Areca nut extract up-regulates prostaglandin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes

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There are about 600 million betel quid (BQ) chewers in the world. BQ chewing is associated with increased incidence of oral cancer and submucous fibrosis. In this study, areca nut (AN) extract (200–800 µg/ml) induced the prostaglandin E2 (PGE2) production by 1.4–3.4-fold and 6-keto-PGF1a production by 1.1–1.7-fold of gingival keratinocytes (GK), respectively, following 24 h of exposure. Exposure of GK to AN extract (>400 µg/ml) led to cell retraction and intracellular vacuoles formation. At concentrations of 800 and 1200 µg/ml, AN extract induced cell death at 21–24 and 32–52% as detected by MTT assay and cellular lactate dehydrogenase release, respectively. Interestingly, AN-induced morphological changes of GK are reversible. GK can still proliferate following exposure to AN extract. Cytotoxicity of AN extract cannot be inhibited by indomethacin (1 µM) and aspirin (50 µM), indicating that prostaglandin (PG) production is not the major factor responsible for AN cytotoxicity. PGE2 exhibited little effect on the growth of GK at concentrations ranging from 100–1000 µg/ml. Stimulating GK production of PGs by AN extract could be due to induction of cyclooxygenase-2 (COX-2) mRNA expression and protein production. These results suggest that AN ingredients are critical in the pathogenesis of oral submucous fibrosis and oral cancer via their stimulatory effects on the PGs, COX-2 production and associated tissue inflammatory responses. AN cytotoxicity to GK is not directly mediated by COX-2 stimulation and PG production.

Introduction

It has been estimated that there are about 600 million people who chew betel quid (BQ) regularly throughout their life (1). BQ chewing is the major contributory factor of oral leukoplakia, oral submucous fibrosis and oral cancer in India, Taiwan and many other countries (1,2). However, the precise mechanisms of oral cancer are still not fully elucidated. In Taiwan, BQ usually consists of areca nut (AN), the inflorescence of Piper betle Linn. and lime, with or without the leaves of Piper betle Linn. Most of the previous studies have focused on evaluating the mutagenicity, genotoxicity and cytotoxicity of various BQ ingredients using mammalian cells or bacterial testing systems (1–5). Recently, chemical carcinogenesis has been considered to be a multi-step process where numerous complex non-genotoxic factors are also involved. Exogenous carcinogens have been suggested to induce the progression of cancer by promoting tissue inflammation (6). Interestingly, connective tissue inflammation is a consistent histopathological finding in tissues obtained from BQ chewers with oral leukoplakia and submucous fibrosis (2). Since aberrant and persistent tissue inflammation are crucial to the occurrence of cancer and tissue fibrosis (6–8), we propose that inducing the oral mucosal inflammation by BQ ingredients can be critical in the pathogenesis of oral cancer and oral submucous fibrosis.

What type of cells can be the target of BQ ingredients? Keratinocyte-derived mediators have been suggested as the initiators for skin and mucosal inflammation (9). Dermal changes such as inflammation or dermal papilla atrophy are considered to be secondary effects due to cytokines or other mediators produced by activated keratinocytes (10). Among numerous inflammatory mediators, prostaglandins (PGs) are considered to be important for tumor initiation, promotion and metastasis (11). PGs can suppress the humoral, cellular immune responses and the killing of malignant cells (11,12). These well-established findings prompted us to elucidate whether BQ ingredients could take part in the oral carcinogenic and fibrotic processes by inducing PG production of gingival keratinocytes (GK). In the present study, we therefore tested the effects of AN extract on the PG production, cyclooxygenase-2 (COX-2) mRNA and protein production of cultured human GK.

