 h (B, D) were analyzed. Results are shown as mean ± standard error of values from five or six independent experiments. Statistically significant differences between the individual experimental sample and media-treated control are shown by different symbols: $^{**}p < 0.01$; $^{*}p < 0.05$; $^{\dagger}p = 0.05$; $^{\ddagger}p = 0.053$, Mann–Whitney test. IL-1β, interleukin-1β; rANE, extracts of ripe areca nut; tANE, extracts of tender areca nut; TNF-α, tumor necrosis factor-α.

The viability of cells treated with 160 μg/mL of extracts of ripe areca nut was 57.6 ± 19.3% and 34.1 ± 13.7% for 4 and 24 h of incubation, respectively. The reduced production of tumor necrosis factor-α when the concentrations of extracts of ripe areca nut were 80 and 160 μg/mL at both 4 and 24 h of incubation, and the decreased level of interleukin-1β when the extracts of ripe areca nut concentrations was 160 μg/mL at 24 h of incubation, might be caused by the toxic effects of extracts of ripe areca nut. However, concentrations of extracts of tender areca nut up to 160 μg/mL revealed little cytotoxicity (viability: 80.9 ± 12.3% and 74.8 ± 18.0% for 4 h and 24 h of incubation, respectively). Therefore, subtoxic concentrations of extracts of ripe areca nut or extracts of tender areca nut could increase the production of tumor necrosis factor-α and interleukin-1β in a dose-dependent and time-dependent manner.

mRNA expression of tumor necrosis factor-α and interleukin-1β in peripheral blood mononuclear cells after treatment with extracts of ripe areca nut or extracts of tender areca nut

The kinetics of mRNA production of tumor necrosis factor-α and interleukin-1β in peripheral blood mononuclear cells after treatment with areca nut extracts was examined. Compared with freshly

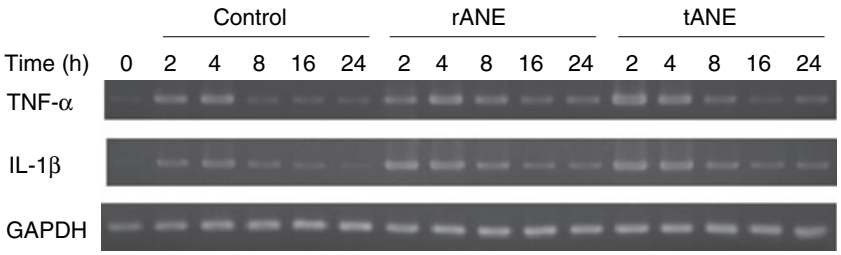

Fig. 2. Effects of areca nut extracts on mRNA expression of cytokines in peripheral blood mononuclear cells as examined using the reverse transcription–polymerase chain reaction assay. Peripheral blood mononuclear cells were treated with medium only (control), extracts of ripe areca nut (20 μg/mL) or extracts of tender areca nut (80 μg/mL) for 2, 4, 8, 16, or 24 h. Analysis of freshly isolated peripheral blood mononuclear cells without incubation (0 h) was also performed in each experiment. Representative results of amplified sequences specific for tumor necrosis factor-α, interleukin-1β and glyceraldehyde-3 phosphate dehydrogenase of three independent experiments are shown. GAPDH, glyceraldehyde-3 phosphate dehydrogenase; IL-1β, interleukin-1β; rANE, extracts of ripe areca nut; tANE, extracts of tender areca nut; TNF-α, tumor necrosis factor-α.

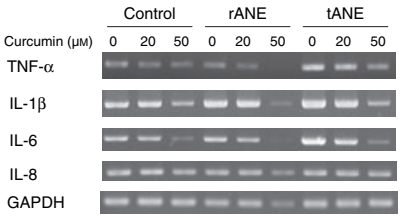

Fig. 3. Effects of curcumin on mRNA expression of cytokines in treated peripheral blood mononuclear cells as measured using the reverse transcription–polymerase chain reaction assay. Cells were pre-incubated for 1 h with 0, 20, or 50 μM curcumin followed by 4 h of incubation with medium only (control), extracts of ripe areca nut (20 μg/mL) or extracts of tender areca nut (80 μg/mL). Representative results of amplified sequences specific for tumor necrosis factor-α, interleukin-1β, interleukin-6, interleukin-8 and glyceraldehyde-3 phosphate dehydrogenase, of three independent experiments, are shown. GAPDH, glyceraldehyde-3 phosphate dehydrogenase; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; rANE, extracts of ripe areca nut; tANE, extracts of tender areca nut; TNF-α, tumor necrosis factor-α.

