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Areca nut extracts reduce the intracellular reactive oxygen species and release of myeloperoxidase by human polymorphonuclear leukocytes

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Background and Objective: Polymorphonuclear leukocytes (PMN) represent the first line of host defense. Areca nut extract inhibits the bactericidal activity of, and the release of superoxide anion (O_2^-) by, PMN. This study investigated the effects of areca nut extract on the intracellular production of reactive oxygen species (ROS) and on the extracellular release of lysosomal enzyme, myeloperoxidase (MPO), by PMN. The effects of arecoline, a principal component of areca nut, were also examined.

Material and Methods: Human PMN were treated with various concentrations of areca nut extract or arecoline followed by treatment with Hanks' balanced salt solution, with or without cytochalasin B and fMet-Leu-Phe (CB/fMLP). The viability of PMN was determined using propidium iodide staining and flow cytometry. The presence of intracellular ROS was determined using 2′,7′-dichlorofluorescin diacetate and fluorometry. MPO release was determined using a substrate assay.

Results: Areca nut extract (25 and 50 μg/ml) significantly decreased the viability of PMN. The intracellular levels of ROS and the extracellular release of MPO were induced in PMN by CB/fMLP. Exposure of PMN to areca nut extract (up to 25 μg/ml) or to arecoline (up to 2 mg/ml) did not directly affect the levels of ROS and MPO activity. However, under conditions that did not affect the viability of PMN, the ability of CB/fMLP to trigger production of intracellular ROS and release of MPO in human PMN was significantly suppressed by areca nut extract and arecoline.

Conclusion: Areca nut impaired the activation of PMN by CB/fMLP that might decrease the effectiveness of PMN in the host defense. Alternatively, exposure of PMN to areca nut extract could decrease the capacity of PMN to damage tissues.

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Areca (betel) chewing is the fourth most commonly addicted habit in the world and is associated with an increased risk of oral submucous fibrosis and oral squamous cell carcinoma (1). Several studies have demonstrated an association between areca chewing and periodontal diseases (2–7). 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. Epidemiological and experimental studies have revealed the cytotoxic, genotoxic, and carcinogenic potencies of areca nut (1). Areca nut extract and its major areca alkaloid, arecoline, inhibit the growth, attachment, and matrix protein synthesis of human gingival fibroblasts *in vitro* (8,9). Moreover, arecoline inhibits protein synthesis in human periodontal ligament fibroblasts (10). Areca nut may also compromise the periodontal health of areca chewers by suppression of alkaline phosphatase gene expression and elevation of RANKL (receptor activator of nuclear factor-kappa B ligand) gene expression in osteoblasts (11).

Polymorphonuclear leukocytes (PMN), the most abundant circulating leukocytes, represent the first line of the host defense mechanism. The main functions of PMN include adherence, chemotaxis, phagocytosis, and bactericidal activity (12–14). PMN within the gingival crevice are able to protect the gingiva against microbial invasion through mechanisms such as the secretion of hydrolytic enzymes and the production of oxygen radicals (12–14). The production of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radial, through the respiratory burst in PMN, is an important pathway involved in oxidative killing (15,16). Moreover, superoxide may function intracellularly by activating proteases within the phagolysosome (17,18). Activation of PMN may also lead to the release of enzymes harbored within the cytoplasmic vesicles of PMN. One of the principal enzymes released from the azurophilic granules is myeloperoxidase (MPO), which can catalyze the formation of a potent oxidant, hypochlorous acid (19,20). Patients with an altered number or function of circulating PMN tend to suffer severe periodontal diseases (16,21–25). Amplified activity of PMN may also be responsible for the tissue destruction in periodontal disease (16,26).

Areca chewing seems to favor subgingival infection with the periodontal pathogens *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, which may lead to greater severity of periodontal disease (4). areca nut extract interferes with the release of superoxide anion by PMN and inhibits the bactericidal and phagocytic activity of PMN (27,28). In this report, the effects of areca nut extract and arecoline on the human PMN functions, with respect to intracellular production of ROS and extracellular release of MPO, are presented.

