14TH ANNUAL
PACIFIC
RESEARCH DAY

Wednesday, May 23, 2012

Abstracts

Faculty, Student and Staff Presentations
Second-Year Student Research Competition
Senior Research Competition
IDS Student Presentations
Orthodontics Resident Presentations
Dental Hygiene Student Presentations
Stockton Campus Student Presentations
UCSF Invited Presentations
14th ANNUAL
PACIFIC RESEARCH DAY
AND
STUDENT RESEARCH
COMPETITIONS

ABSTRACTS

WEDNESDAY, MAY 23, 2012

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PACIFIC-DUGONI SCHOOL OF DENTISTRY
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MICONAZOLE ACTIVITY AGAINST CANDIDA BIOFILMS ON ACRYLIC DISCS

Barbara Dorocka-Bobkowska¹, Senait Gebremedhin², Nejat Düzgünesh² and Krystyna Konopka²*

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OBJECTIVES: Miconazole is commonly used in the treatment of Candida-associated denture stomatitis (CaDS). New research into CaDS is focused on the reduction of Candida biofilms developed on the denture surface. We investigated the activity of miconazole towards in vitro grown mature Candida biofilms formed on denture acrylic discs.

MATERIALS AND METHODS: The effect of miconazole on Candida biofilms developed on acrylic discs was determined for: C. albicans MYA-2732 (ATCC), C. glabrata MYA-275 (ATCC) and four clinical isolates, C. albicans 6122/06, C. glabrata 7531/06, C. tropicalis 8122/06, and C. parapsilosis 11375/07. The MICs of miconazole were determined using the broth dilution susceptibility method. Biofilms were developed on poly(methyl methacrylate) discs incubated for 90 min at 37°C (adherence). After removal of non-adherent cells, discs were submerged in YNB/100 mM glucose and incubated for 48 h at 37°C (biofilm formation). Miconazole (1-200 µM; 0.5-96 µg/ml) was added in YNB medium and the biofilms were incubated for 24 h at 37°C. The metabolic activity of the biofilms was measured by the XTT assay.

RESULTS: MICs for miconazole for the investigated strains ranged from 0.016-32 µg/ml. Miconazole demonstrated significant activity against all Candida biofilms studied. Treatment with miconazole in the range 10-200 µM resulted in a significant reduction of biofilm metabolic activity for all strains; the highest reduction (84%-48%) was observed at 200 µM. For C. albicans MYA-274, C. glabrata 7531/06 and C. parapsilosis 11375/07, 1 µM miconazole had no effect. Biofilms of C. albicans 6122/06, C. glabrata MYA-275, and C. tropicalis 8122/06 were susceptible to 1 µM miconazole, which reduced the metabolic activity of the biofilms by 14%, 47% and 45%, respectively.

CONCLUSIONS: Miconazole exhibits high antifungal activity against Candida biofilms developed on acrylic discs. Our findings suggest that miconazole may be useful for the treatment of biofilm-related CaDS.

This work was presented at the 41th Annual Meeting & Exhibition of the American Association for Dental Research, March 21-24, 2012, Tampa, FL J. Dent. Res. Vol. 91 (Special issue A) Abstract No. 1124, Seq. #148
OSTEONECROSIS OF THE JAW IN RELATION TO BISPHOSPHONATE THERAPY

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OBJECTIVES: Bisphosphonates (BPs) are generally given in two forms, oral and intravascular (IV), to manage bone metabolic disorders. As the use of bisphosphonate increases, clinically reported complications increase as well, specifically osteonecrosis of the jaw (ONJ). The American Association of Oral and Maxillofacial Surgeons (AAOMS) defines BRONJ as “an area of exposed bone in the maxillofacial region that does not repair in eight weeks and affects patients who are receiving or have received BPs systemically and who had no history of radiation therapy to the maxillo-mandibular complex.” The aim is to review multiple studies and journals to better understand and inform dental professionals of the physiological cause of bisphosphonate-related osteonecrosis of the jaw (BRONJ), the mechanism of action, and the management of patients who undergo bisphosphonate therapy.

METHODS: Clinical trials, systematic reviews, journals providing relevant studies relating to osteonecrosis of the jaw due to bisphosphonate therapy were reviewed through use of the electronic database PubMed.

CONCLUSION: Bisphosphonates were found to reduce bone turnover rate through inhibition of osteoclast activity. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) was reported to occur more frequently in patients that have received intravascular (IV) amino-bisphosphonate over a long-term period (three or more years) compared to those patients that received oral bisphosphonates for a similar length of time. Additionally, there is a clear linear relationship between the dosage administered and the levels of risk for the patient to develop BRONJ.

Studies have shown a distinct difference between the incidences of BRONJ in oral versus IV administered bisphosphonate. While oral therapy is estimated to have 1 in 10,000 incidences of BRONJ, the incidence greatly increases for IV-administered bisphosphonates, about 1-10 in 100 people. However, these studies have used smaller sample sizes to determine the incidences of BRONJ. Even with low occurrences, it is essential for the dental professional to know the specific bisphosphonate drug, method administered, dosage, and frequency in order to evaluate whether some dental procedures should be completed. Ideally, the dental professional should complete invasive procedures prior to bisphosphonate therapy. Otherwise, the patient should be watched with care after a procedure and given antibiotics to prevent infection. With a dentist’s foresight, the patient can be managed safely for both medical and dental needs.
CANDIDA BIOFILMS ON DENTURE ACRYLIC DISCS: EFFECTS OF FLUCONAZOLE AND MICONAZOLE

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OBJECTIVE: Candida species not only adhere to mucous surfaces, but also to acrylic resins of dentures. Candida-associated denture stomatitis (CaDS) is a significant problem in denture wearers. Treatment of CaDS necessarily includes the reduction of Candida biofilms that develop on denture acrylic. We examined the effects of fluconazole and miconazole on mature Candida biofilms developed on denture acrylic discs.

METHODS: Biofilms of C. albicans MYA-2732 (ATCC), C. glabrata MYA-275 (ATCC), and clinical isolates C. albicans 6122/06, C. glabrata 7531/06, C. tropicalis 8122/06, and C. parapsilosis 11375/07 were formed on polished poly(methyl methacrylate) discs in 48-well plates. The cells were first allowed to adhere for 90 min at 37 °C in YNB/100 mM glucose. The non-adherent cells were removed by washing twice with PBS. The discs were then placed in wells of 96-well plates in the same medium and incubated for 48 h at 37 °C for biofilm development. The biofilms were incubated with varying concentrations (1-200 µM) of fluconazole or miconazole for 24 h at 37 °C, and their metabolic activity was measured by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay.

RESULTS: The viabilities of C. albicans MYA-273, C. glabrata MYA-275 were reduced to 75.5% and 47% of controls, respectively, at 200 µM fluconazole. These values were 48.7% and 16% for 200 µM miconazole. At the same fluconazole concentration, the viabilities of the patient isolates C. albicans 6122/06, C. glabrata 7531/06, C. tropicalis 8122/06, and C. parapsilosis 11375/07 were reduced to 89.1%, 56.6%, 50.4% and 30.5% of controls, respectively. At 200 µM miconazole, the viabilities of these isolates were reduced to 40.6%, 40.3%, 24.6% and 52.3% of controls, respectively. Even 1 µM fluconazole reduced the viability of C. parapsilosis 11375/07 to 56.9%, but had no significant effect on the other isolates. By contrast, 1 µM miconazole was ineffective against C. parapsilosis 11375/07, but reduced the viabilities of C. glabrata MYA-275 and C. tropicalis 8122/06 to 52.8% and 55.1%, respectively.

CONCLUSIONS: Biofilms of various Candida species developed on denture acrylic discs are resistant to fluconazole. C. parapsilosis 11375/07 is the most susceptible isolate among those investigated. Miconazole, however, is effective against the fluconazole-resistant isolates in biofilms, but ineffective against C. parapsilosis 11375/07. Testing the susceptibilities of biofilms on acrylic discs of Candida isolates that are associated with denture stomatitis to fluconazole and miconazole may be useful in the successful treatment of this condition.

This work was supported by funds from the Arthur A. Dugoni School of Dentistry, and presented at the 11th ASM Conference on Candida and Candidiasis, March 29 - April 2, 2012, San Francisco, CA
TIME AND CONCENTRATION DEPENDENT XYLITOL-INDUCED S. MUTANS DISSOCIATION FROM HYDROXYAPATITE

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OBJECTIVES: We are characterizing the efficacy of xylitol to inhibit the adherence of S. mutans (SM) to pure hydroxyapatite (HA), and to cause the dissociation of SM from monolayer SM already adhered to HA (SM.HA).

HYPOTHESES: 1. Exposure of HA to xylitol will inhibit SM adherence. 2. Exposure of SM to xylitol will inhibit HA adherence. 3. Exposure of SM.HA to xylitol will cause SM to dissociate. 4. Reduced or reversed adherence will correlate with increased negative magnitudes of SM and HA zeta potentials, ζ.

METHODS: Our model comprises commercial SM (UA159) and 20 μm spheres of HA. SM.HA is made by incubating excess SM in the presence of HA in phosphate buffer and washing away excess SM. Preparations of HA, SM and SM.HA are exposed to 0 – 1.3 M xylitol for 0 – 90 minutes, and SM adherence is assayed by fluorescence microscopy after adding SYTO-9. ζs are calculated by the Smoluchowski equation from electrophoretic mobilities measured by phase analysis light scattering at 25°C, and are taken as estimates of relative surface charge.

RESULTS: The [xylitol] dependence was measured for 30’ exposure. Exposure of SM or HA to xylitol and washing before mixing inhibits monolayer binding. Decreased binding is seen at 0.3 M (~5%) and no binding is seen above 0.7 M (~10%) xylitol. The time dependence was measured for 0.3 M xylitol. Reduced binding begins at 10’ and no binding is seen 30’ after addition of 0.3M xylitol.

Adding xylitol to SM.HA induced dissociation. For 30’ exposure, partial dissociation is observed at 0.3 M xylitol and complete dissociation at 0.7 M xylitol.

0.7 M Xylitol had negligible effects on ζ for SM or HA. It also did not affect ζ for SM.HA, which is inconsistent with the dissociation observed by fluorescence microscopy.

CONCLUSIONS: Xylitol inhibits and reverses the first step in bilayer formation by SM and HA. For our conditions, inhibition is complete for 30 minute exposure to ~0.3 M xylitol. Reversal is complete for 60 minute exposure to ~0.6 M xylitol. These effects do not appear to correlation with xylitol-induced changes in surface charge.
RAIN-DROPLET: A NOVEL 3D IN VITRO ANGIOGENESIS MODEL

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OBJECTIVES: The aim of this study was to determine the utility of an in vitro 3D model of endothelial cell (EC) angiogenic sprouting for the microscopic study of EC microvesicle (MV) release and cellular interaction. Few protocols allow simple observation of cell interaction with MVs in vitro. For those that do, the focus is primarily on cellular response to MVs from other cell types. ECs are well known to release both MVs and exosomes when activated. Also, non-EC derived extracellular vesicles may stimulate angiogenesis but less is known of the action of EC-derived MVs on other ECs.

METHODS: Herein, ECs were encapsulated in solid droplets of a peptide matrix. ECs formed networks in the droplets prior to embedding in collagen. Pro-angiogenic mediators induced planar cell invasion and growth of capillary-like sprouts. MV release and cellular interaction were observed microscopically using either differential interference contrast (DIC), Nikon Advanced Modulation Contrast (NAMC), normal or confocal fluorescence.

RESULTS: The EC release and uptake of MVs, estimated to be ~1 μm in diameter, were readily observable by eye using the 3D planar model with either DIC or NAMC. Actin probes clearly defined vesicles by confocal fluorescence. Evidence was seen of angiogenic sprouting towards MVs released into the collagen matrix.

CONCLUSIONS: This planar 3D in vitro model of angiogenesis is a useful tool for investigating the interaction of ECs with MVs around 1μm in diameter. It also allows for fluorescent labeling for specific markers of both cells and MVs of ~1μm or potentially smaller.

Pacific Dugoni School of Dentistry Research Enhancement Award Grant (BDZ) Grant P50-CA97248 (University of Michigan Head & Neck SPORE) from the NIH/NCI, and grants R01-DE14601, R01-DE15948, R01-DE16586, R21-DE19279 and R01DE021139 from the NIH/NIDCR (JEN). This work was presented at ISEV 2012, the first annual general meeting of the International Society for Extracellular Vesicles.
PREFERRED DIET- GOT INSECTS OR LEAVES? A COMPARISON OF C. GUEREZA AND S. OERSTEDI

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OBJECTIVES: New World monkeys, found in Central and South America, and Old World monkeys, found in Africa and Asia, are both part of the Order Primates, but they have been geographically separated for 35 – 40 million years. Having reached the same grade of evolution, they share some of the same characteristics, but different monkey species also have unique traits that make them distinct. The Old World Black and White Colobus monkey (Colobus guereza) and New World Red-Backed Squirrel monkey (Saimiri oerstedii) were compared in this study, portraying differences between monkeys of the Cercopithecidae and Cebidae primate families.