isolated cells, the mRNA expression of both tumor necrosis factor-α and interleukin-1β was increased at all time points, but this increase was particularly apparent at 2 and 4 h in all three groups (i.e. control cells treated with medium only, extracts of ripe areca nut-treated cells and extracts of tender areca nut-treated cells) (Fig. 2). When compared with the control cells treated with medium only at each time point, the tumor necrosis factor-α transcripts persisted longer in both extracts of ripe areca nut-treated group and extracts of tender areca nut-treated group at 8–24 h and showed a slight increase in extracts of tender areca nut-treated cells at 2 h. The levels of interleukin-1β transcripts were increased at 2–24 h time points in cells treated with extracts of ripe areca nut or with extracts of tender areca nut when compared with the control cells. Thus, both extracts of ripe areca nut and extracts of tender areca nut increased the mRNA expression of tumor necrosis factor-α and interleukin-1β in peripheral blood mononuclear cells.

Effects of curcumin on mRNA expression of tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8 in peripheral blood mononuclear cells after treatment with extracts of ripe areca nut or extracts of tender areca nut

The antioxidant, curcumin, was used to examine whether the enhancing effects of areca nut extracts on cytokine expression in peripheral blood mononuclear cells were caused by the generation of reactive oxygen species. After pre-incubation of peripheral blood mononuclear cells with 0, 20, or 50 μM curcumin followed by 4 h of incubation with various areca nut extracts, the viability in each group was about 90%. Compared with the control cells, pre-incubation with 50 μM curcumin decreased the mRNA expression of tumor necrosis factor-α, interleukin-1β and interleukin-6 in both extracts of ripe areca nut-treated and extracts of tender areca nut-treated cells (Fig. 3). By contrast, curcumin had a minimal effect on the levels of interleukin-8 transcripts. The results suggested a possible role of areca-associated oxidative stress in cytokine gene expression of immune cells. The results also indicated that the inhibitory effects of curcumin on different cytokines may be varied.

Effects of curcumin on protein production of tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8 in peripheral blood mononuclear cells after treatment with extracts of ripe areca nut or extracts of tender areca nut

The possible effects of curcumin on the secretion of tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8 in areca nut extracts-treated peripheral blood mononuclear cells at 4 h of incubation were examined. Both 20 and 50 μM curcumin significantly decreased the secretion of all four cytokines in extracts of ripe areca nut-treated or extracts of tender areca nut-treated peripheral blood mononuclear cells (Fig. 4). However, only interleukin-1β induced by extracts of tender areca nut, but not by extracts of ripe areca nut, could be obviously attenuated by 10 μM curcumin (Fig. 4B). Curcumin also reduced cytokine secretion by the cells treated with medium only.

Effects of curcumin on cytokine accumulation in treated peripheral blood mononuclear cells at 24 h

The curcumin effects were examined at 24 h to investigate the role of oxidative stress over a longer incubation time. After pre-incubation of peripheral blood mononuclear cells with 0, 2.5, or 10 μM curcumin followed by 24 h of incubation with areca nut extracts, the cell viability in each tested sample was greater than 80% except in extracts of

