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Effects of Paan Extracts on Periodontal Ligament and Osteosarcoma Cells

Seetha Venkatachalam[1] and K. V. Venkatachalam[2]

[1] Nova High School, Davie, Florida

[2] College of Medical Sciences and Allopathic Medicine, Nova Southeastern University, Ft. Lauderale, Florida

*Correspondence: venk@nova.edu (K.V.V.)

Summary

Oral cancer is a subset of head and neck cancer. There are various types of oral cancers, including cancer of the periodontal ligament (PDL) and osteosarcoma (OS) of jawbones. In South Asian countries, the major cause of oral cancer is reported to be chewing paan, or betel leaf daubed with slaked lime paste and areca nut. To investigate how paan may contribute to the onset of cancer, we evaluated how treating cells with areca nut extract, betel leaf extract, or lime (better known as calcium hydroxide) affected cell proliferation and cell death. Two oral cell lines were treated with solutions of areca nut, young betel leaf, old betel leaf, and lime, either individually or in combination, and we evaluated cell survival and cell growth. While treatment with old betal leaf alone caused marginal slowing of cell growth of both PDL and OS cell lines (12 ± 4% and 10 ± 10%, respectively), effects of young betal leaf and lime on cell growth were minor. Treatment with areca nut caused no significant change in PDL (10 ± 10%), but an increase in cell number for OS (37 ± 19%). Extract combinations of either young or old betal leaf with areca nut and lime resulted in increased cell proliferation of PDL and OS. These initial results indicate that the enhancement of cell growth by areca nut can overcome potential growth inhibition by betal leaf, suggesting that areca nut may promote cell growth. These data warrant further investigation of the effect of areca nut exposure on the onset of oral cancer in humans.

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Introduction

Betel leaf (*Betel piper*) dabbed with slaked lime paste and wrapped along with areca nut is called paan (1). Chewing paan is common among Southeast Asians (1), and it imparts a reddish stain of the mouth that is considered attractive among chewers, an associated cultural trait. Betel leaves (**Figure 1A**) contain a key phenolic compound called chevibetol (8,9). The medicinal properties of the betel leaf have long been recognized in Southeast Asia (10). The boiled extracts of the betel leaves are often used for treating sore throats and stomachaches, as well as other pharmacological purposes (11). The areca nut, *Areca catechu*, belongs to the palm family (**Figure 1B**). The habit of chewing areca alone is many centuries old due to the stimulatory effect that it imparts on the chewer. In many countries, areca is grown as a stimulant; however, the nut contains the harmful compounds arecoline and arecaidine (1). Slaked lime, another ingredient of paan, is better known as calcium hydroxide [$Ca(OH)_2$]. Limestone forms quicklime (CaO) when heated to high temperatures, and water added to CaO forms slaked lime [$Ca(OH)_2$]. The lime paste applied to betel leaves gives the red coloration of the mouth seen in Paan chewers.

However, chewing paan has also been associated with a higher incidence of oral cancer among chewers. Oral cancer can appear in many tissues of the mouth, including the jawbone (2). Worldwide incidence of oral cancer is ~0.5 million (~0.01%) and it is noted that more than 50% of the cases are due to betel quid chewing, especially among the high-prevalence areas of Southeast Asia (3). There are various types of oral cancers, including Osteosarcoma (OS) (4-6), or cancer of the jawbone, and cancer of the periodontal ligament (PDL) (7). The frequency of these cancer types due to paan chewing is not known.

For this study, two cell lines, periodontal ligament (PDL, a noncancerous cell line), and osteochondrosarcoma (OS, a cancerous cell line), were used. The rationale behind using these two different cell types is to see

Figure 1. Plant components of Paan. A) Image of betel leaves. O=Old betel leaf; Y=young betel leaf. B) Photograph of areca nuts. They are round. The top has ring marks and the bottom part is tapered and conical.

how normal and cancerous cell types would respond to treatments with extracts of paan components. PDL is very important for a healthy mouth, and each tooth has its own PDL. It is a soft, specialized, fibrous connective tissue present in the periodontal space, and is situated between roots of teeth and the bone that forms the socket wall. The capacity to adapt, renew and repair itself despite changing physical forces like speech and orthodontic tooth movement is a remarkable quality of the PDL. The soft tissue casing helps protect the vessels and nerves from injury. PDL is mostly made up of fibroblasts and other cells that have nutritional and sensory functions. OS is among the most uncommon types of all cancers. OS causes rare deforming lesions in the face, most commonly in the alveolar ridge and body in maxilla and mandible. Widening of PDL and mandibular canal are distinctive features of this disease.