METHODS: Ecology, life history, diet, locomotion, behavior and anatomy for these two species was determined from published sources. Comparable cranial and dental measurements were taken on two adult skulls, C. guereza (CV-91) and S. oerstedii (CV-63), found in the P & S Comparative Anatomy collection of the Institute of Dental History and Craniofacial Study at Pacific’s Dugoni School of Dentistry. The dentition and occlusion were observed for clues to their respective diets. There was only one specimen of each species, so the collected data is based on the one skull available.

RESULTS: The ratio of coronoid process to ramus height gives an idea of the size of the chewing muscle, temporalis. The higher value in C. guereza, suggests a relatively larger temporalis in this species. Considering that 70% of C. guereza’s diet consists of C. durandii leaves, a larger, stronger temporalis is an adaptation to chewing tough fibers. Both species have four quadrangular (squarish) molars, a sign of evolutionary similarity. However, the high-crowned, steep-edged bilophodont cusps of C. guereza, specialized for processing their folivorous diet, are mechanically advantageous for the cutting and subsequent digestion of fibrous materials. S. oerstedii, with its more omnivorous diet of insects and fruit, has evolved less specialized molars. Their moderately high cusps puncture insect exoskeletons and break fruit skin or shells, and a basin area serves for crushing. The shorter cusps of S. oerstedii help break apart these foods, not just cut them, and the shearing action of high cusps is unnecessary.

CONCLUSIONS: Both visual observations and quantitative skull data show correlations between form and function in these two species. Published field studies confirm distinctions in their diet, behavior and other lifestyle variables. These data indicate that Saimiri oerstedii is the smaller species, occupying an arboreal, frugivorous and insectivorous niche in Central America and Colobus guereza is larger, occupying an arboreal, folivorous niche in Africa.
BACKGROUND AND PURPOSE: Cleft lip and palate anomalies are one of the most common congenital anomalies, affecting 1 in every 500-1000 births. Nonsyndromic cases represent approximately 70% of all clefts and are caused by a combination of genetic and environmental factors. Among candidate genes for nonsyndromic cleft lip with or without cleft palate (NCLIP), methylene tetrahydrofolate reductate (MTHFR) gene polymorphisms have been identified as possible contributors due to their role in folate metabolism. Point mutations at the 677 position replace thymine (T) for cytosine (C) results in a substitution of valine for alanine. As a result, individuals with homozygous TT have impaired MTHFR enzyme function and a reduced ability to form the methyl form of folate by 35%-50%. To study a possible involvement of this gene in etiology of NCLIP in Udaipur, India, we analyzed 677CT MTHFR polymorphism in the sample of patients and compare with the sample of control individuals from the same location.

METHODS: Cases (individuals affected with NCL/P; n=57) and controls (n=30) for this study were identified during Rotaplast medical missions to Udaipur, India in 2011. DNA was isolated from blood and saliva and MTHFR 677CT genotypes were established using polyacrylamide gel electrophoresis (PAGE).

RESULTS: There was no significantly different proportion of genotypes between cases and controls. Interestingly, no TT homozygotes were found in cases, and only 2 among controls (cases: 87.7% CC, 12.3% CT; controls: 73.3% CC, 20.0% CT, 6.7% TT). Higher T allele frequency in controls compared to cases was observed and this difference was close to being significant (cases T allele frequency 0.061, controls 0.167; p=0.051).

CONCLUSION: There was very low frequency of mutated allele T of 677CT MTHFR polymorphism found in Udaipur population. Our samples of cases and controls are rather small and more data are needed for final conclusion, however results of this pilot study suggest that the 677CT variant of MTHFR gene is not associated with NCL/P in Udaipur population.

Rotaplast International, Inc., funded and supported field work for this study. Pacific Craniofacial Genetics Team and Cleft Prevention Program and Department of Orthodontics funded and supported molecular genetics analysis.
CYTOTOXICITY OF LIPOSOMAL C6-CERAMIDE IN ORAL SQUAMOUS CELL CARCINOMA CELLS

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OBJECTIVES: C6-ceramide is a sphingolipid metabolite, with antiproliferative and proapoptotic activity in vitro and in vivo. Its use as a therapeutic agent has been limited because of its insolubility. In this study, we have utilized liposomes as a drug carrier to deliver C6-ceramide to target cells. Survivin is a known inhibitor of apoptosis and is overexpressed in oral squamous cell carcinoma (OSCC) cells. Recent studies with C6-ceramide have shown the down-regulation of survivin in large granular lymphocytic leukemia. We therefore investigated the cytotoxicity and anti-survivin activity of liposomal C6-ceramide in HSC-3 OSCC cells as a potential novel therapeutic agent.

METHODS: Palmitoyloleoylphosphatidylcholine (POPC):dioleoylphosphatidylethanolamine (DOPE) or POPC:DOPE:C6-ceramide liposomes were added to HSC-3 cells in the concentration range 0.1–50 μM of C6-ceramide. After incubation for 24 h at 37°C, cell survival was evaluated by the Alamar Blue assay. Survivin levels were measured by ELISA. The morphology of the treated and control cells were examined by scanning and transmission electron microscopy, and phase contrast microscopy.

RESULTS: Cells treated with liposomal C6-ceramide resulted in dose-dependent, decreased cell viability, measured by Alamar Blue. The viability with plain POPC:DOPE liposomes was 93±5% of the control. For 5 and 10 μM liposomal C6 ceramide, the viability was reduced to 72±3% and 44±0% of untreated cells. Survivin levels decreased from 1226±5 ng/mg protein to 346±6 ng/mg protein at 10 μM C6-ceramide. Electron microscopy indicated deformation of nucleoli by C6 ceramide treatment.

CONCLUSIONS: HSC-3 cells are vulnerable to liposomal C6 ceramide in a dose-dependent manner. Liposomal C6-ceramide reduced cell proliferation in HSC-3 cells probably because of a decrease in the levels of the anti-apoptotic protein, survivin. Further studies will focus on whether liposomal C6 ceramide and the reduced survivin levels will increase the susceptibility of HSC-3 cells to various anti-cancer agents such as doxorubicin and tamoxifen.

Presented at the 41st Annual Meeting and Exhibition of the AADR, March 21-24, Tampa, FL.
ASSOCIATION OF REDUCED FOLATE CARRIER ONE GENE 80AG POLYMORPHISM WITH NONSYNDROMIC CLEFT LIP AND PALATE

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INTRODUCTION: Mutations in folate pathway genes have been shown to be associated with several birth defects including neural tube defects, conotruncal heart defects, and nonsyndromic cleft lip and palate (NCLP). For the last decade, we studied polymorphisms of those genes in cleft populations. The Reduced Folate Carrier One gene (RFC1, also known as SLC19A1) encodes for a cellular surface transmembrane protein that provides transport of folate from diet through the cell membrane. Several single nucleotide polymorphisms (SNP) of the RFC1 gene exist and one common SNP in the RFC1 gene – 80AG has been studied in NCLP populations.

OBJECTIVES: To study association of RFC1 80AG polymorphism with NCLP in ethnically diverse populations and discuss the results with our previous findings and findings of others.

MATERIALS AND METHODS: A case control study design was used. During the past 12 months, three datasets of cases (individuals affected with NCLP) and controls (unaffected individuals with no history of clefts or other birth defects in the family from the same location) from Egypt, India and Venezuela were analyzed - altogether, 245 cases (Sohag, Egypt 116; Udaipur, India 57; Cumana, Venezuela 72) and 168 controls (Sohag, Egypt 81; Udaipur, India, 30; Cumana, Venezuela 57). Venous blood or saliva was collected from cases and controls during Rotaplast medical missions. Specimens were spotted on filter paper, allowed to dry, and transported to the Pacific Craniofacial Genetics Laboratory where DNA analyses were performed using laboratory protocol [DNA isolation, PCR, agarose gel electrophoresis for confirmation of both sufficient amplification and absence of contamination, PAGE (polyacrylamide gel electrophoresis) for genotype analysis].

RESULTS: Statistically significant differences between cases and controls were found in Sohag samples for genotype distribution (p=0.039) and also for allele frequencies (p=0.009). Samples from two other locations (Udaipur and Cumana) showed statistical difference neither in genotype distributions nor in allele frequencies.

CONCLUSIONS: When compared with our previous studies on RFC1 80AG polymorphism associated with NCLP, the Egyptian results support our findings in populations studied in Argentina and Central America. One (Nagamangala) of two other Indian populations showed association. Two Venezuelan populations that we have previously studied showed associations. In summary, our research results reflect a genetic diversity of different populations in the world. It seems that the same anomaly can be caused by different genetic and environmental factors.

Rotaplast Intl., Inc. supported field work and Department of Orthodontics, University of the Pacific, Arthur A. Dugoni School of Dentistry, supported molecular genetic analyses.
A TAIL OF TWO MONKEYS: DIETARY CHOICE IN RELATION TO CRANIAL AND DENTAL ANATOMY IN THE RED HOWLER MONKEY AND OLIVE BABOON

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OBJECTIVES: Monkeys, along with prosimians, apes and humans, are classified in the Order Primates. Members in this order share certain characteristics while each species exhibits unique traits. The dental and cranial characteristics of two monkeys, the New World red howler monkey (Alouatta seniculus) and the Old World olive baboon (Papio anubis), were compared to study the effect of dietary adaptation on dentition and skull morphology.

METHODS: Literature searches resulted in comparative information on the distribution, ecology, life history, diet, locomotion and anatomy of these two monkey species. Cranial and dental observations were made and measurements taken on three skulls, a male red howler monkey (CV-65), and male (CV-94) and female (MP5P-45) olive baboon from the P&S Comparative Anatomy collection of the Institute of Dental History and Craniofacial Study at Pacific’s School of Dentistry.

RESULTS: Published materials indicated dietary and body size differences, with the smaller howler monkey species eating mostly leaves, supplemented seasonally by fruit, and the larger baboon species eating a varied diet, including fruit, flower, seeds, vertebrates and invertebrates. The howler monkey had shorter cranial, neurocranial, molar row and molar measurements than either the male or female olive baboon, confirming smaller body size in the howler. The male baboon had significantly long canines, for social purposes, and a pronounced muzzle. Cusps were high and sharp on the howler molars, while, comparatively, low and rounded on the baboon molars. Mandibular ramus height, angle and overall shape were observably distinct between the two species. Based on origin and insertion measurements for the temporalis and masseter muscles, proportionally, the olive baboon, especially the male, had a notably larger temporalis, in contrast to a notably larger masseter in the howler monkey.

CONCLUSIONS: The higher cusps of red howler molars are efficient at shearing leaves while the lower cusps of olive baboons are better adapted for crushing. The male baboon’s large canines, long tooth row and overall robusticity have led to development of a large temporalis muscle, while the howler’s dietary specialization of chewing leaves has led to a unique mandible shape and development of the masseter muscle. Overall findings suggest that anatomical differences between these two species have resulted from adaptation to an arboreal niche with a primarily folivorous diet (howler monkey) verses a terrestrial niche with an omnivorous diet (olive baboon).
DOSE RESPONSE STUDY OF CYTOPROTECTIVE EFFECT BY LUTEIN AND ZEAXANTHIN ON RETINAL PIGMENT EPITHELIUM FROM OXIDATIVE STRESS INDUCED CYTOTOXICITY

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OBJECTIVES: Lutein (LUT) and Zeaxanthin (ZEA), naturally occurring antioxidants and major components in macular pigment, are currently under investigation in clinical trials as prophylactic nutritional agents for age related macular degeneration (AMD). However, dose used in these trials is empirical and not been investigated in vitro studies. In this study, we investigated the dose response effect of LUT and ZEA in protecting retinal pigment epithelium (RPE) from oxidative stress, a common underlying pathology in AMD.

METHODS: Different concentrations of hydrogen peroxide (H₂O₂), a common oxidant, were applied to the cultured human retinal pigment epithelial cells (ARPE-19) cells for 24 hours to generate a dose response curve. 3000 cultured human retinal pigment epithelial cells (ARPE-19) were plated in 72-well plate and after 24hrs, cells were exposed to four different concentrations of LUT (4, 2, and 0.5 μg/ml) and ZEA (0.8, 0.4, 0.2 and 0.1 μg/ml). After 24 hours incubation, cells were subjected to oxidative stress induced with hydrogen peroxide. Cultures containing saline solution and trichloromethane served as controls. Cell viability was assessed using the WST assay and cell counts were measured using Vi-cell counter. Mechanistic pathways were evaluated by measuring caspase-3 levels as an indicator of apoptosis induction since different upstream pathways leading to apoptosis depend on caspase-3 induction for final apoptotic execution.