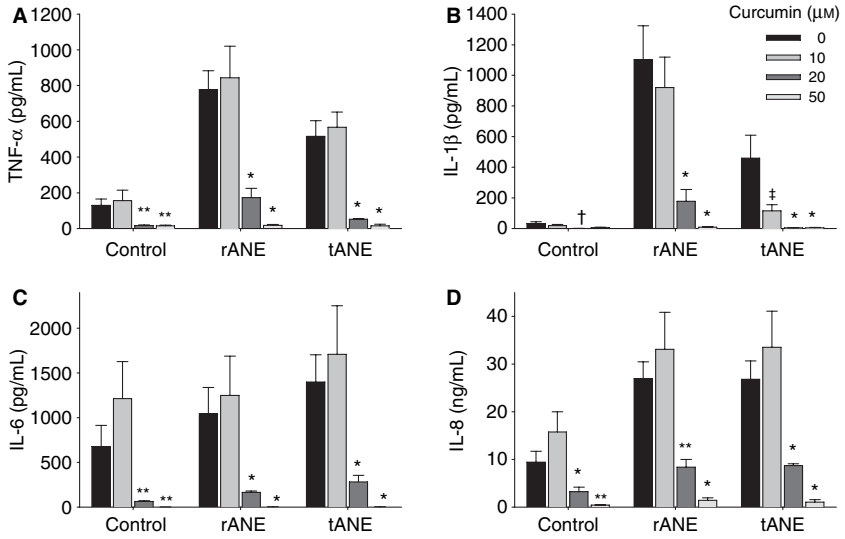

Fig. 4. Effects of curcumin on cytokine production in treated peripheral blood mononuclear cells incubated for 4 h. Peripheral blood mononuclear cells were pre-incubated with various concentrations of curcumin (0, 10, 20, or 50 μM) for 1 h followed by incubation with medium only (control), or with extracts of ripe areca nut (20 μg/mL) or extracts of tender areca nut (80 μg/mL) for 4 h. The levels of tumor necrosis factor-α (A), interleukin-1β (B), interleukin-6 (C), and interleukin-8 (D) in culture supernatants were assayed using enzyme-linked immunosorbent assay. Data are presented as mean ± standard error of values of at least three independent experiments. Statistically significant differences between cells pre-incubated with curcumin and with medium only (control) in each group are shown by different symbols: $^{**}p < 0.01$; $^{*}p < 0.05$; $^{\dagger}p = 0.05$; $^{\ddagger}p = 0.062$, Mann–Whitney test. IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; rANE, extracts of ripe areca nut; tANE, extracts of tender areca nut; TNF-α, tumor necrosis factor-α.

ripe areca nut-treated cells pretreated with 10 μM curcumin (72.2%). When compared with the control cells, 10 μM curcumin significantly reduced the production of tumor necrosis factor-α, interleukin-1β and interleukin-6 in extracts of ripe areca nut-treated or extracts of tender areca nut-treated peripheral blood mononuclear cells (Fig. 5), while 2.5 μM curcumin exhibited only small effects on cytokine production. Though not significant, the accumulated levels of interleukin-8 induced by extracts of tender areca nut, but not by extracts of ripe areca nut, at 24 h could also be reduced by 10 μM curcumin (Fig. 5D). The data showed that the subtoxic dose of curcumin (10 μM) could reduce the enhancing effects of longer exposure to extracts of ripe areca nut or to extracts of tender areca nut. However, the effects of curcumin on interleukin-8 secretion were limited when compared with other cytokines.

Discussion

Sustained and/or greater production of cytokines, such as tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8, in inflamed tissues is responsible for the progression of inflammatory disorders, including periodontal diseases (10,25). Interleukin-1β, the more potent form of interleukin-1 in mediating effects on bone (10), is involved in stimulating the production of protease-like metalloproteinase and a variety of inflammatory mediators, and inducing bone resorption (15). Tumor necrosis factor-α shares the remarkable similarities of biological properties with those of interleukin-1 and has synergistic effects with it (15). Interleukin-6 is a pleiotropic cytokine that can induce bone resorption and B-cell differentiation (10). Interleukin-8, a chemokine, possesses potent chemotactic functions for granulocyte accumulation at sites of inflammation (10). Increased expression of interleukin-6 and interleukin-8 in peripheral blood mononuclear cells following treatment with extracts of ripe areca nut or extracts of tender areca nut has been reported previously (30). The present study demonstrated that secretion of tumor necrosis factor-α and interleukin-1β in extracts of ripe areca nut-stimulated or extracts of tender areca nut-stimulated peripheral blood mononuclear cells was increased in a dose-dependent and time-dependent manner. The kinetics of transcripts of these two cytokines was also enhanced after treatment with areca nut extracts. To the best of our knowledge, this is the first study that clearly defined the expression of these two cytokines in areca nut extracts-activated immune cells. The findings strongly suggest the role of areca in enhancing oral inflammation and perhaps in modulating the host immune responses.