Material and methods

Areca nut extracts

Areca nut extract was prepared from dried ripe areca nuts without husk, as previously described (29,30). Areca nut extract was first dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St Louis, MO, USA) before being diluted in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS) (Gibco BRL Laboratories, Grand Island, NY, USA). The final concentration of DMSO in each sample was less than 0.5%.

Isolation of PMN

PMN were freshly purified from human venous peripheral blood of healthy nonsmokers by dextran sedimentation followed by ficoll density-gradient centrifugation, as described previously (27). Informed consent was obtained from each volunteer. This research proposal was approved through the Institutional Review Board of the Taipei Veterans General Hospital, Taiwan. The PMN/red blood cell pellet was transferred to a new tube and washed once with HBSS. Residual red blood cells were ruptured by hypotonic lysis. After washing and resuspension with HBSS, PMN were counted with a hemocytometer.

Viability of PMN

Viability of PMN after areca nut extract or arecoline treatment was determined by detecting the influx of propidium iodide (PI) into PMN (31). This assay is based on the fact that PI stains nucleic acids in dead cells that have lost their membrane integrity (32,33). Freshly isolated PMN (2×10^6 cells/ml) were incubated with various concentrations of areca nut extract or arecoline (Sigma) for 30 min at 37°C. PMN treated with HBSS only, or with HBSS containing 0.5% DMSO, under similar conditions, served as controls, whereas PMN fixed in 3% paraformaldehyde served as a control for dead cells. HBSS containing 0.5% DMSO did not affect the viability of PMN. Treated PMN were washed and incubated at 2×10^6 cells/ml in HBSS or HBSS containing 4 μg/ml of PI (Sigma) at 37°C for 10 min. After washing twice with HBSS, PMN 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, Cockeysville, MD, USA) equipped with an argon laser operating at an excitation wavelength of 488 nm. The instrument settings were as follows: sideward scatter threshold set at 52; and detector set at E00, 320 and 520 for forward scatter, sideward scatter and fluorescence 2 (FL2, red fluorescence), respectively. Data were analyzed using analytical software programs (CELLQUEST® and WINMDI 2.8; Becton Dickinson). The fluorescence intensities of a total of 10,000 cells were measured. Viability of PMN in each sample was determined by the following formula:

$$\text{Viability of PMN} = \text{number of unstained cells} \div \text{total number of cells.}$$

The relative viability of PMN compared with the control was determined by the following formula:

Relative viability of PMN = (viability of treated PMN ÷viability of control PMN) × 100%.

Detection of intracellular ROS

The intracellular ROS was determined as described by Chen *et al.* (34), with modification. The principle of the test is based on the diffusion of nonpolar 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) into the cells through the cell membrane. The H_2DCFDA is then hydrolyzed to nonfluorescent dichlorofluorescin (H_2DCF) (35). ROS caused oxidation of H_2DCF to a measurable fluorescent product, dichlorofluorescein (36). Freshly isolated PMN were treated with various concentrations of areca nut extract or arecoline in the presence of H_2DCFDA (50 μM) (Molecular Probes, Inc., Eugene, OR, USA) for 30 min at 37°C and then washed with ice-cold phosphate-buffered saline (PBS). Treated PMN (2×10^6 cells/ml) were further incubated with HBSS, or with 5 μg/ml of cytochalasin B and 10 μM of fMet-Leu-Phe (CB/fMLP) in HBSS, for 15 min at 37°C (37,38). Cells were finally resuspended in 100 μl of PBS to a final concentration of 1×10^6 cells/ml for measurements of fluorescence using the Fluorescence Measurement System (Wallac 1420 multilabel counter Victor[2]; Perkin Elmer, Norwalk, CT, USA) (excitation 485 nm, emission 535 nm). The relative level of intracellular ROS was calculated as follows:

Relative level of intracellular ROS = fluorescence intensity of experimental sample ÷ fluorescence intensity of HBSS-treated PMN with no exposure to areca nut extract or to arecoline.