RESULTS: Using the WST assay, a dose dependent cytoprotective effect was observed in ARPE-19 cells exposed to hydrogen peroxide after pretreatment with both LUT and ZEA. (Cell viability as a percentage of control was 81.3, 81.1, and 88.8% at 4, 2, and 1 μg/ml, respectively of Lutein, p<0.001). LUT at 2 μg/ml and ZEA at 0.1 μg/ml were optimum concentrations at which protective effect was observed. At higher doses, there was a reversal of cytoprotective effect. Capase-3 showed a corresponding decrease in levels with Zeaxanthin (0.2 and 0.4 μg/ml) and Lutein (4 μg/ml) and with levels rising at higher doses used in the study.

CONCLUSIONS: Lutein at 2 μg/ml and Zeaxanthin at 0.2 μg/ml provide optimum cytoprotective effect in retinal pigment epithelium from oxidative stress induced cytotoxicity.
MYCOBACTERIUM TUBERCULOSIS-INDUCED SKELETAL CHANGES IN A JUVENILE FROM PREHISTORIC CENTRAL CALIFORNIA

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OBJECTIVES: Mycobacterium tuberculosis infections reached epidemic levels in the Americas following European contact, with tuberculosis becoming the most serious health problem affecting native peoples in the 1900s. Once considered to have European origins, the presence of M. tuberculosis has been confirmed in the Americas prior to the influx of Europeans. Numerous issues remain, however, including the patchy geographic distribution of precontact cases. No cases are known from the west coast of North America; there are only suggested occurrences in prehistoric Central California.

METHODS: We describe a partial juvenile skeleton that derives from Contra Costa County, California (CCo-138). The remains are assigned to the Late Horizon, Phase 1c (1300-1500 AD). For descriptive and reconstruction/restoration purposes we scanned the individual with a GE Lightspeed VCT® scanner (slices 0.1mm). All reconstructions, including mirror-imaging, were made using Amira (5.3.3)® 3D visualization software. Comparison of diseased skeletal elements was made to a sample of normal juveniles from prehistoric Central California. Skeletal remains are housed in the Phoebe Hearst Museum of Anthropology, University of California, Berkeley.

RESULTS: Disease-associated destruction is confined to the T5-T11 vertebrae (C1-C6 and T12-L5 are missing). While pathology is not apparent in the C7-T4 vertebrae or sacrum, the T12 was probably involved. Vertebral changes consist of gibbus, an extensive series of conical drainage pathways, and near-complete loss of the centra of T6, T8-9, and T11 due to osteolysis. Perifocal reactive bone formation is minimal. Vertebral and rib articular facets were involved via modified biomechanical forces. The pedicles and transverse processes show resorption related only to paravertebral abscess formation.

CONCLUSIONS: Seventeen other disease conditions overlap the osseous expression of tuberculosis and have been suggested as potential sources of similar bony changes. However, only two of these, congenital malformation of the spine and coccidioidomycosis are potential differential diagnoses. Neither of these conditions account for the total morphological pattern expressed in this individual. Tuberculosis is the only condition that accounts for the observed distribution of lesions and bony modifications. This case extends the geographic range of precontact tuberculosis to the West coast of the United States in the time range of 1100-1700 A.D.

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ISOLATION, GROWTH AND ODONTOGENIC DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS IN VITRO

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BACKGROUND & PURPOSE: Adult dental pulp-derived stem cells (DPSC’s) can differentiate into mesoderm-derived cell types and nerve cells. DPSC’s are excellent candidates for regenerative cell therapy restorations in the craniofacial region. For this to be possible, the DPSC’s must be efficiently expanded in vitro with no loss of their differentiation potential. We examined effects of combinations of platelet-derived factors (PDF) and fibrin substrate on multiplication and odontogenic differentiation of DPSC’s in vitro. A response of DPSC’s to low oxygen was also tested.

METHODS: DPSC’s were isolated from vital asymptomatic third molars and were grown on the following substrates: polystyrene, fibrin and fibrin with PDF. The growth medium consisted of basal medium (Lonza) supplemented with human adult serum (10%), L-glutamine and antibiotics. The odontogenic differentiation medium consisted of basal medium (Lonza) supplemented with human adult serum (10%), L-glutamine, dexamethasone, L-ascorbic acid, KH₂PO₄ and antibiotics. The cells were stained for markers of differentiation and mineralization.

RESULTS: We constructed growth curves showing that the substrate containing both fibrin and PDF shortened the lag phase of growth and increased the multiplication and differentiation rate of the DPSC’s most efficiently. The fibrin substrate was second in its efficiency. Strong staining with alizarin red S for mineralization of extracellular matrix and strong staining of cellular alkaline phosphatase documented a successful differentiation. Hypoxia significantly increased proliferation rate of DPSC’s.

CONCLUSIONS: Fibrin, PDF and hypoxia – factors that are normally present in a healing wound - can be utilized to accelerate preparation of DPSC’s for clinical applications.
MTHFR – GENE THAT KEEPS US GROWING (AND GOING!) AND ITS ASSOCIATION WITH OROFACIAL CLEFTS

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INTRODUCTION: From the second we are conceived, we start growing – becoming a new human being born nine months later. We are also kept going by a perpetual renewal of cells worn out. The circle of cells’ life and death goes on and on. At all times, methylenetetrahydrofolate reductase (MTHFR) must be doing its job right. As a key player in folate pathway by forming active folate, it is important for synthesis of pyrimidines and methylation of DNA and keeps under control a level of homocysteine. MTHFR is essential for building new cells. If there are not enough bricks (cells) to build a wall, an unfilled gap will remain. Several congenital anomalies and several diseases have been associated with mutations in the MTHFR gene. While the most known is relation of 677CT polymorphism to neural tube defects, also nonsyndromic cleft lip and/or palate (NCLP) showed associations in several studies.

OBJECTIVES: Including our most recent data to evaluate results of studies on associations between NCLP and MTHFR 677CT polymorphism in samples from India (three locations), Argentina (three locations), Venezuela (three locations), and Guatemala done in the Pacific Craniofacial Genetics Laboratory and compare them with studies in the literature.

MATERIAL AND METHODS: Most of our studies on association MTHFR 677CT polymorphism and NCLP have case-control design. Cases are individuals affected with NCLP and controls are unaffected individuals from the same location. Data and specimens for DNA analysis were collected during Rotaplast medical missions. DNA was isolated from venous blood or saliva. MTHFR 677CT genotypes were established by PCR amplification and single nucleotide conformational polymorphism detection using polyacrylamide gel electrophoresis.

Altogether we are evaluating results based on analysis of 1277 cases and 684 controls.

RESULTS: The difference in distribution of genotypes between cases and controls was statistically significant for Guatemala (p=0.005), all three locations in Argentina (p=0.05, 0.041, 0.026), and all three locations in Venezuela (p=0.041, 0.042, 0.037). No statistically significant difference was found in any of three Indian locations. Very low frequencies of T allele and CT and TT genotypes were observed in both cases and controls in Indian population.

CONCLUSIONS: Results of our studies of the 677CT polymorphism of MTHFR gene suggest association with NCLP in Central and South American countries (Guatemala, Argentina, Venezuela). No association was found in any of three populations we studied in India.

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HOW TO CRACK YOUR GENETIC CODE FROM ONE DROP OF SALIVA

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INTRODUCTION: Molecular genetics is without any doubt an important diagnostic tool in medicine and rapidly becoming more and more used in dentistry. Many common conditions in dentistry have a specific genetic background: not only craniofacial anomalies, but also root resorption, periodontitis, hypodontia. Numerous efficient tests for specific single nucleotide polymorphisms are available. However, sometimes the very first step – to obtain a specimen suitable for DNA analysis - may close the door to clarify genetic diagnosis and thus help with treatment planning, prognostic assessment, and counseling about prevention. An excellent source for DNA analysis is the buccal epithelial cells that exfoliate from the inner epithelial linings of the oral cavity.

OBJECTIVE: To review techniques using buccal epithelial cells done by others and compare with our previous study.

MATERIALS AND METHODS: An extensive review of studies published in recent literature was performed and combined with results of our own studies to determine a kind of sample and the most suitable method of sample collection for genetic testing of dental patients.

RESULTS: Intraoral collection of cells from buccal mucosa seems to be a non-invasive method of choice. Three methods of collection were considered: buccal swab, oral rinse and saliva collection. Saliva was shown to give the highest yield of DNA of a good quality. In our own study (Pitigoi-Aron et al., 2005) three methods of DNA isolation from saliva were tested: REDExtract-N-Ampa (Sigma) for fresh saliva, saliva dried on filter paper, and Oragene saliva kit. The most efficient and practical was the use of saliva dried on filter paper (safe for shipping and storage) and also giving a good yield of DNA for molecular genetic testing. Since that study, this method has been successfully used in our laboratory.

CONCLUSIONS: Collection of saliva is acceptable noninvasive technique that can be used safely in children and adults. We introduced and tested for several years relatively inexpensive protocol for collection, transport, and storage of saliva using dried saliva spots on filter paper that yield a good quality of DNA suitable for genetic analysis. This technique can be used on-site as well as off-site, in dental office, or even on medical missions.
AUTOPHAGY IN THE ULTRASTRUCTURE OF C6-CERAMIDE-TREATED HSC-3 ORAL CANCER CELLS

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OBJECTIVES: Autophagy is the mechanism by which cells break down and recycle organelles and proteins. Autophagy pathways are important in stress conditions, such as disease and nutrient deprivation, and help maintain a homeostatic process that can be both detrimental and beneficial to the organism. Autophagy may be involved in cancer, neurodegenerative diseases, as well as immunity and inflammation. We have shown previously that liposome-encapsulated C6-ceramide inhibits the proliferation of HSC-3 oral squamous cell carcinoma cells and reduces the levels of intracellular survivin, an inhibitor of apoptosis. We examined the ultrastructure of these cells, with particular emphasis on autophagy.

METHODS: HSC-3 cells were plated at a density of 1.5x10⁶ in 6-well plates. Following treatments, they were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, with 30 mg calcium chloride. They were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, treated with 1% tannic acid in 0.1 M sodium sulfate buffer, and rinsed in 0.05 M sodium sulfate buffer and en bloc stained in 3% uranyl acetate. Cells were dehydrated in a graded series of alcohol, infiltrated with Spurr resin, polymerized at 60°C oven and sectioned by ultramicrotomy. Sections were post-stained in uranyl acetate and lead citrate and viewed with a Philips 201 transmission electron microscope.

RESULTS: Electron micrographs showed the U-shaped “omegasome” which appears to be derived from both the Golgi and the endoplasmic reticulum. There were chains of ribosomes where endoplasmic reticulum may be located which may also contribute to the formation of the autophagosome and amphisome. There were short chains of ribosomes with cisternae out of the plane of section. The ultrastructure showed the autolysosome with degraded organelles inside, as well as a lysosome fusing on its outside. These lysosomes contain the hydrolytic enzymes which degrade the organelles and release them to the cytoplasm back into the metabolic pathways. C6-ceramide-treated cells had more vacuoles indicating apoptosis than control and plain liposome-treated cells. C6-ceramide caused more cytotoxicity in the cells, which had many more pinocytotic vesicles.

CONCLUSIONS: The ultrastructure shown in this study follows the model of autophagy. C6-ceramide promotes decreased cell growth and apoptosis in oral cancer cells and the ultrastructure shows autophagy occurring in the cells. C6-ceramide may work to facilitate cell death in cancer cells by causing more cytotoxicity with increased pinocytotic vesicles while autophagy proceeds to clean up the cells and recycle cell components.
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STUDENT PRESENTATIONS
PFM, EMPRESS, LAVA AND PROCERA: A SYNOPSIS OF FACTORS INFLUENCING DECISION MAKING OF INDIRECT RESTORATIVE MATERIALS

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OBJECTIVES: Ceramics play an integral role in dentistry. Numerous dental porcelain compositions have been developed for use in fixed restorative dentistry. Metal ceramic crown consists of a metal sub structure on to which porcelain is baked to mimic the natural appearance of tooth. Empress is a leucite-reinforced ceramic. The use of CAD/CAM technology spurred a whole new generation of ceramic substances like Lava and Procera with substructures consisting of zirconium oxide and aluminium oxide with ceramic overlay. The main objective of the paper is to review and compare four different materials PFM, Empress, Procera and Lava available for use in fixed restorative dentistry and also discuss the factors influencing the decision process.

METHODS: We compared the materials based on their physical properties, longevity, fabrication method, marginal fit and biocompatibility, material of choice in terms of patient expectations, previous restorations and amount of remaining tooth structure. We gathered the information from 40 different articles using Journal of American Dental Association, Journal of Esthetic and Restorative Dentistry, Inside Dentistry, Journal of Canadian Dental Association, European Journal of Oral Sciences, British Dental Journal and compared the materials based on the information presented in the Journals and in their case studies.