Oxidative stress plays a key role during the development and progression of inflammatory diseases (24,25). Several studies report that areca nut extracts can generate reactive oxygen species and induce DNA damage (18–23). Reactive oxygen species, such as hydroxyl radical, can be formed in the oral cavity during chewing of areca quid containing ripe or tender areca nut (28,37,38). The ability of areca nut extracts to increase the cellular reactive oxygen species levels in mouse splenocytes is attenuated by the antioxidant *N*-acetyl-L-cysteine (23). Curcumin, a major active component of turmeric (*Curcuma longa*) and a spice in curries, has been reported to be an effective antioxidant and is known traditionally for its anti-inflammatory effects (39). Curcumin is able to scavenge reactive oxygen species (40), increase glutathione content (41,42) and inhibit the activation of nuclear factor-κB (NF-κB) (39). The properties of curcumin might be useful for the treatment of inflammatory conditions. In the present study, at 4 h of culture the secretion of tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-8, and the mRNA expression of tumor necrosis factor-α, interleukin-1β and interleukin-6, in either extracts of ripe areca nut-stimulated or extracts of

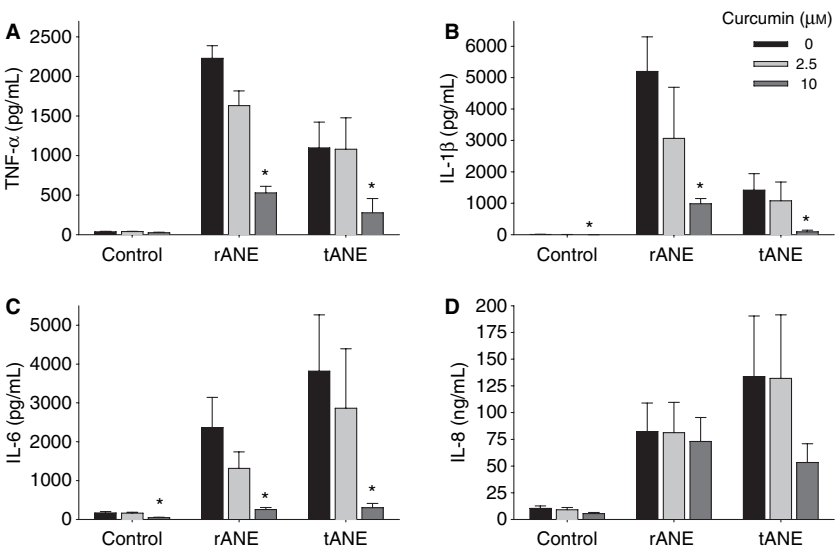

Fig. 5. Effects of curcumin on cytokine accumulation in treated cells incubated for 24 h. Peripheral blood mononuclear cells were pre-incubated with various concentrations of curcumin (0, 2.5, or 10 μM) for 1 h followed by incubation with medium only (control), or with extracts of ripe areca nut (20 μg/mL) or extracts of tender areca nut (80 μg/mL) for a further 24 h. The levels of tumor necrosis factor-α (A), interleukin-1β (B), interleukin-6 (C), and interleukin-8 (D) in culture supernatants were assayed using enzyme-linked immunosorbent assay. Data are presented as mean ± standard error of values of four independent experiments. Statistically significant differences between cells pre-incubated with curcumin and medium only (control) in each group are shown. $*p < 0.05$, Mann–Whitney test. IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; rANE, extracts of ripe areca nut; tANE, extracts of tender areca nut; TNF-α, tumor necrosis factor-α.

tender areca nut-stimulated peripheral blood mononuclear cells, were reduced by curcumin. However, the finding that the mRNA expression of interleukin-8 was only minimally affected by curcumin indicated that the effects of curcumin on different cytokines may be varied. In hepatic stellate cells, the antioxidant property of curcumin (10–30 μM) mainly results from elevated cellular glutathione levels after incubation for 10–24 h, but not before 5 h (41). The results of the present study showed that 10 μM curcumin markedly reduced the secretion of a variety of cytokines in areca nut extracts-treated peripheral blood mononuclear cells after incubation for 24 h. Whether levels of cellular glutathione in areca nut extracts-treated peripheral blood mononuclear cells were affected by curcumin remains to be determined. Our previous study revealed that the antioxidant pyrrolidine dithiocarbamate could inhibit the expression of interleukin-6 by extracts of ripe areca nut-treated peripheral blood mononuclear cells (30). Together, these findings suggest that the increased expression of numerous inflammatory cytokines by peripheral blood mononuclear cells might result from areca-associated oxidative stress.