Myeloperoxidase released from PMN

The effects of areca nut extract or arecoline on extracellular release of the lysosomal enzyme, MPO, were determined (38). Freshly isolated PMN (4×10^6 cells/ml) were incubated with various concentrations of areca nut extract or arecoline for 30 min at 37°C. After washing and resuspension in HBSS, treated PMN (2×10^6 cells/ml) were further incubated with HBSS or CB/fMLP for 15 min at 37°C. The supernatants of the reaction mixtures were harvested by centrifugation at 1700 *g* for 2 min at 4°C. The reaction mixture (in a total volume of 200 μl), which consisted of PMN supernatant, 1.6 mM tetramethylbenzidine, 0.3 mM H_2O_2, 30 mM sodium citrate buffer (pH 5.4), 8% *N*,*N*-dimethylformamide, and 40% PBS, was incubated for 3 min at 37°C. The reaction was terminated by cooling in an ice bath and by the addition of sodium citrate buffer (pH 3.0) to a final concentration of 155 mM. The absorbance (*A*) of the MPO product was measured in a spectrophotometer (DU® 640 spectrophotometer; Beckman Instruments, Inc., Fullerton, CA, USA) at a wavelength of 655 nm. The relative activity of MPO was calculated as follows:

Relative activity of MPO = *A* of experimental sample ÷ *A* of HBSS-treated PMN with no exposure to areca nut extract or to arecoline.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). Differences between the experimental sample and HBSS-treated PMN in the absence or presence of CB/fMLP were analyzed using the paired *t*-test. Differences between PMN that were treated with HBSS or with CB/fMLP at the same concentration of areca nut extract or arecoline were also compared using the paired *t*-test. A *p*-value of < 0.05 was considered significantly different.

Results

The effects of areca nut extract and arecoline on size, granularity and viability of PMN were analyzed using flow cytometry. Changes in light scatter profiles were observed when PMN were treated with areca nut extract (Fig. 1A), but not with arecoline (Fig. 1B). The size and granularity of areca nut extract-treated PMN increased slightly when compared with the control. Viable cells exhibit low background fluorescence (Fig. 2). Mean background fluorescence increased from 2.12 to 6 when treated with 50 μg/ml of areca nut extract (Fig. 2A). A permeabilized membrane allows PI to diffuse to the nucleus, where firm binding to nucleic acids occurs with concomitant appearance of bright cell fluorescence. Treatment of PMN with areca nut extract impaired the viability of PMN in a dose-dependent manner (Fig. 2A). The relative cellular viability was reduced to 55.94 ± 9.54% ($p = 0.023$) and to 33.55 ± 4.11% ($p = 0.002$) when 25 μg/ml and 50 μg/ml of areca nut extract were used, respectively (Fig. 3A). However, there was no difference in PI exclusion between PMN exposed to arecoline and nonexposed control cells (Figs 2B and 3B), suggesting that the integrity of the plasma membrane was not affected after exposure to arecoline at the concentrations examined.

The effects of areca nut extract and arecoline on intracellular levels of ROS in PMN were determined. Intracellular production of ROS in control PMN was significantly induced by CB/fMLP activation ($p < 0.05$) (Fig. 4). In the absence of CB/fMLP, treatment with various concentrations of areca nut extract (0.781–12.5 μg/ml) (Fig. 4A) or arecoline (0.016–10 mg/ml) (Fig. 4B) did not significantly alter the intracellular levels of ROS in PMN. However, in the presence of CB/fMLP, the intracellular levels of ROS in areca nut extract-treated PMN decreased when compared with HBSS-treated PMN. In the presence of CB/fMLP, the intracellular levels of ROS in PMN were significantly reduced when treated with 12.5 μg/ml of areca nut extract ($p = 0.03$), 2 mg/ml of arecoline ($p = 0.044$) or 10 mg/ml of arecoline ($p = 0.003$) (Fig. 4). The levels of ROS production after activation by CB/fMLP decreased to ≈50% when 1.563 μg/ml of areca nut extract or 16 μg/ml of arecoline was used. Thus, areca nut extract treatment interfered with the activation of PMN by CB/fMLP. The results indicate that areca nut extract abolished the activation of PMN by CB/fMLP in terms of intracellular ROS production (Fig. 4).