RESULTS: PFM has the highest flexural strength of $1895 \pm 317\text{N}$ and highest longevity with a poor marginal fit of $64\mu\text{m}$. Lava has the best marginal fit of $40\mu\text{m}$ and highest fracture toughness of $5-10\text{MPa/m^2}$. Procera has moderate flexural strength of $687 \text{MPa}$ and can be used in either the anterior or posterior teeth. Empress has good esthetics and poor flexural strength of $215-220 \text{MPa}$ and has a marginal fit of $62\mu\text{m}$.

CONCLUSION: There are many factors that play a critical role in the decision making process. Single anterior crowns may be best created using Empress in the presence of a non-disclored tooth. Because of the masking capacity a zirconia core ceramic should be chosen to treat nonvital discolored anterior teeth or in the presence of metal posts that cannot be removed and a metal free restoration is desired. Posterior single crowns may be fabricated using a PFM, Procera or Lava. Fixed partial dentures with no more than two pontic elements should be fabricated using a zirconia substructure or PFM. In patients with parafunctional habits like bruxism PFM with metal occlusal is indicated. Finally we would like to conclude by saying that there is no single factor that decides which material is the best and numerous clinical studies have demonstrated that more than one material is ideal for a given situation.
DETERMINING THE RESTORABILITY AND PROGNOSIS OF A TOOTH: WHAT FACTORS COME INTO THE DECISION MAKING PROCESS

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BACKGROUND: The principal reason for providing dental therapy is to achieve optimal oral health and retention of the dentition. This objective includes the restoration of form and function, esthetics, and the avoidance of further disease. Restoring a tooth is an important aspect of dental practice involving treatment options of varying complexity. The clinician must be able to predict the probability of restoring the teeth successfully. This process involves addressing numerous factors regarding the health of the tooth in question. Since determining the prognosis helps to estimate the longevity of the tooth, this review focuses on factors that help in this decision making processes.

METHODS: More than 100 articles published in peer reviewed journals were reviewed. The databases used for such searches were PubMed, the Cochrane review, and the ADA Evidence Based Dentistry website. Book sources were also utilized. Patient and individual tooth related factors were reviewed.

RESULTS: This review identifies factors that should be considered when choosing between treatment options. The options considered should include:
Patient factors: medical conditions, behavioral habits, esthetics, finances, and decay activity.

Periodontal factors: probing depth, bone loss, furcation involvement, tooth mobility, gingival biotype, biologic width, and periodontal maintenance.

Endodontic factors: root resorption, canal calcification, endodontic anatomy, periapical lesions, re-treatment considerations, and potential complications during endodontic treatment.

Individual tooth factors: The height, width and placement of the ferrule, and the crown to root ratio of the remaining tooth structure.

CONCLUSION: The decision as to whether to extract or retain a tooth may not be straightforward. It is multifactorial and includes the evaluation of the remaining tooth structure, periodontal stability, endodontic health, esthetic demands, occlusal factors and cost.
CYTOKINE INDUCTION BY *PORPHYROMONAS GINGIVALIS* DEPENDS ON EPITHELIAL CELL TYPE

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OBJECTIVES: Infection of epithelial cells with *Porphyromonas gingivalis* (Pg) results in production of pro-inflammatory cytokines involved in the initiation and progression of periodontal disease. Interleukin-6 (IL-6) is a pleiotropic cytokine that stimulates immunoglobulin secretion and plays an important role in regulating immune responses to periodontal pathogens. Interleukin-8 (IL-8) is a potent chemoattractant inducing the influx of neutrophils into periodontal lesions. The objective of the study was to compare IL-6 and IL-8 responses of three human oral epithelial cell lines to two Pg strains.

METHODS: Pg strains, 2561 and W83, were sub-cultivated on blood agar plates and suspended in Medium 199. Non-tumor-derived immortalized oral epithelial GMSM-K cells, and oral squamous cell carcinoma, HSC-3 and H413 cells, were exposed to live and heat-inactivated Pg at 10⁸ bacteria/well (inoculation ratio ~500 bacteria per cell), and incubated at 37°C for 6 and 24 h. IL-6 and IL-8 were determined by ELISA.

RESULTS: The levels of IL-8 produced by GMSM-K cells were much lower than that produced by HSC-3 and H413 cells. Live Pg induced significant IL-6 and IL-8 secretion in GMSM-K and HSC-3 cells. Heat-inactivation of Pg enhanced greatly IL-6 and IL-8 stimulation in these cells. Uninfected H413 cells produced higher levels of IL-6 and IL-8 than HSC-3 and GMSM-K cells, but these cells were not responsive to live Pg. However, heat-inactivated Pg-2561 and Pg-W83 resulted in a 2- to 4-fold increase in IL-6 and IL-8 secretion in H413 cells.

CONCLUSIONS: The amount of IL-6 and IL-8 secreted by control cells and their response to Pg was strongly dependent on the cell type; thus, conclusions on cytokine responses to Pg should not be based on studies with a single cell type. Heat-inactivated Pg strains stimulated higher IL-6 and IL-8 secretion, suggesting an effect of Pg cysteine proteinases (gingipains) on both cytokines.

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TOOTH WHITENING: INDICATIONS AND CONTRAINDICATIONS: TYPES OF SYSTEMS: FOR VITAL AND NON-VITAL TEETH: WHEN AND HOW?

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OBJECTIVES: Tooth whitening is one of the conservative treatments to achieve esthetic results. The question is if tooth whitening is actually a reliable method or not? What are the criteria for case selection? How does whitening address various degrees of tetracycline stains? This paper will give a detailed insight into these issues along with methodologies of various whitening procedures and their merits and demerits.

MATERIALS AND METHOD: The dental literature was searched using the MEDLINE, PUBMED, WILEY and OVID databases. Using specific inclusion and exclusion criteria, 4 reviewers evaluated titles, abstracts, and full articles to identify articles relevant to this review. All searches were conducted for articles published for last 20 years. There were 100 articles, which were sorted to look further into. The topic was decided into introduction, in office, at home and non-vital bleaching. Different methods and systems were analyzed based on literature and explained.

RESULT: Bleaching is indicated for all kinds of extrinsic stains and developmental conditions in vital and non-vital teeth. Contraindications include caries, exposed dentin and recent trauma etc. At home bleaching products include night guard vital bleaching (NGVB) and over the counter products (OTC). NGVB, a proven safe method, uses custom bleaching trays and bleaching gels (carbamide peroxide or hydrogen peroxide). Over the counter products include direct to consumer trays, whitening strips, paint on gel, whitening dentifrices, mouthwashes, chewing gums etc. The disadvantages are sensitivity of teeth and gingival hyperemia and being unsupervised. In-office bleaching seems to be a short-term solution. In office methods include, Conventional (chemical only), Gel and Light and Laser, with 30-40%H2O2. Widely used Non-vital whitening techniques include walking bleach, inside/outside bleaching and in-office bleaching where Hydrogen peroxide, carbamide peroxide, and sodium perborate is used as bleaching agents.

CONCLUSION: Case selection is extremely important with attention to origin, tooth vitality and severity of stains. In-office bleaching seems to be short-term solution, the effects of which are largely attributed to dehydration of teeth. At home night guard vital bleaching is the most conservative esthetic procedure and has a long history of safety and efficacy. The use of OTC bleaching agents could increase the risk of adverse effects, which is less likely in a supervised treatment. In office tooth whitening is recommended for immediate results, with consideration to high conc. of H2O2. In non-vital tooth bleaching, walking bleach technique is the best with history of efficacy. Moreover, No matter what system is used, studies show that relapse occur invariably and repeated application is necessary to achieve patient satisfaction and maintenance.
ROLE OF ORTHODONTICS IN THE TREATMENT OF PEG LATERALS, DIASTEMAS AND CONGENITALLY MISSING LATERAL INCISORS PRIOR TO RESTORATIVE TREATMENT

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OBJECTIVE: The purpose of this article is to present an interdisciplinary orthodontic and restorative approach for the treatment of diastemas, peg laterals or congenitally missing lateral incisors. Patient autonomy also has an influence when deciding about the inclusion of orthodontics into the treatment plan.

DISCUSSION: We will discuss how orthodontic treatment helps in redistribution of the restorative space into a more favorable position prior to restorative treatment to produce optimal esthetic and occlusally functional results in various clinical scenarios. The traditional treatment of canine mesialization is also discussed in terms of esthetics, occlusion and conservation of tooth structure and how treatment may have been influenced by the existing restorations and crown to root ratio. Restorative approach alone may not be able to produce ideal results with anterior spacing cases. Orthodontic treatment may be required to assist the restorative plan in producing optimum outcome. Once the spacing issues have been addressed the restorative treatment can be administered with two main approaches: direct approach including the resin restoration; or the indirect approach including the porcelain laminate veneers, porcelain fused to metal crowns and all ceramic crowns; and implants with their corresponding crowns. While considering the various treatment options, it is important to evaluate the numerous esthetic parameters such as dental and facial midline, tooth dimension and shape, gingival zenith, axial inclinations, golden proportion, interdental embrasures and contact point, sex, personality, age and RED proportion. All possible clinical scenarios have been discussed in the article as well as unpleasant outcome by instant orthodontics and no-prep restorations such as resin veneers, lumineers and snap-on smile.

CONCLUSION: The scientific literature suggests that the ideal treatment plan for a complex esthetic cases due to anterior diastema, peg laterals and congenitally missing teeth should include interdisciplinary orthodontic and restorative management to get the optimum esthetic and functional results. Moreover, Instant orthodontics by restorative dentist without orthodontist as well as space closure by orthodontist without consultation with restorative dentist can cause irreversible tooth structure reduction, not appropriate occlusion and unesthetic result. The combined efforts of restorative dentist and orthodontist will help to insure the most esthetic and functionally appropriate final treatment result.
DIMINISHED SERUM AGGREGATIBACTER ACTINOMYCETEMCOMITANS ANTIBODY AND PORPHYROMONAS GINGIVALIS ANTIBODIES ARE ASSOCIATED WITH CADMIUM EXPOSURE IN US ADULTS: THE THIRD NATIONAL HEALTH AND NUTRITION EXAMINATION SURVEY (NHANES III, 1988–94)

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OBJECTIVES: Epidemiologic data suggest that there is an association between smoking and periodontal disease. There is also evidence that the smoking is also related to diminished number of antibodies in the periodontal disease due to depressed immune response. This study explored the hypothesis that cadmium from cigarette smoke is associated with diminished serum Aggregatibacter Actinomycetemcomitans antibody and Porphyromonas gingivalis antibodies.

METHODS: The current study investigated associations between urinary cadmium and the prevalence of decreased serum serum Aggregatibacter Actinomycetemcomitans antibodies and porphyromonas gingivalis antibodies using data from a sample of 6,497 participants aged 17-80 in the Third National Health and Nutrition Examination Survey. Logistic regression model was used to investigate existing associations while adjusting for confounding variables.

RESULTS: Both simple and covariate-adjusted models indicated that urinary cadmium was associated with diminished serum Aggregatibacter Actinomycetemcomitans antibody and porphyromonas gingivalis antibodies ($P_{trend} < 0.05$ for both). Adjusted odds ration for diminished antibodies showed the effect of cadmium in the mechanism.

CONCLUSIONS: This analysis demonstrates that cadmium exposure is associated with high prevalence of diminished serum Aggregatibacter Actinomycetemcomitans antibody and porphyromonas gingivalis antibodies. Additional studies will be required to determine whether the increased risk derives from cadmium per se or from the other component of cigarette smoke.
ENDODONTIC THERAPY VS THREE UNIT FIXED PARTIAL DENTURE VS SINGLE UNIT IMPLANT

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OBJECTIVES: To comprehend the factors that drive the decision-making process when opting for endodontic therapy vs. three-unit fixed partial denture (FPD) vs. single unit implant as applicable to day-to-day clinical settings.

METHODS: Articles were gathered using PubMed, EBSCO, and Cochrane Reviews. A thorough reading and interpretation of these articles assisted in classifying the factors involved in the decision making process. They fall into four categories namely, systemic factors, local factors, patient and clinician related factors.

RESULTS: There are no absolute systemic contraindications to endodontic therapy. Patients with xerostomia are at high risk for coronal and root caries, impacting the tooth’s prognosis, hence, posing as a relative contraindication to endodontic therapy and retaining the tooth in question. Local factors also affect prognosis for endodontic success. The root anatomy/presence of calcification, remaining clinical coronal structure available for ferrule, need for orthodontic extrusion, periodontal condition including furcation involvement, crown to root ratio, history of endodontic failure and quality of treatment, periapical radiolucency >5 mm, tooth position in the arch are all such factors.

The absolute contraindications to implant placement include IV bisphosphonates, uncontrolled diabetes, bleeding dyscrasias, terminal disease, immunosuppression, incomplete growth, and pregnancy. Relative contraindications include smoking, history of radiation therapy, controlled diabetes, and oral bisphosphonates.