The effects of chewing paan on periodontal ligament and the jawbone are not well understood. Most scientists who have worked on PDL and OS have studied the etiology of the cancer, the pathological features, the disease progression. This is the first study to look at the effects of paan ingredients individually or in combination on PDL and OS cells. The purpose of this research is to better understand how the ingredients of paan affect PDL and OS cancer cells. The data revealed that the extracts individually or in combination had varying effects on cell viability, assessed using an MTT assay.

Results

PDL and OS cells were treated with Phosphate Buffered Saline (PBS), Young betel (Yb) and Old betel (Ob) leaf extracts, areca extracts, or lime [$(Ca(OH)_2$] solutions individually and in combination, and incubated overnight. The following day, cells were visually assessed for viability using a microscope (data not included). Multiple qualitative microscopic observations lead us to perform the quantitative MTT.

Effects of the individual extracts and their combinations on PDL and OS cells were tested by an MTT assay to quantify the number of viable cells and examine cell proliferation. For this experiment, the same batch of extract of AR, Yb, Ob, L were used for replicate treatments. The raw absorbance (570 nm) values from each treatment (more than eight data points/treatment) were averaged. We measured the absorbance of the PBS-treated control cells and used that value to establish 100% viability. Based on that control measurement, the averaged absorbance value was converted into relative percent viability.

None of the treatments individually or in combination had a significant effect on PDL cell viability. For the PDL cells, the normalized PBS-treated control was 100 ± 9% (mean ± standard deviation). Cells treated with 1 mg Yb had a viability of 104 ± 6% and cells treated with 0.2 mg L had a viability of 90 ± 10%. Cells treated with 1 mg OB had a viability 88 ± 4%, slightly lower than the other groups. Treatment with 0.2 mg AR resulted in a normalized viability of 110 ± 10%. For the combinations,