The effects of areca nut extract and arecoline on the extracellular release of MPO from PMN were analyzed

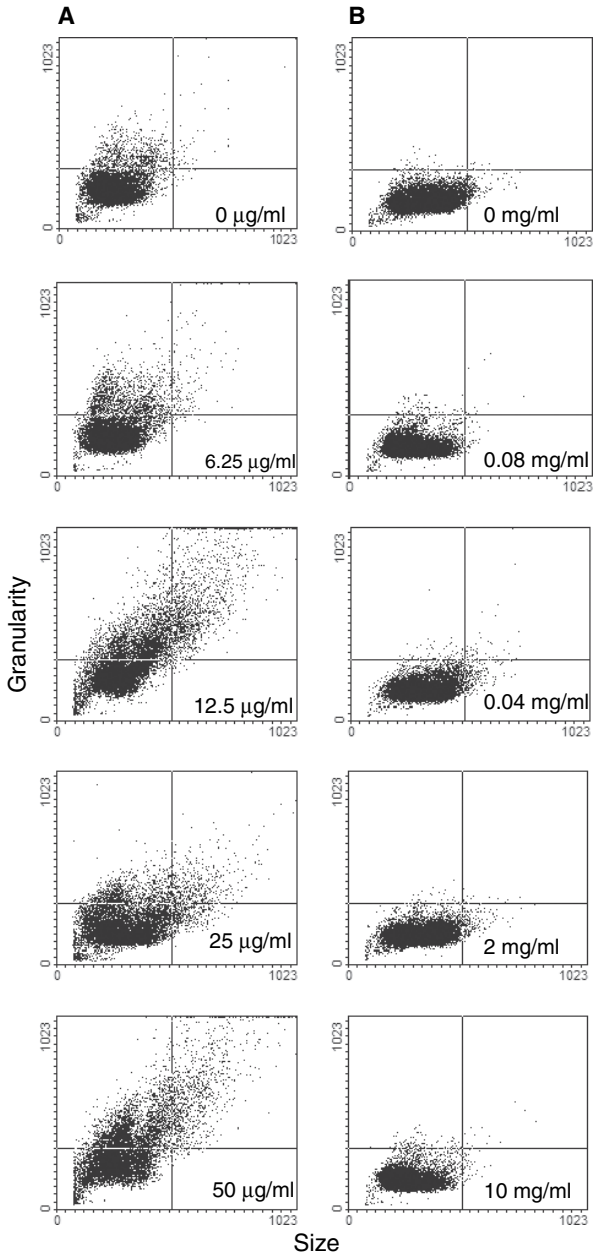

Fig. 1. Effects of areca nut extract on patterns of scattered light of polymorphonuclear leukocytes (PMN). PMN were incubated with (A) areca nut extract (0, 6.25, 12.5, 25 or 50 μg/ml) or with (B) arecoline (0, 0.08, 0.4, 2 or 10 mg/ml) for 30 min, washed, and examined using flow cytometry. The forward scatter (size) and sideward scatter (granularity) signals of each treated sample are shown. The figures illustrated are the representative results of three independent experiments performed with PMN obtained from different donors.