FPDs are favored over implants when adjacent teeth need large restorations or there are large existing restorations. If bone quality or quantity is poor due to a composite defect, pink porcelain can be placed to mask the area if surgery is contraindicated. Achieving esthetic results with implants becomes more challenging than a conventional FPD in thin gingival biotype, low crest and high lip line. The papillary fill is 44% greater around a pontic of a FPD than around a single implant.

CONCLUSION: A thorough understanding of how various systemic, local and dentist related factors impact the survival and success of endodontic therapy, fixed partial denture and implants has led us to understand that every case needs to be treated differently.

Endodontic therapy and retention of the tooth should be the first line of treatment if there are no contradictory factors. Endodontic retreatment is the second line of treatment. If the retreatment fails and the tooth in question does need to be extracted, a thorough evaluation of the systemic, local, patient and dentist related factors should help the clinician judge whether an implant or FPD would be the better option.
POLYMORPHISMS OF THE RFC1 80AG AND MTHFR 677CT GENES IN PATIENTS WITH CLEFT LIP AND PALATE IN EGYPT

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INTRODUCTION: Nonsyndromic cleft lip and/or palate (NCL/P) is one of the most common congenital anomalies affecting 1 in every 500-1000 births. Nonsyndromic cases represent approximately 70% of all orofacial clefts and are caused by a combination of genetic and environmental factors. Among candidate genes for NCL/P, reduced folate carrier 1 (RFC1) and methylenetetrahydrofolate reductase (MTHFR) genes have important roles in folate metabolism.

OBJECTIVE: To determine the roles of RFC1 80AG and MTHFR 677CT gene polymorphisms in etiology of NCL/P in Sohag, Egypt.

METHODS: Case control study design was used. Cases (individuals affected with NCL/P; n=134) and controls (unaffected individuals with no birth defect in family history; n=106) were identified during Rotaplast medical mission to Sohag in 2010. DNA was isolated from blood or saliva. RFC1 80AG and MTHFR 677CT genotypes were established by polyacrylamide gel electrophoresis of PCR-amplified fragments.

RESULTS: Statistically significant difference in proportions of RFC1 80AG genotypes (p=0.0039) and also significantly higher frequency of mutated allele G (p=0.009) were found when cases and controls were compared (cases: 23.3% AA, 32.7% GG, 44% AG, G allele frequency 0.547; controls: 37.5% AA, 21.2 % GG, 41.3% AG, G allele frequency 0.418). Also in MTHFR 677CT, statistically significantly higher proportion of TT homozygotes (p=0.018) and also significantly higher T allele frequency in cases compared to controls (p=0.011) was found (cases: 40.3% CC, 14.9% TT, 44.8% CT, T allele frequency 0.373; controls: 52.8% CC, 4.7 % TT, 42.5% CT, T allele frequency 0.259).

CONCLUSIONS: Results of this pilot study suggest that folate pathway-related genes RFC1 and MTHFR may be involved in etiology of NCL/P in Sohag, Egypt. We analyzed RFC1 80AG and MTHFR 677CT polymorphisms and both showed statistically significant differences in genotype distributions and in allele frequencies when cases and controls were compared. Mutated alleles and genotypes with mutated alleles were more prevalent in individuals with NCL/P than in controls.

Rotaplast International, Inc. funded and supported field work for this study. Pacific Craniofacial Genetics Team and Cleft Prevention Program and Department of Orthodontics funded and supported molecular genetic analysis. This study was presented at the 112th Annual Session of the American Association of Orthodontists in Honolulu, HI, May 5-8, 2012.
A CASE REPORT: TWO BILATERAL CLEFT LIP AND PALATE PATIENTS

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INTRODUCTION: Oral clefts commonly affect the lip, alveolar ridge, and hard and soft palates. Three fourths of clefts are unilateral; one fourth is bilateral. Bilateral Cleft lip and Palate (BLCLP), while is characterized by symmetry, is a far more challenging defect to treat. The bilateral cleft lip is reconstructed in infants between the age of 3 and 6 months. Palatal repairs are performed on patients between 9 and 18 months of age, which satisfies a balance between optimizing the child’s anatomy for proper speech development and waiting long enough to minimize the surgical insult on facial growth. During the mixed dentition, Phase 1 orthodontics can be undertaken to minimize incisor malalignment, thus facilitating normal speech development, palatal expansion, and space maintenance. Bone Grafting to Alveolar Cleft. Some patients may also benefit from orthognathic surgery. Lip revision is delayed until after orthognathic surgery. Formal rhinoplasty is undertaken as the final step. Two BLCLP patients were referred from Children’s Hospital to the orthodontic clinic at the University of the Pacific in January, 2011. One is in early mixed dentition, the other is in adolescence. This report shows how different approaches apply to the patients in terms of developmental stage, successful previous treatment experience and prognosis.

CASE PRESENTATION: T.W. is 7yr 7m year old very cooperative male with bilateral cleft lip and palate. The need for pre-surgical orthodontic treatment was the chief complaint. Lip surgery and palatal surgery were performed at 3 month and 9 month years old, respectively. Dentally, he presented with the following problems: V-shaped upper arch form, retroclined, extruded, rotated and spaced upper incisors, two supernumerary teeth, canine cross bite, deep bite, midline discrepancy and dental caries on the upper right second primary second molar and upper left primary first molar while permanent premolars on the upper right are missing. Treatment goal is to establish a favorable environment for the maxillary graft before eruption of permanent canines. The objectives are to: relieve crossbite with expansion, level and align upper incisors, and remove supernumerary teeth. Treatment Plan includes: caries control, expansion in canine area with Quad helix, align of upper incisors, extraction of supernumerary teeth and iliac bone graft to the alveolar cleft.

H.M. is a 15y 2m years old male with BLCLP with skeletal Class III with mid-face deficiency without any bone graft history or Phase 1 orthodontic treatment. Also he showed maxillary deficiencies in the transverse and sagittal planes, and mandibular vertical excess in vertical plane with class III molar relationship, bilateral posterior cross bite, anterior crossbite, retroclined upper and lower incisors, missing teeth, supernumerary canines, and poor oral hygiene including multiple dental caries. Treatment goals focus on improving oral hygiene, facial esthetics, sagittal relationship, transverse relationship, and vertical relationship.

Treatment Plans include the following: bone graft to alveolar cleft, pre and post orthodontic treatment, and two jaw orthognathic surgery with pharyngeal flap surgery.

CONCLUSION: Parents and patients with CLP should be prepared for a protracted course of therapy based on their developmental stage, previous treatment history, and anticipated prognosis to correct the cleft deformities
ISOLATION, GROWTH AND ODONTOGENIC DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS IN VITRO

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BACKGROUND & PURPOSE: Adult dental pulp-derived stem cells (DPSC's) can differentiate into mesoderm-derived cell types and nerve cells. DPSC's are excellent candidates for regenerative cell therapy restorations in the craniofacial region. For this to be possible, the DPSC's must be efficiently expanded in vitro with no loss of their differentiation potential. We examined effects of combinations of platelet-derived factors (PDF) and fibrin substrate on multiplication and odontogenic differentiation of DPSC's in vitro. A response of DPSC's to low oxygen was also tested.

METHODS: DPSC's were isolated from vital asymptomatic third molars and were grown on the following substrates: polystyrene, fibrin and fibrin with PDF. The growth medium consisted of basal medium (Lonza) supplemented with human adult serum (10%), L-glutamine and antibiotics. The odontogenic differentiation medium consisted of basal medium (Lonza) supplemented with human adult serum (10%), L-glutamine, dexamethasone, L-ascorbic acid, KH2PO4 and antibiotics. The cells were stained for markers of differentiation and mineralization.

RESULTS: We constructed growth curves showing that the substrate containing both fibrin and PDF shortened the lag phase of growth and increased the multiplication and differentiation rate of the DPSC's most efficiently. The fibrin substrate was second in its efficiency. Strong staining with alizarin red S for mineralization of extracellular matrix and strong staining of cellular alkaline phosphatase documented a successful differentiation. Hypoxia significantly increased proliferation rate of DPSC's.

CONCLUSIONS: Fibrin, PDF and hypoxia – factors that are normally present in a healing wound - can be utilized to accelerate preparation of DPSC's for clinical applications.
PHOTODYNAMIC THERAPY OF CANDIDA BIOFILMS

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OBJECTIVES: Photodynamic therapy (PDT) utilizes light to activate a photosensitizing agent (photosensitizer) in the presence of oxygen. The exposure of the photosensitizer to light results in the formation of toxic oxygen species causing localized photo-damage and cell death. Candida-associated denture stomatitis is a common recurrent disease in denture wearers. The formation of C. albicans biofilms contributes to the failure of antifungal therapy and to recurrent infections. In this study, the photodynamic activity of the porphyrin-based photosensitizer TMP-1363 towards Candida biofilms, formed on denture acrylic discs, and planktonic Candida was investigated in the absence and presence of the azole antifungal, miconazole.

METHODS: The effect of TMP-PDT ± miconazole on Candida biofilms was determined for two clinical isolates, C. albicans 6122/06 and C. tropicalis 8122/06. Biofilms were developed on poly(methyl methacrylate) discs over 2-day period. Candida biofilms were incubated with miconazole for 2 h at 37°C, following by treatment with TMP-1363 (10 μg/ml) for 30 min at 37°C. The plates were exposed to broadband visible light (350-800 nm) from a light bulb at a distance of 10 cm from the light bulb to the plate, for 30 min. The irradiance at the surface of the plate was 32.5 mW/cm². Infrared radiation was minimized using a 1 cm water filter. The metabolic activity of the biofilms was measured by the XTT assay. The suspension of planktonic Candida was added to wells of 96-well plates, and the cells were treated and irradiated as described for Candida biofilms. The effect of PDT against planktonic Candida was tested by the microdilution plate assay.

RESULTS: The photodynamic activity of TMP-1363 alone towards Candida biofilms reduced metabolic activity of biofilms by ~40%, while miconazole alone reduced metabolic activity by ~30%. Combined treatment with miconazole + TMP-PDT was more effective, reducing metabolic activity by 55% and 73% for C. tropicalis and C. albicans biofilms, respectively. The photodynamic activity of TMP-1363 alone towards both planktonic Candida species reduced colony forming units (CFU) by ~72%. Treatment with miconazole + TMP-PDT and miconazole alone was slightly less effective for planktonic C. tropicalis, while C. albicans was not sensitive to this treatment.

CONCLUSIONS: The biofilms formed by C. tropicalis and C. albicans on denture acrylic discs were susceptible to the photodynamic activity of the porphyrin-based photosensitizer TMP-1363. Miconazole increased the sensitivity of biofilms to TMP-PDT. Planktonic Candida was sensitive to TMP-PDT, but miconazole did not enhance the efficacy of TMP-PDT.
MTHFR 677CT POLYMORPHISM AND CLEFT LIP AND PALATE ANOMALIES

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INTRODUCTION: A sufficient bioavailability of active folate seems to be important for normal early development of the embryo. Maternal folate insufficiency has been found more frequently in spina bifida and nonsyndromic cleft lip and/or palate (NCLP). It may be due to a mutation of a folate pathway gene or a low dietary intake.

OBJECTIVES: Compare and discuss proportions of MTHFR 677 CT genotypes and C and T allele frequencies in samples from Guatemala City, Guatemala, and Cebu City, Philippines.

MATERIALS AND METHODS: Guatemala study (DeLurgio et al.2009) and Philippines study (Chandwani et al, 2009) used case control study design comparing in each population a sample of cases (individuals affected with NCLP) with controls (unaffected individuals with no family history of any birth defect). Altogether, venous blood specimens were obtained from 242 cases and 218 controls from Guatemala and 145 cases and 120 controls from Philippines and DNA was analyzed using our standard laboratory protocol.

RESULTS: In the Guatemala sample, statistically significant difference (p = 0.0045) was found between genotype distributions in cases and controls (genotypes in cases - CC =7.4 %, CT=46.7%, TT =45.9%; in controls CC=16.1 %, CT=48.6 %, TT=35.3 %) and also in allele frequencies (p = 0.03). The T allele frequency was 0.692 in cases and 0.596 in controls. In the Philippine sample, no statistically significant differences between genotype distributions and allele frequencies of cases and controls were found. The T allele frequency was 0.09 in cases and 0.10 in controls.

CONCLUSIONS: Etiology of NCLP is multifactorial comprising both genetic and environmental factors. Association of NCLP with C677T polymorphism of MTHFR was found in the Guatemalan sample but not in the Philippine sample. However, Tolar found in the same Philippine population an association with a low maternal plasma folate levels (Tolar et al. 2007). Both examples document an important role of folate in early embryonal development of the face. In Guatemala, a folate insufficiency could be related to a higher frequency of T allele, in Cebu City, it was probably related to a low nutritional intake of folate by mothers. Studies of this kind support prevention of NCLP by folate supplementation.