Materials and methods

Materials

Lactate dehydrogenase (LDH) assay kits, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), calf skin type I collagen and bovine plasma fibronectin were obtained from Sigma Chemical Co., St Louis, MO. PGE2, 6-keto-PGF1a and thromboxane B2 assay kits and PGE2 were purchased from Cayman Chemical Co. (Ann Arbor, MI). Keratinocyte growth medium (KGM-SFM), pituitary gland extract and epidermal growth factor were obtained from Gibco BRL, Life Technologies (Grand Island, NY). Ethidium bromide, agarose and kits for reverse transcription (RT) and polymerase chain reaction (PCR) were purchased from HT Inc., UK. Total RNA isolation kits were from Qiagen Inc. (Santa Clarita, CA). AN extract was prepared and weighed as described previously (4,5). Specific PCR primer sets for COX-2 and β-actin were synthesized by Genemed Biotechnologies, Inc. (San Francisco, CA). Mouse anti-human COX-2 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Protein assay kits were obtained from Bio-Rad (Hercules, CA).

Culture of GK

Human GK were established as described previously (5) except that calf skin type I collagen (80 µg/ml) and bovine plasma fibronectin (20 µg/ml)-coated culture dishes were used for cell culture. GK were cultured in KGM-SFM containing 5 ng/ml epidermal growth factor, 50 µg/ml pituitary gland extract, 100 U/ml penicillin and 100 µg/ml streptomycin. The culture passage numbers between one and three were used for this study.

Effects of AN extract on the PG production by GK

Briefly, 2.5×105 GK were seeded into 6-well culture plates. When confluence was reached, these cells were exposed to KGM-SFM overnight and then to 1 ml of fresh KGM-SFM containing AN extract at 100, 200, 400, 800 or
1200 µg/ml concentration for 24 h. The culture medium was collected for analysis of PGE₂, 6-keto-PGF₁α, and thromboxane B₂ production using enzyme-linked immunosorbent assay (ELISA). Morphological alterations of GK were photographed under a phase-contrast microscope. Concomitantly, the cultured medium and cell lysates were collected for cytotoxicity assay using LDH assay kits. The cell lysates were prepared by disrupting cells with 1 ml of 0.1% Triton X-100 for 10 min which was subjected to LDH assays following the manufacturer’s instructions. The LDH activity in the culture medium is used as an indicator of chemical cytotoxicity. In some experiments, culture medium was collected, cells washed several times with PBS and finally cytotoxicity was measured with the MTT assay (5). Moreover, indomethacin (1 µM) or aspirin (50 µM) was added 30 min prior to AN extract to study the roles of PGE₂ in AN cytotoxicity.

For elucidation of the growth capacity, GK were exposed to AN extract for 24 h and then moved to fresh medium for cell recovery. Viable cells were measured after 1 and 5 days. Briefly, cells were trypsinized and the number of cells that excluded trypan blue dye were counted under a phase-contrast microscope.

**Effects of PGE₂ on the growth of GK**

GK were seeded at an initial density of 5x10⁵ cells/well into 24-well culture plates. After 24 h, various amounts of PGE₂ were added into each well to reach a final concentration of 100-1000 µg/ml. Cells were cultured for a further 5 days. Cell number was measured with the MTT assay (5).

**Chemical exposure, RNA isolation and preparation of cell lysates**

Confluent GK were incubated in KGM-SFM for 24 h and then exposed to various concentrations of AN extracts (100-800 µg/ml) for 24 h. In another set of experiments, GK were exposed to AN extract (800 µg/ml) for 0.5, 1, 2, 4, 8 and 24 h. Total RNA was isolated using RNA isolation kits.

For western blot analysis, cell lysates of GK were collected as described previously (13). Briefly, GK were exposed to AN extract (800 µg/ml) for 0.5–8 h. Cells were then washed three times with ice-cold PBS, scraped off with a rubber policeman and pelleted at 800 µg for 5 min. Cells were then lysed in 250 µl of freshly prepared lysis buffer (10 mM Tris–HCl pH 7, 140 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 2 mM phenylmethylsulfonyl fluoride, 1% aprotinin and 5 mM dithiothreitol) for 20 min on ice. The cell lysate was cleared by centrifugation at 4°C for 30 min at 10,000 g and used for western blot analysis. Protein concentration was determined with Bio-Rad protein assay kits.