by measuring enzyme activity. The relative release of MPO by PMN in the presence of CB/fMLP was $\approx 2.31 \pm 0.43$ times higher than PMN in the absence of CB/fMLP ($p = 0.049$) (Fig. 5A). Exposure of PMN to areca nut extract (3.125–25 μg/ml) did not significantly affect the extracellular release of MPO from PMN in the absence of CB/fMLP (Fig. 5A). Little difference was observed between PMN that were treated with HBSS or with CB/fMLP at all concentrations of areca nut extract examined (Fig. 5A), suggesting that areca nut extract inhibited the inducing effects of CB/fMLP to release MPO. For arecoline experiments, the relative release of MPO by PMN in the presence of CB/fMLP was $\approx 2.66 \pm 0.25$ times higher than PMN in the absence of CB/fMLP ($p = 0.011$) (Fig. 5B). In the absence of CB/fMLP, exposure of PMN to arecoline (0.08–2 mg/ml) did not alter the release of MPO. The release of MPO was increased when PMN were treated with 10 mg/ml of arecoline. Exposure of PMN to arecoline interfered with the activation of PMN by CB/fMLP to release MPO. The relative release of MPO decreased to 1.64 ± 0.27 ($p = 0.007$), 1.67 ± 0.25 ($p = 0.006$) or 1.60 ± 0.18 ($p = 0.008$) when 0.4 mg/ml, 2 mg/ml or 10 mg/ml of arecoline was used, respectively (Fig. 5B). Therefore, areca nut extract and arecoline reduced the release of MPO from PMN that were induced by CB/fMLP.

Discussion

PMN are important immune cells and kill invading bacteria via oxygen-dependent and oxygen-independent mechanisms. However, excess release of ROS from PMN may damage periodontal tissues. This *in vitro* study determined the effects of areca nut extract and arecoline on the functions of PMN regarding intracellular production of ROS and extracellular release of MPO. In our findings, areca nut extract (up to 25 μg/ml) and arecoline (up to 2 mg/ml) did not have a direct effect on the intracellular production of ROS and release of MPO by PMN. However, under conditions that did not affect the viability of PMN, areca nut extract and arecoline suppressed the activation activity of PMN induced by CB/fMLP. These observations indicate that areca nut extract and arecoline might affect the normal activation activity of PMN.

The antimicrobial efficiency of human PMN depends on certain events, including the generation of ROS by assembly and activation of the NADPH-dependent oxidase, and the release of enzymatic or antimicrobial protein content in the granules. The combination of CB and fMLP enhances the production of ROS in PMN (39). This study showed that the levels