Rotaplast International, Inc., funded and supported field work for this study. Pacific Craniofacial Genetics Team and Cleft Prevention Program and Department of Orthodontics funded and supported molecular genetics analysis. This study has been presented as the Table Clinic Student Competition at the CDA Scientific Session in Anaheim, May 3-6, 2012.
IMPLICATIONS OF MASTICATOR (PTERYGMANDIBULAR) SPACE VARIATIONS FOR INFERIOR ALVEOLAR INJECTION EFFICACY

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OBJECTIVES: Inferior alveolar injections are cited as having a high variation in efficacy. A better understanding of the anatomy of the masticator space, and especially structures such as the buccinator muscle, inferior alveolar foramen and neurovascular bundle, lateral pterygoid muscle, lingual nerve, medial pterygoid muscle, mylohyoid muscle, sphenomandibular ligament, superior constrictor muscle, and temporalis muscle.

METHODS: Conducted a literature search to better understand the anatomy of the masticator space and the difficulty in delivering IANB. Ten cadaver heads were obtained from DDS 2014’s anatomy lab course. Heads were selected for minimal disruption of structures in question. Heads ranged from partially to fully edentulous. A protocol was established to view structures by gross dissection. This protocol was modified in select cadavers to provide specific views of interest. Anatomical variations were recorded qualitatively and photographed.

RESULTS: Gross dissection demonstrated variations in prominence of pterygomandibular raphe, medial pterygoid muscle attachment and volume, sphenomandibular ligament size, and temporalis muscle attachment.

CONCLUSIONS: Inferior alveolar nerve block (IANB) protocol dictates the use of intraoral soft and hard tissue landmarks. The high amount of anatomic variation seen in the masticator space demonstrates that the use of intraoral soft tissue landmarks to deliver anesthetic to the IA foramen may not be efficacious. This may also explain the high failure rate recent studies have reported.
THE ROLE OF ANIMAL PROTEINS IN OROFACIAL CLEFTING

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INTRODUCTION: Orofacial clefts are one of the most common birth defects in humans, occurring in approximately 1 of every 700 live births. The psychological burden on children and families affected by clefts can be great, and the surgeries and rehabilitative services available to treat clefts are often prohibitively expensive or geographically limited and may take years to complete. Prevention of orofacial clefts can save much psychosocial and economic hardship. Existing research suggests that certain components of the maternal periconceptional diet may help to prevent orofacial clefting, however little attention has been paid to the role of animal protein specifically.

OBJECTIVES: The purpose of this research was to review the existing literature and pose new theories for how maternal periconceptional animal protein consumption and orofacial clefting may be related.

METHODS: Electronic searches were conducted on the topic of maternal periconceptional animal protein consumption and orofacial clefting. Given limited articles on this topic, additional searches were conducted on specific nutrients and chemical compounds found primarily in animal proteins.

RESULTS: Studies have shown that nutrients such as iron and zinc serve as protective factors against clefting. While animal protein is rich in these nutrients, the same studies fail to show any significant differences between cases and controls with respect to animal protein intake. Plant protein intake is significantly higher, however, in mothers of children without clefting, suggesting that protective nutrients like iron and zinc come primarily from non-animal protein sources. Choline is also plentiful in animal protein and helps protect against neural tube defects, but it is largely destroyed during the cooking process. Plant protein, including soy products and numerous green vegetables, may actually serve as a richer source of dietary choline and can be consumed without cooking. Finally, cooked animal protein has been shown to produce mutagenic HCAs and PAHs, which have been linked to cancer in humans and could conceivably increase the risk of birth defects through similar effects on the DNA.

CONCLUSIONS: These findings suggest that animal protein - although rich in iron, zinc and choline - may not play as protective a role in the periconceptional diet as other sources of these nutrients and vitamins, namely plant proteins. What is more, cooked meat may introduce mutagens that alter DNA in ways that actually increase the risk of orofacial clefts. The mechanism by which animal protein ultimately acts on DNA has yet to be explored, but further research on this topic could prove especially valuable in identifying ways in which nutrient supplementation and dietary counseling could be used to prevent clefting.
LIPOSOME TARGETING TO *PORPHYROMONAS GINGIVALIS*: POTENTIAL APPLICATION TO PHOTODYNAMIC THERAPY

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OBJECTIVES: Photodynamic therapy (PDT) exploits visible light and photosensitizers to inactivate cells and has been used for the treatment of several types of malignancy. *Porphyromonas gingivalis* is one of the most significant periodontal pathogens. The use of photosensitizer and light as an antimicrobial agent against periodontal microbial biofilms represents an attractive method of eliminating oral bacteria. Liposomes can encapsulate photosensitizers and may be a potential delivery vehicle to target periodontal bacteria. We investigated the binding specificity of different types of liposomes (positive, neutral and negative) to *P. gingivalis* at different lipid concentrations, using fluorescence spectroscopy. We also examined the photosensitizer, zinc phthalocyanine, for its effect on *P. gingivalis* and on oral epithelial cells. Zinc phthalocyanine has advantages of chemical and photochemical stability, and strong absorption in the red region, which can more readily penetrate tissues.

METHODS: Liposomes were composed of Palmitoyloleoylphosphatidylcholine (POPC): phosphatidylglycerol (PG), POPC, or dioleoyltrimethylammoniumpropane (DOTAP): POPC, and contained the fluorescent probe rhodamine-phosphatidylethanolamine. The liposomes were added to *P. gingivalis* in suspension in the concentration range 10-100 µM. After incubation for 30 min at room temperature, *P. gingivalis* were centrifuged at 14,000 rpm for 10 min. Fluorescence levels in the supernatant and pellets were measured using a Perkin-Elmer Luminescence Spectrometer. Zinc phthalocyanine was incubated with *P. gingivalis* in suspension for 2 h. The bacteria were irradiated with red light for 20 min and grown on blood agar plates for 48 h. Zinc phthalocyanine was also examined for its dark and light toxicity against the oral epithelial cell line, HSC-3, and its ability to be encapsulated in liposomes.

RESULTS: DOTAP:POPC liposomes at 100 µM were ~100% bound to *P. gingivalis*. POPC:PG and POPC liposomes were ~17% and ~23% bound, respectively. Zinc phthalocyanine was partially toxic to *P. gingivalis* at 5 and 10 µM in the dark. When exposed to light, the cytotoxicity was increased further. Zinc phthalocyanine could be encapsulated in liposomes, and was not toxic to HSC-3 cells in the dark.

CONCLUSIONS: Among the three different types of liposomes, the positively charged ones exhibit the best binding specificity to *P. gingivalis*. Zinc phthalocyanine may be used to selectively kill *P. gingivalis* under conditions that do not affect oral epithelial cells. Further studies will focus on whether zinc phthalocyanine encapsulated in liposomes is more cytotoxic to *P. gingivalis* than free zinc phthalocyanine.
SUBMANDIBULAR GLAND VASCULAR RESPONSES TO SYMPATHETIC STIMULATION: EFFECTS OF INHIBITING B- AND A-ADRENERGIC RECEPTORS

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INTRODUCTION: The response to sympathetic stimulation in the rat submandibular gland is biphasic (vasoconstriction followed by vasodilatation). Vasoconstriction is thought to be due to the activation of α-adrenoceptors, whereas endothelial-derived relaxing factors are primarily responsible for the vasodilatory phase. However, little is known about β-adrenergic vascular responses in the submandibular gland. Thus, the aims of this study where to examine the vascular responses to sympathetic stimulation in the submandibular glands of male and female rats, before and after β- and α-adrenoceptor blockade.

METHODS: Male and female Sprague-Dawley rats were anesthetized using pentobarbital (35 mg/kg, i.p.) followed by chloralose (80 mg/kg, i.v.). Blood flow was measured using laser-Doppler flowmetry, and vascular responses to sympathetic stimulation (2 and 4 Hz continuously, or 20 Hz and 40 Hz in bursts of 1s every 10s) were determined. To block β-adrenergic receptors, propranolol was administered (2 mg/kg, i.p.), and α-adrenergic receptors were inhibited using phentolamine (2 gm/kg, i.v.). Differences were analyzed for statistical significance using one-way ANOVA for repeated measures.

RESULTS: The preliminary results (mean ± SEM, % change in basal perfusion) are given in the table below:

<table>
<thead>
<tr>
<th></th>
<th>20 Hz</th>
<th>40 Hz</th>
<th>2 Hz</th>
<th>4 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.1 ± 3.8</td>
<td>22.3 ± 7.1</td>
<td>-20.5 ± 2.9</td>
<td>-30.5 ± 6.4</td>
</tr>
<tr>
<td>Propranolol</td>
<td>21.2 ± 8.3</td>
<td>16.8 ± 9.7</td>
<td>-27.4 ± 6.0</td>
<td>-36.3 ± 12.5</td>
</tr>
<tr>
<td>Propranolol + Phentolamine</td>
<td>20.1 ±9.8</td>
<td>26.6 ± 11.6</td>
<td>7.0 ± 9.3*</td>
<td>4.5 ± 12.3*</td>
</tr>
<tr>
<td>Females (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>27.0 ± 3.6</td>
<td>18.0 ± 6.0</td>
<td>-23.0 ± 7.4</td>
<td>-43.9 ± 8.8</td>
</tr>
<tr>
<td>Propranolol</td>
<td>23.0 ± 3.0</td>
<td>8.1 ± 6.8</td>
<td>-39.2 ± 9.2</td>
<td>-50.9 ± 9.3</td>
</tr>
<tr>
<td>Propranolol + Phentolamine</td>
<td>13.8 ± 8.3</td>
<td>7.2 ± 5.8</td>
<td>-21.8 ± 9.8</td>
<td>-20.3 ± 13.7</td>
</tr>
</tbody>
</table>

*Propranolol + Phentolamine vs control or propranolol alone, P < 0.01

CONCLUSIONS: Propranolol had little effect on the vascular response to sympathetic stimulation in either male or female rats, suggesting that β-adrenergic receptors are not prominent on vascular smooth muscle in the submandibular gland. After the addition of phentolamine to block α-adrenoceptors, a prominent vasoconstriction remained. This was most likely due to the co-release NPY from sympathetic terminals, which has been shown to have a vasoconstrictor effect nearly equal to that of noradrenaline in the dog submandibular gland (McCloskey and Potter, 2000).
HIV-SPECIFIC PROMOTERS TO ELIMINATE HIV-INFECTED CELLS BY GENE THERAPY

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OBJECTIVE: Current therapies against HIV infection are unable to eradicate the chromosomally integrated proviral genome. Successful therapy of HIV/AIDS requires a method to specifically kill HIV-infected cells. We are developing HIV-specific promoters to drive the expression of suicide genes that will kill HIV-infected cells, but not uninfected cells. Here we examined the expression of a reporter gene, luciferase, driven by the HIV promoter, LTR, and its mutants. Our aim is to design and synthesize a promoter that responds to the HIV transcriptional activator, Tat, but not to cellular transcription factors.

METHODS: The full-length LTR and five progressively truncated versions of the promoter were generated using PCR-based cloning techniques (Bionexus), and designated LTR1–LTR6. These promoters were inserted into the pGL3 Basic Vector (Promega) encoding luciferase. These plasmids were transfected into HeLa cells, and HeLa-tat-III cells that constitutively express Tat, using the cationic liposome, Metafectene (Bionex). Luciferase activity (relative light units (RLU)/ml cell lysate) was measured 48 h later, using the Luciferase Assay System (Promega).

RESULTS: Luciferase expression from the LTR2 construct increased from 1,060 RLU/ml in HeLa cells to 108,187 RLU/ml in HeLa-tat-III cells, a 102-fold increase. In LTR2, the LTR modulatory region is truncated, but the NF-κB binding region is maintained. Luciferase expression from LTR1 containing the wild type HIV promoter, was 595 RLU/ml in HeLa cells, and 30,373 in HeLa-tat-III cells. In LTR3 without the NF-κB binding region the corresponding values were 498 and 25,387 RLU/ml. The other constructs resulted in much lower gene expression.

CONCLUSIONS: HIV-specific cell killing may be possible by generating a suicide gene construct driven by the LTR2 promoter. It is expected that the incorporation of this, or a more Tat-specific construct, into a lentiviral vector may lead to the therapeutic transduction of all HIV-harboring cells.

This work was funded by Research Pilot Project Awards 03-Activity 071 and 03-Activity 076 from the University of the Pacific, Arthur A. Dugoni School of Dentistry

This work was presented at the 41st Annual Meeting & Exhibition of the American Association for Dental Research, Tampa, FL, March 21-24, 2012. J. Dent. Res. Vol. 91 (Special issue A) Abstract No. 1275, Seq. #170
INTRODUCTION: The etiology of nonsyndromic cleft lip with or without cleft palate (N/CLP) is multifactorial (genetic and environmental factors). Among the most commonly studied genes are two genes - MTHFR and/or RFC1 - that are related to folate metabolism. When their function is altered due to mutations, a decreased utilization of folate slows down cell multiplication and it may contribute to orofacial clefting. RFC1 (Reduced Folate Carrier 1) gene that encodes a cell membrane protein essential for internalizing folate bound to a folate-binding protein from circulating blood into cells.