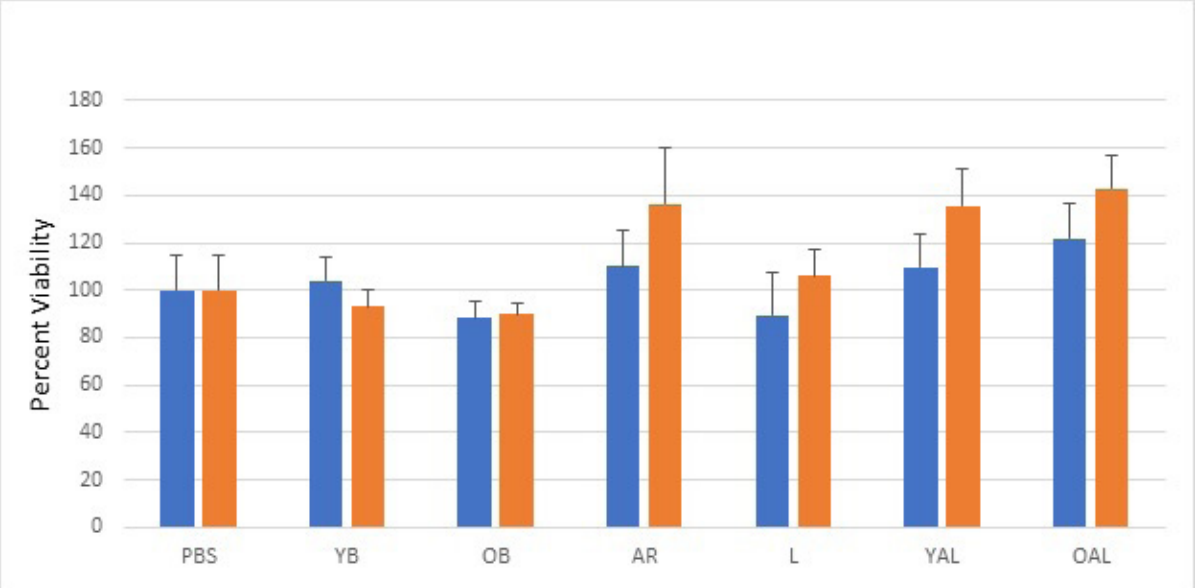

Figure 2. Results of MTT assay. From the MTT assay, the OD-595 readings were converted into percent viable cells using a PBS control as 100%. For OS cells, each value represents an average of 8 replicates of the MTT assay. For PDL each value represents an average of 16 replicates of the MTT assay. The standard deviations were calculated for the average values. Error bars indicate standard deviation (SD). Yb=young betel leaf; Ob=old betel leaf; AR=Areca; L=Lime; YAL= Yb+AR+L; OAL=Ob+AR+L . The blue bar corresponds to PDL and orange bar to OS cells.

0.067 mg AR combined with 0.33 mg Yb and 0.067 mg L resulted in a normalized viability of 110 ± 10%. 0.067 mg AR combined with 0.33 mg Ob and 0.067 mg L resulted in a measured viability 122 ± 10%.

For OS cells, individual treatments of all extracts did not cause dramatic effects, except AR, which promoted cell growth. The PBS-treated OS cell viability was 100 ± 9%. All treatment values were normalized to the control PBS. Treating cells with 1 mg Yb and 0.2 mg L resulted in a viability of 93 ± 4% and 106 ± 7%, respectively. Treatment with 1 mg Ob resulted in a cell viability of 90 ± 10%. Treating cells with 0.2 AR alone promoted cell growth, resulting in a viability effect of 137 ± 19%. Combinations of the other extracts with AR did not further promote cell growth. OS cells treated with 0.067 mg AR combined with 0.33 mg Yb and 0.067 mg L had a viability of 136 ± 12%. Treatment with 0.067 mg AR combined with 0.33 mg Ob and 0.067 mg L resulted in a cell viability of 143 ± 12% (**Figure 2**).

Statistical analysis was done as follows. For each treatment, the values represented an average of 8 replicates for OS cells and an average of 16 for PDL cells. Standard deviation (SD) was calculated. One-way repeated measures ANOVA calculator was used to calculate the R-squared values along with *p*-values. The R-squared values for all the PDL cell treatments (PBS, Yb, Ob, L, Yb+AR+L and Ob+AR+L) was 13.73. The *p*-value was 0.00006. Similarly, R-squared values for all the OS cell treatments (PBS, Yb, Ob, L, Yb+AR+L and Ob+AR+L) was 14.04. The *p*-value for all OS cell conditions was 0.00062.

In addition, separate statistical comparisons were done to test each of the experimental groups to one another using ANOVA calculator of socscistatistics.com. The results are as follows for OS cells:

1. ***Ob vs. PBS control:*** *F*-ratio is 3.452, *p*-value is .084.
2. ***AR vs. PBS control:*** *F*-ratio is 8.7, *p*-value is .011.
3. ***Yb vs. PBS control:*** *F*-ratio is 1.42, *p*-value is .253.
4. ***AR vs. YAL:*** *F*-ratio is 0.006, *p*-value is .938.
5. ***AR vs. OAL:*** *F*-ratio is 0.232, *p*-value is .637.
6. ***YAL vs. OAL:*** *F*-ratio is 0.22, *p*-value is .482.

When extracts were combined in equal volumes (2 µL 0.33 mg Yb, 2 µL 0.067 mg AR, and 2 µL 0.067 mg L), mixed, and applied to cells, both PDL (p=0.0176) and OS (0.0017) cells showed more viability and growth compared to 6 µL of 1 mg Yb alone. Similarly, Ob+AR+L mixed treatment also showed higher number of PDL (p<0.0001) and OS (p=0.0001) cells relative to individual treatments.

Discussion

Paan is chewed for its stimulating effects. Paan is composed of the nut of the areca palm (*Areca catechu*), the leaf of the betel pepper (*Piper betle*), and lime (calcium hydroxide). Approximately 200 million people chew betel regularly throughout the western Pacific basin and south Asia. Betel use is associated with oral leukoplakia, sub mucus fibrosis, and squamous cell carcinoma (12). We designed experiments to examine the cancer-causing effects of paan. Two proliferating cell lines, PDL and OS cells, were chosen for experiments to test the effects of old and young betel leaves, areca nut, and lime on cell proliferation and viability. OS and PDL lines are readily available and easily cultured. OS was chosen for comparative purposes since it is a cancer cell line and the response of these cells to various treatments can be compared with normal immortalized PDL cells. One disadvantage is that OS cells are physiologically distinct from PDL cells, and therefore direct comparisons should be made with caution.