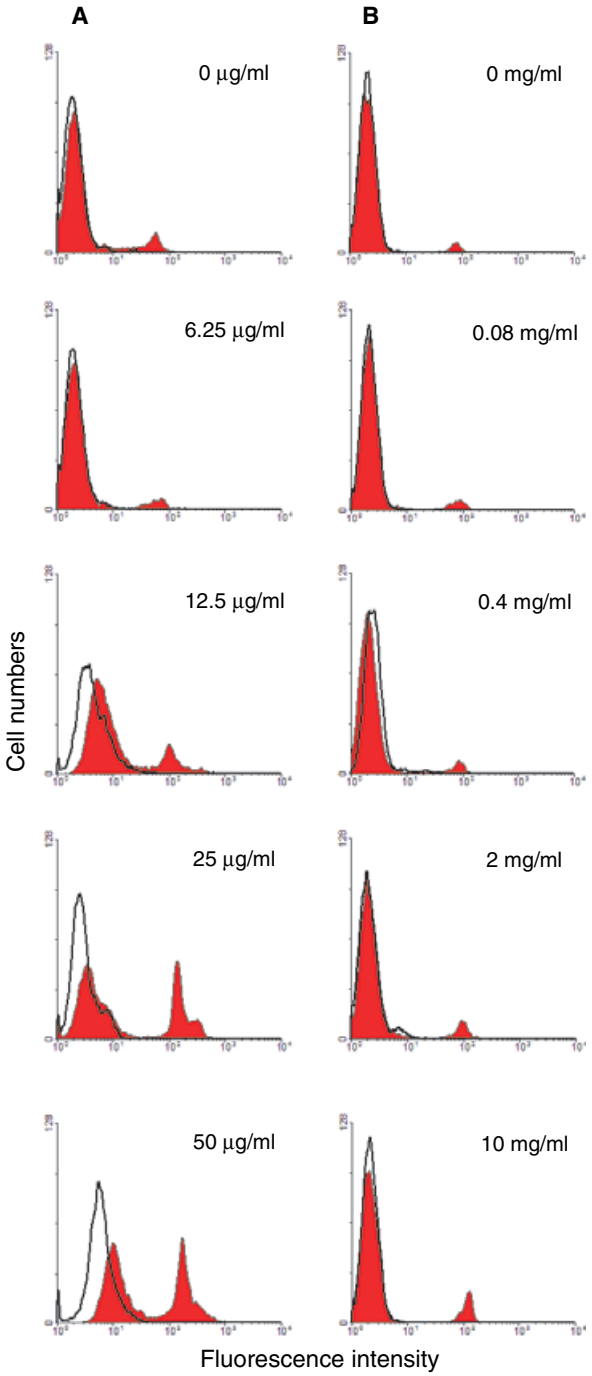

Fig. 2. Effects of areca nut extract and arecoline on the viability of polymorphonuclear leukocytes (PMN) as determined using propidium iodide (PI) staining. PMN were incubated with (A) areca nut extract (0, 6.25, 12.5, 25 or 50 μg/ml) or with (B) arecoline (0, 0.08, 0.4, 2 or 10 mg/ml) for 30 min, washed, and then tested for PI staining followed by flow cytometry. Logarithmic parameters were shown for fluorescence intensity. Black lines represent the fluorescence of PMN incubated without PI, whereas the red areas represent the fluorescence intensity of PMN incubated with PI. The histograms illustrated are the representative results of three independent experiments performed with PMN obtained from different donors.

of ROS production, after activation by CB/fMLP, decreased to ≈50% when 1.563 μg/ml of areca nut extract or 16 μg/ml of arecoline was used. Superoxide may function intracellularly by activating proteases within the phagolysosome (17,18). Thus, areca nut extract and arecoline may inhibit the production of intracellular ROS that lead to inhibition of the bactericidal activity. The concentration of arecoline in saliva during areca chewing is ≈140 μg/ml (40). Concentrations of areca nut extract sufficient to impair the function of PMN would be present in the gingival tissues and crevicular fluids of areca chewers. Further studies will be of value to define the contribution of areca nut in the functions of PMN *in vivo*.

Secretion of antimicrobial compounds, including β-glucuronidase, elastase and MPO, is one mechanism performed by PMN to deliver antimicrobial substances (41). MPO is a hemoprotein, present in the azurophil granules of PMN, which is released upon cell activation into the phagolysosome or into the extracellular space (42). MPO reacts with H_2O_2, formed by the NAPDH oxidase, and increases the toxic potential of this oxidant. Treatment of areca nut extract and arecoline inhibited the release of MPO in CB/fMLP-activated PMN. Many of the areca chewers also smoke and drink alcohol. Moreover, areca quid may contain tobacco as an additive. Similarly to the results observed for areca nut extract and arecoline in this study, nicotine does not affect directly the release of MPO from PMN, but inhibits the release of MPO when PMN are treated with CB/fMLP (38). In addition, nicotine reduces the activation ability of CB/fMLP regarding ROS production (38). Ethanol also inhibits the release of MPO by CB/fMLP-activated PMN (43). These findings indicate that nicotine, ethanol, arecoline, and areca nut extract can inhibit the release of MPO from activated PMN. All these components may affect the immune functions of PMN. Whether there is an additive effect of nicotine, ethanol, and areca nut remains to be examined.