OBJECTIVES: The purpose of our study was to determine whether RFC1 A80G polymorphism is associated with NCLP in a sample of patients from Sohag, Egypt and compare it with MTHFR C677T polymorphism in same samples.

MATERIAL AND METHODS: A case-control study design was used. Cases (individuals affected with NCL/P; n=116) and controls (n=104) for this study were identified during Rotaplast medical missions to Sohag, Egypt in 2009. Diagnosis of NCL/P was determined by physical examination of each individual and venous blood and saliva was obtained for DNA analysis. RFC1 A80G genotypes were established by PCR amplification and single nucleotide conformational polymorphism detection using polyacrylamide gel electrophoresis (PAGE).

RESULTS: The difference in distribution of genotypes between cases and controls was statistically significant (p=0.039). Genotypes at nucleotide 80 of the RFC1 gene among 116 cases revealed 27 (23.3%) cleft patients homozygous for the wild-type allele (AA), 51 (44%) heterozygous (AG), and 38 (32.7%) homozygous for the mutation (GG). Even more statistical significant was difference in allele frequencies between cases and controls (p=0.009). Allele frequencies in the cases were 45.3% for the A allele and 54.7% for the G allele. Among 104 controls, 39 individuals (37.5%) were homozygous for the wild-type allele (AA), 43 (41.3%) were heterozygous (AG), and 22 (21.2%) were homozygous for the mutation (GG). Allele frequencies in the control group were 58.2% for the A allele and 41.8% for the G allele.

CONCLUSION: Results of this pilot study suggest that the RFC1 A80G polymorphism of may be involved in the etiology of NCL/P in Sohag population.

EVALUATION OF ULTRACET® VERSUS VICODIN® FOR POST OPERATIVE PAIN CONTROL FOLLOWING REMOVAL OF BILATERAL SYMMETRICAL PARTIAL BONY IMPACTED THIRD MOLARS

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OBJECTIVES: Study the efficacy and side effects of Ultracet® (37.5mg Tramadol/325mg Acetaminophen) versus Vicodin® (5mg Hydromorphone/500mg Acetaminophen) in clinical patients following the removal of bilateral symmetrical partial bony impacted third molars.

METHODS: Patients were given a post-operative pain medication form to fill out and returned to researcher. Pain scale was dictated on form and patients were allotted an area to fill in any side effects they experienced. Patients were evaluated for four days following intravenous sedation, recording their pain level on a scale from 0-10 four times per day at 8:00am, 12:00pm, 4:00pm and 8:00pm. The prescription of the pain medication was random. Patients were evaluated based on their radiographs to qualify to participate in the study.

RESULTS: This study on the efficacy Ultracet® versus Vicodin® is currently ongoing and under review, results are expected to be completed by June 4, 2012.

CONCLUSIONS: Up to this point we have evaluated that Ultracet® has been shown in our patients to have less side effects (including: dizziness, sleepiness, nausea) and more effective analgesia than observed in Vicodin®. Upon completion of the study, more concrete data and conclusions will be evaluated and determined.
THE IMPLICATIONS OF BONY AND SOFT TISSUE ANATOMICAL VARIATION FOR SUCCESS RATES IN MANDIBULAR NERVE (V₃) ANESTHESIA

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OBJECTIVES: Application of anesthesia into the region where the inferior alveolar nerve (IAN) enters the mandibular foramen (MF) relies on the positioning of a series of defined hard and soft tissue landmarks. However, these landmarks are variably identifiable and result in success rates for mandibular nerve anesthesia that range from 13-98%. This range of rates reflects variation introduced by the technique employed, the level of practitioner experience, and the degree of morphological variation in the landmarks.

Lacking experience, students focus on landmark identification at the expense of understanding the impact of variation. This reliance on ‘stable’ landmarks introduces significantly reduced success rates. Alternatively, we believe that experienced practitioners subconsciously employ modified landmarks on a patient-by-patient basis and that this change underlies their increased success rates. We hypothesize that a strict reliance on available IAN-block landmarks results in low success rates and that this variation underlies most block failures. We test this hypothesis by quantifying the variation in IAN-block related hard and soft tissue landmarks and by documenting the location and course of the IAN.

METHODS: We acquired 2D and 3D landmarks from 190 adult mandibles; this resulted in 25 IAN block-related measurements and angles. These metric data were supplemented by cadaver dissections (n=40). Further, 2D and angular data were acquired from these dissections. These measures describe the course of the IAN relative to the mandibular ramus, foramen, and canal.

RESULTS: The correlations between MF position and all IAN-block related landmarks is extremely weak ($r^2 \approx 0.0$). Only the location of the coronoid notch is consistent and useful in IAN-blocks. Further, ramus breadth was not significantly correlated with MF position. However, we found that ramus breadth values could be divided into three groups such that a consistent maximum depth of injection dimension could be established. We also found the IA neurovascular bundle to be positioned more anterior then generally reported. The IA nerve is generally anteriorly located and that it courses medially on exiting the MF.

CONCLUSIONS: Mandibular shape, in general, and IAN-block related features, specifically, are highly variable and impose a barrier to successful block. We document this high degree of variation in all IAN-block related landmarks. Only the coronoid notch is here considered useful for IAN-block procedures. We also demonstrate that, while highly variable, the division of mandibular ramus breadths into three categories allows consistent maximum values for needle penetration to be established. We also demonstrate that the IAN is generally anteriorly located and that it courses medially. This finding suggests that injections should be shallow, as might be dictated by the use of a short needle. Whereas we establish a landmark for height of penetration and recommend a series of depth of penetration values (based on mandibular ramus breadths), we are currently unable to provide a point of initial injection. The latter value is necessary to restrict the depth of penetration to within plus/minus $\pm 2-3.0$ mm.
A ROLE FOR PKC BETA IN ALTERING CALCIUM HOMEOSTASIS UNDER HYPERGLYCEMIC CONDITIONS IN HUMAN ENDOTHELIAL CELLS

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OBJECTIVES: Cardiovascular diseases (CVD) are leading causes of morbidity and mortality for diabetic patients. Endothelial cell dysfunction is a hallmark of CVD. Intracellular Ca2+ concentration ([Ca2+]i) may regulate endothelial cell function. Here, we investigated the effects of hyperglycemia on [Ca2+]i and a role for protein kinase C (PKC) in regulating [Ca2+]i in the human endothelial cell line, EA.hy926.

METHODS: Cells were cultured in normal (5.5 mM, NG) or high (25 mM, HG) glucose media then treated with either a) vehicle (0.1% DMSO), b) LY341684 (selective PKCβ inhibitor, 100 nM, 24h), or c) phorbol12-myristate 13-acetate (PMA, PKC activator, 200 nM, 4h). Using a spectrofluorometer, [Ca2+]i in cells loaded with Fura 2-AM was monitored in the absence and then presence of extracellular Ca2+.

RESULTS: Thapsigargin (SERCA inhibitor, 1 µM) induced a transient increase in [Ca2+]i which was similar among the experimental groups of cells. However, when 1.5 mM Ca2+ was added, Ca2+ entry was significantly increased in cells cultured in HG compared to NG. This elevated Ca2+ entry in HG-treated cells was attenuated by treatment with LY341684. Cells treated with PMA in NG showed an increased Ca2+ entry similar to cells cultured in HG.

CONCLUSIONS: Our results suggest that hyperglycemia enhanced Ca2+ entry in endothelial cells is PKCβ dependent. Thus, PKCβ inhibitors may restore normal endothelial cell function under hyperglycemic conditions.

This work has been supported by the National Instituted of Health (NIDCR, R15 DE016587). This work was presented at the Federation of American Societies of Experimental Biology 2012, April 21-25, 2012, San Diego, CA
GENDER DIFFERENCES IN AORTIC ENDOTHELIAL FUNCTION OF STREPTOZOTOCIN-INDUCED DIABETIC RATS: POSSIBLE INVOLVEMENT OF PROTEIN KINASE C BETA AND NITRIC OXIDE PRODUCTION

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OBJECTIVES: Little is known of the interaction between diabetes and gender in the vasculature. The objective of this study was to investigate whether there are gender differences in rat aortic endothelial function in streptozotocin (STZ, 60mg/kg, iv)-induced diabetes and the roles of protein kinase C beta (PKCβ) and nitric oxide (NO).

METHODS: Endothelium-dependent vasodilatation (EDV) to acetylcholine (ACh; 10^{-8} to 10^{-5}M) was measured in aortic rings precontracted with phenylephrine (PE; 2 μM) before and after pretreatment with LY341684 (1μM), a selective PKCβ inhibitor. Constrictor response curves to PE (10^{-8} to 10^{-5}M) were also generated before and after incubation with indomethacin (indo, 10 μM), a cyclooxygenase (COX) inhibitor, and L-NAME (200μM), an endothelial nitric oxide synthase inhibitor.

RESULTS: STZ-induced diabetes impaired aortic EDV to ACh only in females. Inhibition of PKCβ increased the sensitivity to ACh in both control and diabetic male rats. However, PKCβ inhibition enhanced the sensitivity to ACh only in aorta taken from diabetic female rats. Addition of L-NAME resulted in a significant potentiation of the contractile responses to PE in all groups. However, aorta from control females had a greater maximal potentiation of the PE responses than others.

CONCLUSIONS: These data suggest that the predisposition of female rat aorta to vascular injury in diabetes is possibly due to differences in enhanced PKCβ activation and decreased basal NO production.

This work was supported by NIH/NIDCR (DE016587). This work was presented at 2012 Experimental Biology, April 21-25, 2012, San Diego, CA.
NOVEL THERAPEUTIC AGENTS FOR TRANTHYRETIN AMYLOID DISEASES

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OBJECTIVES: Design, synthesis, and biological evaluation of novel transthyretin (TTR) stabilizing agents as potential drug candidates for TTR amyloid related diseases.

METHODS: Using a fluorescence polarization high throughput screening (HTS) assay, we have previously discovered small molecules that can bind and stabilize TTR against amyloidosis. A series of analogs of one of the best hits from HTS were synthesized and studied for for their ability to stabilize TTR. All the compounds were identified and characterized by NMR spectroscopy and high resolution mass spectrometry (HRMS). The compounds are used to study and measure the TTR tetramer stability against acid denaturation in serum using Western blot over a period of 72 hours. Also, TTR competitive binding assay (at 10 μM concentration) and dose response studies (between 0.2 – 50 μM concentration ranges) were performed for these compounds by fluorescence spectroscopy. The best compound from aforementioned studies was used to study and monitor the change in TTR tetramer size and volume in PBS (pH 4.0) using dynamic light scattering (DLS) over a period of 72 hours. Tafamidis, a drug currently in clinical trials for treatment of TTR amyloid diseases, was studied concurrently for comparison purposes.

RESULTS: All the compounds were successfully synthesized, identified and characterized using NMR and HRMS. The western blot analysis revealed that compounds few of our analogs stabilized the TTR tetramer complex better than Tafamidis upon acid denaturation over a period of 72 hours. Two analogs were at least 1.5 – 2 times better than Tafamidis. The TTR competitive binding assay shows that these two analogs have higher binding affinities than Tafamidis. Interestingly, one of our new analog (MMA1) has at least 22 times higher binding affinity in comparison to Tafamidis. Finally, the DLS study shows that MMA1 stabilizes the TTR protein over a period of 72 hours without significant changes in the protein size or volume. However, similar effect was not observed in the Tafamidis treated sample after 24 hours.

CONCLUSION: Studies reveal that MMA1 appears to be a better stabilizing agent in comparison to Tafamidis and may be a potential drug candidate against TTR amyloid diseases.

This work was supported by a New Investigator Award (M. Alhamadsheh) from the American Association of Colleges of Pharmacy (AACP).
EVALUATION OF 5-HOUR ENERGY® DRINK ON THE BLOOD PRESSURE AND ELECTROCARDIOGRAPHIC PARAMETERS ON YOUNG HEALTHY VOLUNTEERS: A RANDOMIZED, DOUBLE BLIND, CROSSOVER, PLACEBO-CONTROLLED TRIAL

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OBJECTIVES: To assess the effects of a popular energy supplement beverage, 5-Hour Energy®, on systolic and diastolic blood pressure (BP) and electrocardiographic (ECG) parameters, particularly QTc interval, on young healthy volunteers.