**Expression of COX-2 mRNA in cultured GK**

Briefly, 2 µg of denatured total RNA was subjected to RT in a total volume of 44.5 µl reaction mix containing 4 µl of random primer (500 µg/ml), 8 µl of dNTP (2.5 mM), 4.5 µl of 10× RT buffer, 1 µl of RNase inhibitor (40 U/ml) and 0.5 µl of RT (21 U/ml) at 42°C for 90 min. Four microliters of cDNA were used for PCR amplification in a reaction volume of 50 µl containing 5 µl of 10× Super Taq buffer, 4 µl of dNTP (2.5 mM), 1 µl each of specific primers and 0.2 µl of Super Taq enzyme (2 U/ml). Specific primers for human COX-2 were: 5′-GAA-GTC-AGT-GGT-GG-GTT-TTG-AAG-3′ and 5′-AGA-CTT-CCT-GGT-CAG-ATC-TTT-3′ (14). Primers for ß-actin are 5′-AAGAGAGCCATCCTCAACCTC-3′ and 5′-TACATGGCTGGGGTGTTGGA-3′. The amplified DNA products were 305 and 218 bp for COX-2 and ß-actin, respectively. The reaction mix was placed at 94°C for 5 min in the first cycle. Then the reaction was run for 15–25 cycles with a thermal cycler (Perkin Elmer 4800; PE Applied Biosystems, Foster City, CA) at 94°C for 1 min and 55°C for 5 min. Finally, the reaction was terminated following an extension at 55°C for 10 min. Amplified DNA products were subjected to 1.8% agarose gel electrophoresis and stained with ethidium bromide. The amount of amplified DNA product that is linear with respect to the input RNA was used for presentation.

**SDS–PAGE and COX-2 immunoblotting**

Immunoblotting procedures were performed as described previously (13). Briefly equal amounts of protein (50 µg/lane) were separated by 12% SDS–PAGE on 0.75 mm mini-gels (Midget System; Pharmacia Inc., Piscataway, NJ) and transferred to immobilon P membrane (Millipore Corp., Bedford, MA). After transfer, the semi-dry electrophoretic apparatus (TE70; Hoefer Scientific Instruments, San Francisco, CA). The membrane was blocked overnight at room temperature with blocking reagent (20 mM Tris pH 7.4, 125 mM NaCl, 0.2% Tween-20, 4% non-fat dry milk and 0.1% sodium azide) and then incubated for 1 h with mouse anti-human COX-2 monoclonal antibody (Transduction Laboratories, Lexington, KY). Membranes were washed three times and then incubated with alkaline phosphatase-conjugated rabbit anti-mouse antibody for COX-2 in PBS and 0.5% Tween-20 for another 45 min with gentle shaking. After three final washes, the proteins were visualized by incubating with colorogenic substrates, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co.).

**Results**

AN extract induced the PGE₂ production of GK by a dose-dependent manner. The medium PGE₂ level of untreated GK was measured to be 245 pg/ml. The PGE₂ production of GK was increased by 1.4–3.4-fold following exposure to 200–800 µg/ml of AN extract for 24 h (Figure 1a). The level of 6-keto-PGF₁α, a stable metabolite of prostacyclin (PGI₂), in the culture medium of untreated GK was 100.7 pg/ml, slightly less than that of PGE₂. However, the 6-keto-PGF₁α level was increased by 10 and 72% following exposure to AN extract at concentrations of 400 and 800 µg/ml, respectively (Figure 1b). In addition, the level of thromboxane B₂ in the culture medium of untreated GK was below detectable level (<7.8 pg/ml) (data not shown). Induction of PGE₂ production by AN extract is not directly associated with cytotoxicity as detected by measuring the LDH release into culture medium. At concentrations of 800 and 1200 µg/ml, AN extract caused 21 and 52% of cell death in 24 h (Figure 1c). Simultaneously, cytotoxicity was evaluated by measuring the mitochondrial functions. As analyzed by MTT assay, exposure to 800 µg/ml of AN extract for 24 h led to 24% of cell death (Figure 1d). AN extract, at concentrations <400 µg/ml, induced little cytotoxicity. Indomethacin (1 µM) and aspirin (50 µM), two cyclooxygenase inhibitors, were ineffective in preventing the AN-induced cytotoxicity (Table I) and intracellular vacuole formation (data not shown). In addition, PGE₂ had little effect on the morphology and growth of GK at concentrations of 100–1000 pg/ml (data not shown).