Reactive oxygen species can enhance inflammation via activation of redox-sensitive factors, such as NF-κB and activator protein-1, and then increase the expression of pro-inflammatory molecules (43,44). In oral keratinocytes, activation of NF-κB and mitogen-activated protein kinase (MAPK) by extracts of ripe areca nut (45), and activation of the MAPK/c-Fos pathway by extracts of tender areca nut (46), have been reported. Curcumin exhibits the ability to block the activation of NF-κB and activator protein-1 (39,47). In addition, pyrrolidine dithiocarbamate is also a known inhibitor of NF-κB (48). Inhibitory effects of curcumin and pyrrolidine dithiocarbamate on cytokine expression in areca nut extracts-treated peripheral blood mononuclear cells were noted in the present study and in our previous report (30). It is possible that the greater increase of a variety of cytokines in areca nut extracts-treated peripheral blood mononuclear cells occurs via activation of these transcription factors through oxidative modification and/or stimulation with products like cytokines. However, the detailed mechanism involved is still elusive and more studies are necessary to clarify the pathways underlying areca-associated cytokine expression.

The biological effects of extracts of ripe areca nut and extracts of tender areca nut on cytokine expression in peripheral blood mononuclear cells shared remarkable similarities in this study; however, several discrepancies were noted. First, extracts of ripe areca nut were more toxic than extracts of tender areca nut. Second, under the experimental conditions of this study, the inducing effects of extracts of ripe areca nut on tumor necrosis factor-α and interleukin-1β were higher when compared with the inducing effects of extracts of tender areca nut, whereas the inducing effects of extracts of ripe areca nut on interleukin-6 and interleukin-8 were similar to or lower than the inducing effects of extracts of tender areca nut. These observations suggest that the biological effects of extracts of ripe areca nut and extracts of tender areca nut may be different. Third, the enhancing effects of extracts of tender areca nut, but not of extracts of ripe areca nut, on the production of interleukin-1β were reduced by 10 μM curcumin after 4 h of incubation. The differential inhibition of curcumin on certain cytokines, induced by extracts of ripe areca nut and extracts of tender areca nut, might be related to their different composition and properties, for example, extracts of tender areca nut contain lower amounts of phenolic compounds (28) and induce less oxidative damage (18) when compared with extracts of ripe areca nut. Together, the results suggest that extracts of ripe areca nut and extracts of tender areca nut might create a different pathological microenvironment.

In conclusion, both extracts of ripe areca nut and extracts of tender areca nut enhanced the production of a variety of inflammatory cytokines in peripheral blood mononuclear cells, although several discrepancies between their biologic effects were observed. The results imply that exposure to areca nut as a result of the areca chewing habit might enhance a local inflammatory reaction and produce harmful effects on the host immune system. The potent inhibitory effects of curcumin on the expression of a variety of cytokines in areca nut extracts-treated cells raised the possibility of disease prevention using curcumin or other dietary polyphenols for areca chewers. However, the clinical relevance of these findings remains to be defined.

Acknowledgements

This investigation was supported by Research Grant 91GMP004-4 (NSC93-3112-B010-002) of the National Research Program for Genomic Medicine from the National Science Council, Taiwan, and the Association for Dental Sciences of the Republic of China.