METHODS: This is a randomized, double blind, crossover, placebo-controlled Institutional Review Board approved clinical trial being conducted at David Grant USAF Medical Center. Volunteers are accepted into the study if meeting the following inclusion criteria: healthy, active-duty military between the ages of 18-40 years and willing to abstain from caffeine-containing products during the duration of the trial. Participants are excluded if they are pregnant, taking supplements or over the counter medications, or if their baseline ECG confirmed a QTc interval greater than 440 milliseconds. The study is separated into two 1-week Phases separated by a 7 day washout period to ensure elimination of the 5-Hour Energy constituents from the body. The study timeline is as follows: Phase 1, days 1-7 followed by a washout period during days 8-14 followed by Phase 2, days 15-21. The subjects either receive placebo or active form of the drink to be taken twice a day during Phase 1 and crossed-over to the opposite drink during Phase 2. All subjects have their BP and ECG readings taken by the researcher at baseline, then 1 hour, 3 hours, and 5 hours after drinking the 5-Hour Energy® or placebo on day one and again after 7 days of the study drink. Maximum values are also evaluated to assess any effects independent of time. A student's t-test is utilized for analysis of continuous data.

RESULTS: Eleven subjects have enrolled in the study. Two withdrew due to gastrointestinal side effects and scheduling conflicts. Analysis of the remaining 9 subjects showed that systolic BP increased at the 1 hour time (3±9 mmHg) and for maximum (4±9 mmHg) readings (p-values 0.09 and 0.05, respectively). Diastolic BP increased by 2±8 mmHg at the 1 hour mark (p-value 0.09) and at the maximum reading (p-value 0.06). QTc and heart rate readings did not show statistically significant differences between placebo and active drink at the 1 hour, 3 hour, 5 hour, or maximum values.

CONCLUSION: Our preliminary findings of the 5-Hour Energy® trial display trends towards statistically significant increases in systolic and diastolic BPs at the 1 hour and maximum reading. More subjects are needed to determine if our preliminary findings will be sustained. These results could have important policy implications for the energy drink market.

This study was funded by start-up funds from the department of Pharmacy Practice and an internal grant from David Grant Medical Center.
UCSF
INVITED
PRESENTATIONS
BIOCHEMICAL CHANGES AND STRUCTURAL RESPONSES IN A PERIODONTITIS MODEL

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OBJECTIVE: Spatiotemporal response of a functional bone-PDL-cementum complex to periodontal disease was evaluated by identifying biophysical and biochemical changes in the tension and compression sides of the complex.

METHODS: 4/0 silk ligatures were used to induce periodontitis in 6-week-old male Sprague-Dawley rats for 4 and 15 days; corresponding control rats were flossed (n=3 for each group). Diseased and control maxillae were evaluated using: (1) micro-XCT for PDL-width and alveolar crest recession; (2) histology sections for picrosirius red (PSR), tartrate acid resistant phosphatase (TRAP), and immunofluorescent (IF) labeling of fibronectin (FN), receptor activator of NF-κB ligand (RANKL), and osteopontin (OPN).

RESULTS: In the innately prestrained bone-tooth complex (tension in the mesial complex and compression in the distal complex), increased bone recession irrespective of tension or compression sides was observed. Bone recession was complemented with the increased presence of TRAP positive cells in the diseased complex. Additionally, increased RANKL and TRAP positive cells in the compression-distal complex compared to the tension-mesial complex was observed in diseased specimens. In both controls and diseased, FN expression was identified in secondary cementum. However, in the diseased specimens it was identified on the periphery of cementum resorption pits specifically on the compression side. Higher levels of OPN were identified in control specimens compared to the diseased. Within groups, higher level of OPN was observed in between stratified layers of bone on the tension side of the complex compared to its presence only within the endosteal spaces and around resorption pits on the compression side.

CONCLUSION: No significant disease related trends were observed between earlier (4D) and later (15D) time points. However, altered biochemical expressions of RANKL, FN and OPN could indicate that disease related effects are more distinct on the compression-distal side of the diseased complex.
THE MORPHOLOGICAL AND CELLULAR BASIS OF HYPOXIA INDUCED CRANIOFACIAL MALFORMATIONS

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OBJECTIVE: Craniofacial malformations, ranging from simple cleft lip and palate to complex syndromes including holoprosencephaly, can be caused by genetic and/or environmental factors. One particular environmental factor of interest is prenatal hypoxia. Recent clinical and experimental evidence correlates hypoxia to craniofacial anomalies. However the mechanisms whereby hypoxia causes these defects are not yet understood. The objective of this work was to examine the morphological and cellular changes underlying hypoxia induced craniofacial defects in chick embryos.

METHODS: Chicken eggs were incubated in 9%, 11%, or 13% O2 (hypoxia) or in 21% O2 (normoxic control). Embryos were collected on days 2 through 6 for morphological, cellular behavior and metabolic analyses. Embryos were fixed overnight in 4% paraformaldehyde and photographed. For analysis of craniofacial skeletal development, embryos were collected at day 13, cleared, and stained in alcian blue and alizarin red for cartilage and bone, respectively. Geometric morphometric analyses were performed on images of embryos to quantitate facial shape variation. TUNEL and caspase 3 assays (for apoptosis) and BrdU staining (for cell proliferation) were performed. Immunohistochemical staining for AMP-activated protein kinase (AMPK) was performed for analysis of metabolic stress.

RESULTS: Hypoxia reduced the survival rate of avian embryos and led to developmental delay. Severe malformations included exencephaly, anencephaly, microcephaly, facial anomalies, and absence of the upper jaw anlage. Milder malformations included cephalic asymmetry, anophthalmia, and microphthalmia. Hypoxia led to delayed craniofacial skeletal development, and a number of late-stage hypoxic embryos displayed craniofacial skeletal defects. Hypoxic embryos exhibited abnormal facial shape variation in relation to centroid size and age among individuals in hypoxic groups and between the hypoxic and normoxic groups. Cell proliferation was disrupted in hypoxic embryos. A cohort of early stage hypoxic embryos showed apoptosis of neural crest progenitor cells. Hypoxic embryos also exhibited increased expression of AMPK in sections.

CONCLUSIONS: Hypoxia leads to high mortality, developmental delay, and craniofacial and cephalic malformations in chick embryos. Hypoxia also disrupts cell proliferation and leads to early stage neuroepithelial apoptosis, and may induce cellular oxidative stress. The reduced proliferation, apoptosis, and oxidative stress response primarily occurred in the neuroepithelium, indicating that the brain is most vulnerable to hypoxic stress and that the malformations are a result of neuroepithelial apoptosis and metabolic stress.

This work has been supported by NIDCR grant 1F31DE021964-01.
DEATH NOTE AGAINST ORAL SQUAMOUS CANCER CELLS

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BACKGROUND: Oral squamous cell carcinoma (OSCC) is a well-known malignancy that accounts for more than 90% of all oral cancers. 5-years survival rates for OSCC decrease with delayed diagnosis. So far, there is no sensitive and specific clinical screening method for OSCC. We therefore investigate a less invasive but more specific and sensitive method for screening OSCC.

OBJECTIVE: Our goal is to find ligands that will specifically bind to OSCC cells but not to normal healthy oral cells for clinical screening and subsequent treatment.

METHOD: In this study, we employed “one bead one compound” combinatorial library (OBOC) strategy to identify specific ligands for OSCC. Briefly, 24 random libraries were screened with OSCC derived from lateral side of tongue for 1.5 h to identify library with possible OSCC binding ability. One library named X1 with strong, and fast OSCC binding property was chosen for further study. The selected library has a permutation of 24.3 million compounds. Live OSCC were co-cultured with X1 OBOC libraries for 1.5 hrs. These OSCC cell binding beads were isolated and co-cultured with normal keratinocytes derived from skin. Compound beads that showed strong binding to OSCC but not to normal keratinocytes were identified. The chemical structures of the compounds were determined using Edmund Chemistry.

RESULTS: A final of 12 beads with strong OSCC binding, but no keratinocyte binding ability were identified and a motif was observed.

CONCLUSION: Current work is underway to evaluate these ligands’ cytotoxicity, and possible conjugation to imaging agents for visual inspection of OSCC in vitro. In the future, these OSCC cell binding ligands will be reserved and generated on “one bead two compound” libraries to induce apoptosis of OSCC.
EPITHELIUM-DRIVEN G PROTEIN SIGNALING REGULATES THE MINERALIZATION OF MANDIBLES

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OBJECTIVES: G protein-coupled receptors (GPCRs) regulate many cellular activities related to development and differentiation. However, little is known about the influences of GPCRs on epithelial-mesenchymal interactions required for normal tooth and jaw development. Many GPCRs signal through Gs proteins, which activate the production of second messenger cAMP. Our objective is to determine the effects of increasing Gs signals in epithelia on the formation of mesenchymal and epithelial tissues in murine mandibles.

METHODS: We used an engineered Gs-coupled GPCR known as receptor activated solely by synthetic ligands (RASSL) Rsl to investigate Gs protein activity in vivo with minimal interference from endogenous ligands. Double transgenic mice expressing Rsl in epithelial cells were created by mating K5-tTA mice with TetO-Rsl mice. Mandibles were harvested six weeks after birth, scanned using Micro X-ray Computed Tomography (MicroXCT), and examined histologically. MicroXCT scans were analyzed for relative differences in mineral content using semi-automated algorithms to quantify volumetric pixel intensities of bone, dentin, and enamel.

RESULTS: MicroXCT analyses of 6-week-old transgenic mice showed a significant decrease (p<0.05) in relative mineral content of the enamel of transgenic mice as compared to wild-type mice, and histological analyses showed abnormal ameloblast morphology, suggesting an effect on differentiation of epithelial lineage cells. Surprisingly, the mineral content of cortical bone and radicular dentin (p=0.019 and p=0.048 respectively) were significantly increased compared to wild-type mice.

CONCLUSIONS: Increased Gs signaling in oral epithelia altered ameloblast differentiation resulting in a hypomineralized enamel defect. Increased Gs signaling in oral epithelia also altered the formation of mesenchymally-derived alvelolar bone and dentin suggesting a role for GPCRs in mediating epithelial-mesenchymal interactions during tooth and bone development. Further analyses are underway for specific cellular and molecular changes of epithelial and mesenchymal lineage cells in mandibles.

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Introduction

Uncontrolled and persistent angiogenesis is the hallmark of several diseases including cancer, autoimmune diseases, age related macular degeneration and atherosclerosis. In vitro models for angiogenesis are valuable for isolating physiological mechanisms or for evaluating responses to drugs with a view to therapeutic intervention in systems that have been subverted by disease. Extracellular vesicles including exosomes (30-120 nm dia.) and larger microvesicles (100-2000 nm dia.) are known to carry disease-specific soluble mediators or bear disease-specific markers. Protocols exist for isolation and study of microvesicles in body fluids but investigation of microvesicles or exosomes in tissue matrix or in vitro models of tissue models are not well described. Here we present the Responsive Angiogenic Implanted Network (RAIN)-Droplet model, a planar in vitro 3-D matrix model of angiogenesis that allows live observation of microvesicle and cell interaction as well as easy fixation and staining of cells and released vesicles.

Methods and materials

Human dermal microvascular endothelial cells (HMECs) (Lonza) were maintained in EGM-2-MV medium (Lonza). The RAIN-Droplet was created and embedded in collagen as described in Figure 1. Vascular Endothelial Growth Factor (VEGF) was added to the top collagen layer prior to gelation. Endothelial cells were allowed to form sprouts and to migrate out from the droplet releasing extracellular vesicles throughout which were observed by a variety of methods as described in figure legends.

Results

Figure 3. Secreted extracellular vesicles localised to cells invading into a 3-D collagen matrix. Cellular and vesicular actin stained green with AlexaFlour 488-Phalloidin (Invitrogen), nuclei stained blue with DAPI (confocal microscopy, 200x objective). Actin-bearing vesicles are absent where invading cells are absent (white arrow) but are abundant around the invading cells.

Figure 4. Time lapse of "questing" cell interaction with extracellular vesicle (Differential Interference Contrast, 200x mag). The donor cell (black asterisk in T1) has deposited a vesicle (black arrow) in the extracellular matrix. The questing cell (white asterisk in T1) sends out pseudopodia, one of which clearly detects the shed vesicle. Over the following 12 sequential images (T1-T12) the questing cell makes contact with, attaches to, then appears to absorb the vesicle (white marker in T12).

Figure 5. Filopodia of capillary sprout tip-cell (white arrow) stretching towards deposited microvesicles (black arrow) (Nikon Advanced Modulation Contrast, 400x). Tip cells respond strongly to paracrine release of chemokines which may also be found within microvesicles. Microvesicles deposited in extracellular matrix by endothelial cells, or potentially invading cancer cells for example, may act as "sign-posts to direct angiogenic sprouting."

Conclusions

- RAIN-Droplet model is a flexible platform for analysis of cell-derived microvesicles from angiogenically activated endothelial cells
- Interaction of cells with larger unlabelled microvesicles (~800-1000 nm) may be observed using methods such as Differential Interference Contrast microscopy
- The RAIN-Droplet model lends itself to various fluorescent labeling techniques for analysis of potentially far smaller microvesicles
- Active seeking of cells for tissue bound microvesicles suggests a "quasi-paracrine" role for them in cellular invasion of tissue

Reference