Confluent GK were cuboid or polygonal in appearance. They were in contact with each other and showed bright intercellular spaces (Figure 2a). Exposure of GK to AN extract (>400 µg/ml) for 24 h led to cell retraction and intracellular vacuole formation (data not shown). Cell retraction, increasing of intercellular space and formation of intracellular vacuoles of GK became more evident with exposure to 800 µg/ml of AN extract for 24 h (Figure 2b). Interestingly, these morphological changes of GK were reversible. Intracellular vacuoles became less evident after 5 days of recovery; GK could proliferate and fill the intercellular space (Figure 2c).

Quantitatively, exposure of GK to AN extract led to the death of some cells. After 24 h of recovery, the cell number decreased from 9.6×10⁵ (control) to 6.4×10⁵ and 4.1×10⁵ cells/well with exposure to 800 and 1200 µg/ml of AN extract, respectively. However, these damaged GK exerted the potential for repair and growth. Following 5 days of recovery, cell numbers increased to 8.3 and 5.5×10⁵ cells, respectively (Table II).

Stimulating the PGE₂ production of GK could be partly due to COX-2 mRNA expression by AN extract. COX-2 mRNA expression was stimulated by 200–800 µg/ml AN extract (Figure 3a). Stimulatory effects of AN extract (800 µg/ml) could be observed following exposure of GK for 1–2 h and persisted for >8 h (Figure 3b).

Cell lysate was subjected to western blot analysis to elucidate whether induction of COX-2 mRNA expression by AN extract was related to COX-2 protein production. As shown in Figure 4, AN extract (800 µg/ml) stimulated the COX-2 protein produc-
Betel quid chewing and oral cancer

Fig. 1. Induction of the PGE2 and 6-keto-PGF1α production, LDH release and MTT reduction of GK with AN extract. Confluent GK in 6-well culture plates were exposed to 1 ml of KGM-SFM containing various concentrations of AN extract (100–1200 µg/ml) for different time points. Culture medium was collected following exposure of GK to AN extract (100–800 µg/ml) for 24 h and used for measurement of (a) PGE2 level and (b) 6-keto-PGF1α level by ELISA. Results are expressed as percentage of control (as 100%, 245 pg/ml). (c) GK were exposed to AN extract (100–1200 µg/ml) for different time points. Culture medium and cell lysates were collected. The relative proportions of LDH activities in the medium and cell lysates were measured and used for indicators of cytotoxicity. At concentrations below 200 µg/ml, AN extract has no cytotoxicity and the data are not shown here. (d) Cytotoxicity of GK to AN extract was analyzed with MTT assay. Asterisks denote significant difference ($P < 0.05$) when compared with control group.

Discussion

Betel quid chewing is a popular yet harmful oral habit. There are about 600 million BQ chewers in the world (1). Epidemiological studies have elucidated a strong correlation between the incidence of oral leukoplakia, submucous fibrosis, oral cancer and the heavy consumption of BQ (2). However, the mechanisms underlying these oral mucosal diseases are not completely understood. PGs, as important inflammatory mediators, are present in virtually all mammalian tissues and are crucial molecules for signal transduction with a wide range of biological effects such as wound healing, bone metabolism, blood clotting, immune response, etc. (15). However, PGs also play critical roles in the pathogenesis of cancer and tissue fibrosis (7,9–12). The major products of arachidonic acid metabolism in GK are PGE2 and 6-keto-PGF1α as revealed in this study. Little thromboxane B2 was produced, indicating the differential expression of downstream enzymes synthesizing
Table I. Effects of indomethacin and aspirin on AN extract-induced cytotoxicity on cultured GK

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitochondrial dehydrogenase activity (% of control)</th>
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<tbody>
<tr>
<td>1200 µg/ml AN</td>
<td>68.8 ± 4.4</td>
</tr>
<tr>
<td>1 µM indomethacin + 1200 µg/ml AN</td>
<td>62.8 ± 2.8</td>
</tr>
<tr>
<td>50 µM aspirin + 1200 µg/ml AN</td>
<td>55.2 ± 2.8b</td>
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</table>

GK were exposed to indomethacin or aspirin 30 min prior to the addition of AN extract. Cell number was measured with an MTT assay 24 h later (n = 5).