References

1. Gupta PC, Ray CS. Epidemiology of betel quid usage. *Ann Acad Med Singapore* 2004;**33**(suppl 4)**:**31–36.
2. IARC. Betel-quid and areca-nut chewing. *IARC Monogr Eval Carcinog Risk Chem Hum* 1985;**37:**141–200.
3. Jeng JH, Chang MC, Hahn LJ. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. *Oral Oncol* 2001;**37:** 477–492.
4. Ling L-J, Hung S-L, Tseng S-C *et al.* Association between betel quid chewing, periodontal status, and periodontal pathogens. *Oral Microbiol Immunol* 2001;**16:**364–369.
5. Waerhaug J. Prevalence of periodontal disease in Ceylon. Association with age, sex, oral hygiene, socioeconomic factors, vitamin deficiencies, malnutrition, betel and tobacco consumption and ethnic group. Final report. *Acta Odontol Scand* 1967;**25:**205–231.
6. Teng HC, Lee CH, Hung HC *et al.* Lifestyle and psychosocial factors associated with chronic periodontitis in Taiwanese adults. *J Periodontol* 2003;**74:** 1169–1175.
7. Neely AL, Holford TR, Loe H, Anerud A, Boysen H. The natural history of periodontal disease in humans: risk factors for tooth loss in caries-free subjects receiving no oral health care. *J Clin Periodontol* 2005;**32:**984–993.
8. Amarasena N, Ekanayaka AN, Herath L, Miyazaki H. Association between smoking, betel chewing and gingival bleeding in rural Sri Lanka. *J Clin Periodontol* 2003;**30:**403–408.
9. Gemmell E, Yamazaki K, Seymour GJ. The role of T cells in periodontal disease: homeostasis and autoimmunity. *Periodontol 2000* 2007;**43:**14–40.
10. Okada H, Murakami S. Cytokine expression in periodontal health and disease. *Crit Rev Oral Biol Med* 1998; **9:** 248–266.
11. Teng YT. The role of acquired immunity and periodontal disease progression. *Crit Rev Oral Biol Med* 2003;**14:**237–252.
12. Goldsby RA, Kindt TJ, Osborne BA. Cytokines. In: Goldsby RA, Kindt TJ, Osborn BA, eds. *Kuby Immunology*. New York: W. H. Freeman and Company, 2000: 303–327.
13. Parsonnet J. Molecular mechanisms for inflammation-promoted pathogenesis of cancer. The sixteenth international symposium of the Sapporo cancer seminar (Sapporo, Japan, July 3–5, 1996). *Cancer Res* 1997;**57:**3620–3624.
14. Bours V, Bonizzi G, Bentires-Alj M *et al.* NF-κB activation in response to toxical and therapeutical agents: role in inflammation and cancer treatment. *Toxicology* 2000;**153:**27–38.
15. Dinarello CA. Interleukin-1 and interleukin-1 antagonism. *Blood* 1991;**77:**1627–1652.
16. Kida Y, Kobayashi M, Suzuki T *et al.* Interleukin-1 stimulates cytokines, prostaglandin E_2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-κB in human gingival fibroblasts. *Cytokine* 2005;**29:**159–168.
17. Jeng JH, Wang YJ, Chiang BL *et al.* Roles of keratinocyte inflammation in oral cancer: regulating the prostaglandin E_2, interleukin-6 and TNF-α production of oral epithelial cells by areca nut extract and arecoline. *Carcinogenesis* 2003;**24:** 1301–1315.
18. Liu TY, Chen CL, Chi CW. Oxidative damage to DNA induced by areca nut extract. *Mutat Res* 1996;**367:**25–31.
19. Nair UJ, Floyd RA, Nair J, Bussachini V, Friesen M, Bartsch H. Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA *in vitro* with betel quid ingredients. *Chem Biol Interact* 1987;**63:**157–169.
20. Chang MC, Ho YS, Lee PH *et al.* Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells: association of glutathione, reactive oxygen species and mitochondrial membrane potential. *Carcinogenesis* 2001;**22:**1527–1535.
21. Jeng JH, Wang YJ, Chang WH *et al.* Reactive oxygen species are crucial for hydroxychavicol toxicity toward KB epithelial cells. *Cell Mol Life Sci* 2004;**61:**83–96.
22. Lai KC, Lee TC. Genetic damage in cultured human keratinocytes stressed by long-term exposure to areca nut extracts. *Mutat Res* 2006;**599:**66–75.
23. Wang CC, Liu TY, Wey SP, Wang FI, Jan TR. Areca nut extract suppresses T-cell activation and interferon-γ production via the induction of oxidative stress. *Food Chem Toxicol* 2007;**45:**1410–1418.
24. Halliwell B, Gutteridge JMC. Reactive species and disease: fact, fiction or filibuster? In: Halliwell B, Gutteridge JMC, eds. *Free Radicals in Biology and Medicine*. New York: Oxford University Press, 2007: 488–613.
25. Chapple IL. Reactive oxygen species and antioxidants in inflammatory diseases. *J Clin Periodontol* 1997;**24:**287–296.
26. Hanham AF, Dunn BP, Stich HF. Clastogenic activity of caffeic acid and its relationship to hydrogen peroxide generated during autooxidation. *Mutat Res* 1983;**116:**333–339.
27. Stich HF. The beneficial and hazardous effects of simple phenolic compounds. *Mutat Res* 1991;**259:**307–324.
28. Chen CL, Chi CW, Liu TY. Hydroxyl radical formation and oxidative DNA damage induced by areca quid in vivo. *J Toxicol Environ Health A* 2002;**65:**327–336.
29. Haque MF, Harris M, Meghji S, Barrett AW. Immunolocalization of cytokines and growth factors in oral submucous fibrosis. *Cytokine* 1998;**10:**713–719.
30. Chang LY, Wan HC, Lai YL, Liu TY, Hung SL. Enhancing effects of areca nut extracts on the production of interleukin-6 and interleukin-8 by peripheral blood mononuclear cells. *J Periodontol* 2006;**77:** 1969–1977.
31. Hung S-L, Chen Y-L, Wan H-C, Liu T-Y, Chen Y-T, Ling L-J. Effects of areca nut extracts on the functions of human neutrophils in vitro. *J Periodont Res* 2000;**35:** 186–193.
32. Pennica D, Nedwin GE, Hayflick JS *et al.* Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature* 1984;**312:**724–729.
33. Auron PE, Webb AC, Rosenwasser LJ *et al.* Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *PNAS* 1984;**81:**7907–7911.
34. Hirano T, Yasukawa K, Harada H *et al.* Complementary DNA for a novel human interleukin (BSF-2) that induces B lym-