We performed an MTT assay, an indicator of viable cells, for both PDL and OS cells after different treatments with extracts derived from paan components in order to quantify the percentage of live cells. For both PDL and OS cells, treatment with Yb alone had no significant effect on number of cells that are alive as compared to control. In contrast, Ob treatment slightly reduced both PDL and OS cell viability. Hence, this study suggests that the betel leaf extracts have pharmacological properties; in this case, the inhibition of cell division. The difference observed between young and old leaves could mean that the quantity of the substance that is causing cell death might be different or present in higher concentrations in the mature betel leaves.

AR extracts have been known to contain phenolic compounds such as arecoline and arecaidine. The effects of arecoline and arecaidine have been studied in mice, and the chemical mechanism of action is poorly understood in terms of underlying physiology (14). When AR extract was tested on PDL and OS cells, it caused an increase in growth compared to control. The cell-growth-promoting effects of AR, judged by microscopy and an MTT assay, indicate that the areca nut could be the paan-related cause of oral cancer. Future work will involve testing this hypothesis.

It is possible that AR forms arecadine from arecoline, a more potent carcinogen combination, in the presence of other combined extracts to promote cell growth. AR has been reported to induce tumor growth factor (TGF) that ultimately signals cell division kinases that cause cell cycle progression/growth (15, 16), a hypothesis that should be tested in the future. The dose-dependent effects of the individual extracts of AR on these cells were not tested. However, we intend to study AR dose effects in the future.

Lime had dark staining effect on PDL cells when

observed by light microscopy, and lime-treated cells showed slight differences in cell number when assessed by the MTT assay. Lime itself might be causing some staining in the presence of growth medium, resulting in high contrast in the visual field.

In the combinations of extracts tested, the amount of AR used was only one third of that used for individual treatments (0.067 mg compared to 0.2 mg). However, AR combined with L and Ob or Yb showed an even higher response (p=0.0012 for PDL and p=0.0001 for OS). The type of biochemical transformation that is happening in the combined extract before and after treatment should be investigated to further understand the mechanism behind these observed effects. The conclusion from this study should be taken with caution in that the MTT assay was performed only on one of the representative batches, in multiples. However, light microscopy was performed multiple times using different extracts and batches of cells, and every time the conclusions were the same (data not shown).

Multiple MTT assays, from multiple batches of treated PDL and OS cells, will be performed in the future to strengthen the preliminary observations of this study. In addition, future experiments could investigate treatments with different amounts of the crude extracts and the putative purified components could be tested for dose-dependent effects on the selected cell lines.

Previously, the components of betel leaf extracts were analyzed by gas chromatography and mass spectrometry, and its antimicrobial properties against several oral pathogens were reported (10). In this study, the mature leaves might have a higher concentration of active compounds such as hydroxychavicol, allyl pyrocatechol, eugenol, and others. With their plethora of pharmacological properties, these compounds could perhaps have biochemical effects on the tested cell lines, resulting in cell death. The mechanism of action of the extracts is not understood and could be a direction for future study. Nitric oxide (NO) is a gaseous intracellular hormone that causes cell death of periodontal ligament cells (13). Hence, betel leaf compounds from the treated extracts could enter the cell and perhaps induce the intracellular synthesis of NO by nitric oxide synthase and signal cell death/apoptotic pathway(s).

Because this is only a cell culture study, one should be cautious in extending these observations in terms of actual human applications. Nonetheless, based on the results from this in vitro cell culture study one can suggest that betel leaf extracts may cause cell death and areca nut treatment may promote some cancer cell proliferation. Though testing beneficial effects can be studied in actual humans, studying harmful effects might involve ethical issues. Perhaps, at best, one can warn areca chewers about its possible harmful effects, much like the nicotine warnings of cigarette smoking. Future research will involve chromatography and isolation of extracts to identify the exact chemical(s) that would cause cell proliferation or cell death. Further, the effects of various treatments on gene expression of PDL and OS cells could also be studied. The biochemical, cell biological and molecular biological mechanisms of the effects of the individual and the combined extracts on OS and PDL cells warrants further research.