PGE2, PGI2 and thromboxane B2 in GK. Some tumor promoters, including phorbol-12-myristate-13-acetate, mezerein and anthralin, etc., have been shown to induce PGE2 release of keratinocytes (11,12,16). Inflammatory cytokine, such as interleukin-1β, can induce even more than 50-fold of prostaglandin synthesis in A549 lung cancer cells (17). PGE2 and prostacyclin (PGI2) can induce vasodilatation, increase vascular permeability, and lead to inflammatory cell infiltration (7,15).

In the present study, AN extract stimulated the PGE2 and 6-keto-PGF1α production of GK. BQ chewing therefore contributes to the mucosal inflammation and may promote oral cancer progression by stimulating PG production by GK (8). Consistently, juxtaepithelial inflammation in biopsy specimens obtained from BQ chewers’ mucosa is usually observed during routine histopathological examination (2). Although the roles of PGE2 in the promotion of oral cancer are not well established, AN extract has recently been found to promote tumor formation in 7,12-dimethylbenzanthracene (DMBA)-initiated hamster cheek pouch (18). Persistent inflammatory responses further contribute to the occurrence of tissue fibrosis (7).

Induction of keratinocyte PG production by AN extract can therefore contribute to the occurrence of oral submucous fibrosis.

PGE2 can be induced by various tumor promoters, such as phorbol-12-myristate-13-acetate, mezerein, anthralin and sodium dodecyl sulfate, via interaction with specific cellular components or via non-specific cytotoxic response (16). In the present study, inducing PG production does not underlie the cytotoxicity of AN, as indicated by the LDH release and MTT assay. AN extract (400 µg/ml) induced the PGE2 production by GK even in the absence of cytotoxicity. Similarly, exposure of oral mucosal keratinocytes to AN extract (3–500 µg/ml) also decreased the clonal cell growth, uptake of neutral red, and increased uptake of trypan blue (3). For further elucidation of whether induction of prostaglandin production by AN extract is associated with cytotoxicity, two COX inhibitors (indomethacin and aspirin) were used. Interestingly indomethacin and aspirin at non-toxic concentrations are not able to prevent AN-extract-induced cytotoxicity. This indicates that AN cytotoxicity is not directly mediated by its induction of PGE2 production. AN extract has been shown to induce DNA strand breaks, unscheduled DNA synthesis and differentiation of cultured oral mucosa and GK (3,4,19). Toxic effects of the AN ingredients on GK can partly explain the epithelial atrophy with marked reduction of rete pegs in BQ chewers’ mucosa (2). Interestingly, AN-induced injury to GK is a reversible event as revealed by the disappearance of intracellular vacuoles and recovery of proliferation potential following exposure to fresh medium for 5 days. This is crucial for carcinogenic processes because normal cells should be able to divide and undergo transformation. AN extract has been shown to induce DNA damage and unscheduled DNA synthesis of oral keratinocytes (3,5,19). Proliferation of GK following exposure to AN extract will fix the damaged DNA, lead to formation of initiated cells and finally tumor generation.

Concomitantly, the cytotoxic effects of AN extract on the GK was accompanied by morphological changes. Confluent GK were cuboid or polygonal with evident intercellular space. Exposure of GK to AN extract (400–800 µg/ml) led to

Fig. 2. Morphological changes of GK. (a) Confluent GK are generally cuboid or polygonal in appearance with marked intercellular space. (b) Exposure of GK to AN extract (800 µg/ml) for 24 h. GK became retracted and numerous intracellular vacuoles were found. (c) Exposure of GK to AN extract (800 µg/ml) for 24 h and then incubation in fresh medium for 5 days of recovery. (100× original magnification, bar = 100 µm.)
showed linear relationship with the amplification bp, 19 cycles). The amplification -actin mRNA expression by GK with AN extract. (a) Confluent GK were exposed to solvent (control) or AN extract (100–800 µg/ml) for 24 h. Total RNA was isolated. COX-2 and β-actin mRNA expression was determined by RT–PCR for 25 and 20 cycles, respectively. (b) Confluent GK were exposed to solvent (control) or AN extract (800 µg/ml) for 0.5, 1, 2, 4, 8 and 24 h. Total RNA was extracted and used for semi-quantitative RT–PCR of COX-2 (305 bp, 24 cycles) and β-actin (218 bp, 19 cycles). The amplified DNA products of COX-2 and β-actin that showed linear relationship with the amplification cycles are shown. A DNA ladder of known base pairs was also used for identification of PCR products.