phocytes to produce immunoglobulin. *Nature* 1986;**324:**73–76.
35. Mukaida N, Shiroo M, Matsushima K. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J Immunol* 1989;**143:**1366–1371.
36. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Detection of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994;**368:**753–756.
37. Nair UJ, Nair J, Friesen MD, Bartsch H, Ohshima H. Ortho- and meta-tyrosine formation from phenylalanine in human saliva as a marker of hydroxyl radical generation during betel quid chewing. *Carcinogenesis* 1995;**16:**1195–1198.
38. Chen PH, Tsai CC, Lin YC *et al*. Ingredients contribute to variation in production of reactive oxygen species by areca quid. *J Toxicol Environ Health A* 2006;**69:**1055–1069.
39. Jagetia GC, Aggarwal BB. "Spicing up" of the immune system by curcumin. *J Clin Immunol* 2007;**27:**19–35.
40. Ruby AJ, Kuttan G, nesh Babu K, Rajasekharan KN, Kuttan R. Anti-tumour and antioxidant activity of natural curcuminoids. *Cancer Lett* 1995;**94:**79–83.
41. Zheng S, Yumei F, Chen A. De novo synthesis of glutathione is a prerequisite for curcumin to inhibit hepatic stellate cell (HSC) activation. *Free Radic Biol Med* 2007;**43:**444–453.
42. Strasser EM, Wessner B, Manhart N, Roth E. The relationship between the anti-inflammatory effects of curcumin and cellular glutathione content in myelomonocytic cells. *Biochem Pharmacol* 2005;**70:**552–559.
43. Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* 1999;**85:**753–766.
44. Rahman I, Gilmour PS, Jimenez LA, MacNee W. Oxidative stress and TNF-α induce histone acetylation and NF-κB/AP-1 activation in alveolar epithelial cells: potential mechanism in gene transcription in lung inflammation. *Mol Cell Biochem* 2002;**235:**239–248.
45. Lin SC, Lu SY, Lee SY, Lin CY, Chen CH, Chang KW. Areca (betel) nut extract activates mitogen-activated protein kinases and NF-κB in oral keratinocytes. *Int J Cancer* 2005;**116:**526–535.
46. Chang MC, Wu HL, Lee JJ *et al*. The induction of prostaglandin E_2 production, interleukin-6 production, cell cycle arrest, and cytotoxicity in primary oral keratinocytes and KB cancer cells by areca nut ingredients is differentially regulated by MEK/ERK activation. *J Biol Chem* 2004;**279:**50676–50683.
47. Duvoix A, Blasius R, Delhalle S *et al*. Chemopreventive and therapeutic effects of curcumin. *Cancer Lett* 2005;**223:**181–190.
48. Grandjean-Laquerriere A, Gangloff SC, Le Naour R, Trentesaux C, Hornebeck W, Guenounou M. Relative contribution of NF-κB and AP-1 in the modulation by curcumin and pyrrolidine dithiocarbamate of the UVB-induced cytokine expression by keratinocytes. *Cytokine* 2002;**18:**168–177.