Materials and Methods

Experiments were performed under the supervision of a senior scientist in the laboratories of Nova Southeastern University (NSU). All experiments were carried out in two cell types: Periodontal Ligament (PDL) and Osteosarcoma (OS) cells. The control group consisted of each cell type treated with PBS. The experimental conditions included treatments with young betel leaf extract, old betel leaf extract, areca nut extract, and slaked-lime solution, individually or in combination. The effects of treatment on cell viability were assessed with an MTT assay according to the manufacturer's procedures, microscopy, or a live/dead assay.

Cell Culture

All operations were carried out under sterile conditions. The cells were distributed onto 96-well culture plates by the following method: Propagated stock cultures of PDL cells (immortalized from normal subject; ScienCell, catalogue #2301) and OS cells (cancerous cell line; ATCC, catalogue #CRL-1427) were plated using equivalent cell suspensions (~1x10^5 cells/0.2 mL) in each well. Plates were checked under a microscope, then incubated overnight at 37ºC (95% O2, 5%) CO2 in an incubator. The next day, cells were visually observed for viability prior to treatments. Only healthy, viable cells were used for treatments.

Preparation of Extracts and Reagents

Young and old Betel Leaf Extracts: Betel leaves (3 of each) were washed with water and wiped. Leaves, young and old, were chopped and 0.5 g of each was weighed and uniformly ground with a mortar and pestle using 2 mL of triple-distilled water. The mortar and pestle were rinsed with 1 mL of water and pooled with the rest of the ground materials. Each 3 mL of extract was transferred to a labeled tube and centrifuged at 1000 g for 10 min. The supernatant was filtered with an 0.2 µm filter. This sterile filtrate was used for treatments. Preparation of Areca Extract: Areca nut was broken into pieces mechanically and ground into powder using a heavy-duty grinder. Areca nut powder (0.1 g) was made into a 3 mL extract using triple-distilled water and filtered, using the same procedure as described above for leaf extracts.

Preparation of slaked-lime solution: 0.1 g of lime powder was made into a 3 mL solution and filtered, as described for the other extracts.

Treatment of Cells

6 µL of young or old leaf, areca, and lime solutions were delivered onto respective wells. For controls, 6 µL of PBS were used. For different combinations, the solutions were mixed in equal amounts and a 6 µL aliquot was used for treatments. Treated cells were mixed gently and incubated overnight at 37ºC (95% O2/5% CO2) in an incubator.

Light Microscropy

After overnight treatment, cells were observed under microscope. Live cells had clear shape and cell integrity. Pictures were taken for overall observation and analysis. Microscopy was done for three independent batches of cells treated with extracts that came from three different batches of leaf extracts (Yb, Ob) and three different preparations of L and AR. Each time treatments were done in multiple cell culture wells. All cell culture wells were observed and analyzed for overall assessments of its effects (data not included).

MTT Assay

The MTT assay (M6494, Thermo Fisher Scientific) is a colorimetric assay used to assess cell viability as a function of cell number based on metabolic activity. This test measures the reduction of tetrazolium dye by intact mitochondria present in live cells. The chemically reduced form of the dye appears dark purple when compared to oxidized dye and can be measured at an absorbance of 570 nm. The intensity of the purple color is proportional to intact mitochondrial function, which corresponds to the number of live cells. In this study, the optical density (OD) at 570 nm from the PBS control was used as the standard for 100% viability, to which readings for all treatments were normalized. Our procedures all accorded with the manufacturer's instructions. From each culture, media was removed and tetrazolium dye (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) was added. Live cells with intact mitochondria metabolically oxidize the tetrazolium dye and result in the formation of purple formazan precipitate. The formazan was solubilized and the purple color absorbance at 570 nm was quantified with a plate reader (Synergy microtiter plate reader). Dead cells without functional mitochondria should not form purple formazan precipitate. MTT assays were done in duplicate 96 well plates. Single batches of OS and PDL cells from a single batch of extracts were performed in multiples (at least eight wells) for the MTT assay. For each treatment of PDL cells, 16 different trials were averaged. For each treatment of OS cells, 8 replicates were averaged. From this data, the respective absorbance value of each treatment was converted into relative percent viability.

Statistical Analysis

One-way repeated measures ANOVA calculator was used to calculate the R-squared values along with *p*-values using the program of socscistatistics.com.

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