Table II. Number of GK following exposure to AN extract (800 and 1200 µg/ml) for 24 h and then placement in fresh medium for recovery of 1 and 5 days

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Cell number (×10^5 cells/well)</th>
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<tbody>
<tr>
<td></td>
<td>1 day</td>
</tr>
<tr>
<td>Control</td>
<td>9.6 ± 0.62</td>
</tr>
<tr>
<td>800 µg/ml AN extract</td>
<td>6.4 ± 0.17</td>
</tr>
<tr>
<td>1200 µg/ml AN extract</td>
<td>4.1 ± 0.52</td>
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</table>

Cells that exclude trypan blue dye are counted. Results are expressed as numbers of cells/well (mean ± SEM) (n = 3).

*Significant difference (P < 0.05) when compared with the cell number after 1 day recovery.

can stimulate or inhibit the cell proliferation and differentiation depending on the cell types examined (12). Sundqvist and Graffstrom (19) have recently found that AN ingredients can regulate the differentiation of buccal mucosal keratinocytes. This indicated that induction of PGs by BQ ingredients may be important for the pathogenesis of oral leukoplakia and oral cancer. However, this effect may not be due to direct mitogenesis of PGs, since PGE2 has little effect on the growth of GK. Perhaps PGs participate in the oral carcinogenesis indirectly via inducing inflammatory cell infiltration, cytokine production, reactive oxygen species production (6,8,15) or immune suppression (12). More studies are therefore needed to elucidate the roles of PGs in oral carcinogenesis.

Because glutathione is able to inhibit the isomerase enzyme responsible for converting the PGH2 to PGE2 (20), depletion of cellular glutathione by AN extract can be one possible reason that AN extract induced production of PGs by GK (3) (Jeng et al., unpublished observations). Another potential reason is inducing the expression of COX-2 mRNA by GK with AN ingredients. PGs are produced from arachidonic acid via the COX pathway. COX-2 is induced by various pro-inflammatory cytokines, growth factors, oncogenes, carcinogens and tumor-promoting phorbol esters (11,12,15,21). In the present study, stimulation of production of PGs by GK can be partly explained by the up-regulation of COX-2 mRNA expression by AN ingredients. AN-extract-induced COX-2 mRNA expression of GK was observed following 1–2 h of exposure, indicating that COX-2 expression is an early cellular response and regulated by AN ingredients at transcriptional level. Moreover, production of COX-2 protein by GK was also induced following exposure to AN extract for 2–8 h. Expression of COX-2 mRNA in head and neck tumor tissues has been reported to be 150-fold higher than that of normal mucosa (21). COX-2 protein is also increased in head and neck tumors (21). These results suggest the potentially important roles of COX-2 expression in BQ-chewing-related oral carcinogenesis. In extrahepatic regions like the head and neck, tissues usually lack mixed function oxidase activity. COX-2 has both COX and peroxidase activities that can activate tobacco carcinogens (21). Stimulating the COX-2 protein production by GK with AN extract will therefore potentially activate the tobacco carcinogens. Tobacco extract also stimulates PGE2 production and interleukin-1 production in GK.
(22). This can explain why chewing BQ in conjunction with smoking dramatically elevates the oral cancer risk (23).

In conclusion, PGE$_2$ and COX-2 levels are high in head and neck cancer and are considered to be important for localized immunosuppression, tumor growth and metastasis (11,12,21). Aberrant tissue inflammation can promote the occurrence of cancer and tissue fibrosis (6–8). In the present study, AN ingredients induced the production of PGs, COX-2 mRNA and protein formation. BQ chewing habit therefore contributes to the pathogenesis of oral submucous fibrosis and oral cancer by inducing the production of PGs, COX-2 expression and associated tissue inflammatory responses.

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References

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