The production of ROS has been shown to influence cell cycle progression, apoptosis, and chemical toxicity (44,45). Areca nut extract induces oxidative DNA damage in Chinese hamster ovary cells (CHO-K1) (30). The production of intracellular H_2O_2 in

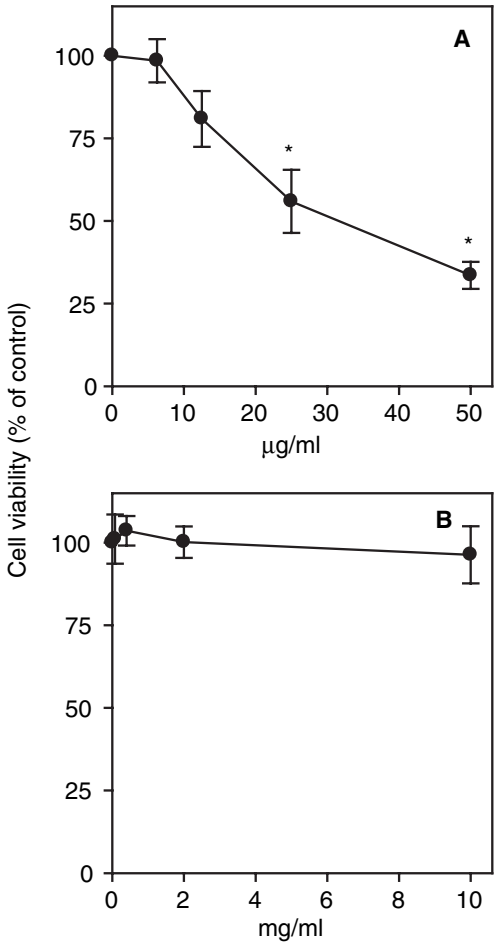

Fig. 3. Effects of areca nut extract and arecoline on the relative viability of polymorphonuclear leukocytes (PMN). The percentages of cellular viability in the presence of (A) areca nut extract or (B) arecoline, relative to that of the control, are shown. The results were averaged from three independent experiments performed with PMN obtained from different donors. The standard deviation (SD) is shown as an error bar. *Significant difference ($p < 0.05$) compared with control PMN.

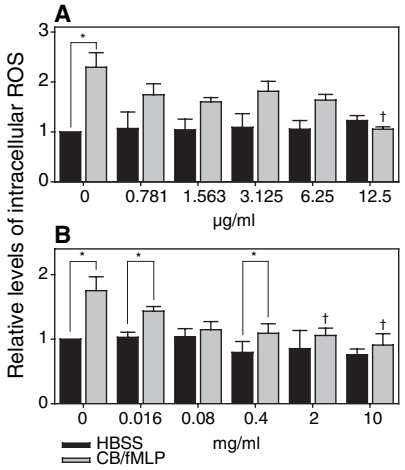

Fig. 4. Effects of areca nut extract and arecoline on the production of intracellular reactive oxygen species (ROS) in polymorphonuclear leukocytes (PMN). In the presence of 2′,7′-dichlorofluorescin diacetate (H_2DCFDA), PMN were treated with various concentrations of (A) areca nut extract or (B) arecoline followed by incubation with Hanks' balanced salt solution (HBSS) or cytochalasin B and fMet-Leu-Phe (CB/fMLP) for 15 min at 37°C. The fluorescence intensity of control PMN treated with HBSS was considered as 1. Each treated sample was expressed as relative levels of intracellular ROS compared with the control sample. The results from three independent experiments performed with PMN obtained from different donors are expressed as mean ± SD. *Significant difference ($p < 0.05$) between PMN that were treated with HBSS or with CB/fMLP at the indicated concentration. †Significant difference ($p < 0.05$) compared with control PMN that were treated with CB/fMLP.

oral carcinoma cells (KB) is enhanced by areca nut extract (800–1200 μg/ml), but inhibited by arecoline (0.1–1.2 mM; equivalent to 23.6–283 μg/ml) (46). Aqueous extracts of areca quid induce the formation of H_2O_2 and MPO activity in mouse skin cells by topical treatment (47), indicating a crucial role in hyperplasia and inflammation. Extracts of areca quid, containing areca nut, flower of *Piper Linn*, and red lime, induce the production of H_2O_2 and the activity of MPO in mouse epidermal cells (JB6) with a long-term treatment (48). However, areca nut extract and arecoline reduced the extracellular release of superoxide anion in PMN that were treated with lipopolysaccharide and phorbol 12-myristate 13-acetate (27). In addition, areca nut extract and arecoline did not have a direct effect on the production of ROS and the release of MPO in PMN in this study. The discrepancy in these studies may be a result of differences in the composition of areca quid, the culture medium, the incubation time, and the cell system examined. The resting levels of H_2O_2 for salivary PMN were elevated when compared with blood PMN (49). The possible effects of areca nut extract on salivary PMN *in vivo* require further analysis.

The functions of PMN induced by fMLP, including oxidative burst or granule secretion, are regulated by mitogen-activated protein (MAP) kinases (50,51). In response to fMLP stimulation, the kinase activity and phosphorylation of MAP kinases, including p38 MAP kinase and extracellular signal-regulated kinase (ERK), are increased (50–52). Stimulation with fMLP also induces intracellular calcium release and extracellular calcium influx (53). Increases in the intracellular concentration of Ca^{2+} may activate protein kinase C, which regulates degranulation and the production of ROS (54,55). The activation of the oxidative metabolism, known as the respiratory burst, first involves the activation of NADPH oxidase, an enzymatic complex composed of cytosolic (p40phox, p47phox and p67phox)

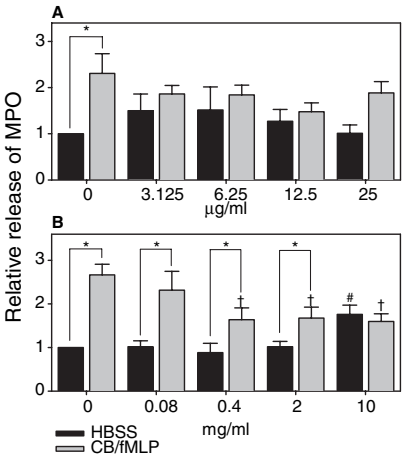

Fig. 5. Effects of areca nut extract and arecoline on the extracellular release of myeloperoxidase (MPO) from polymorphonuclear leukocytes (PMN). PMN were incubated with various concentrations of (A) areca nut extract or (B) arecoline for 30 min followed by treatment with Hanks' balanced salt solution (HBSS) or cytochalasin B and fMet-Leu-Phe (CB/fMLP). The supernatants of the reaction mixtures were analyzed for MPO activity. The absorbance (*A*) of control PMN treated with HBSS was considered as 1. Each enzyme activity was expressed as relative release of MPO compared with the control sample. The results from three independent experiments performed with PMN obtained from different donors are expressed as mean ± standard deviation (SD). *Significant difference ($p < 0.05$) between PMN that were treated with HBSS or with CB/fMLP at the indicated concentration; # significant difference ($p < 0.05$) compared with control PMN that were treated with HBSS; and † significant difference ($p < 0.05$) compared with control PMN that were treated with CB/fMLP.

and membrane proteins (p22phox and gp91phox) (56). After formation of active NADPH oxidase, this enzymatic complex is able to generate superoxide anion (O_2^-), which can dismutate into H_2O_2 (57). Arecoline also depletes intracellular thiols in gingival fibroblasts (58) and periodontal ligament fibroblasts (10). Depletion of thiols (such as glutathione) and ROS production may play crucial roles in the toxicity of areca nut. The detailed molecular mechanisms involved in the inhibitory effects by areca nut extract and arecoline observed in CB/fMLP-treated PMN in this study remain to be defined.

In conclusion, treatment of PMN with areca nut extract or arecoline succeeded in preventing the increase of intracellular ROS and release of MPO caused by CB/fMLP activation. The results here imply that areca nut extract and arecoline could impair substantially the normal functions of PMN. These might result in harmful influences on the host defense mechanism. However, the results also raise the possibility that exposure of PMN to areca nut extract and arecoline could decrease the capacity of PMN to damage tissues. The clinical relevance of these observations remains to be examined.

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