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THE EFFECTS OF THE ND:YAG LASER ON IN VITRO FIBROBLAST ATTACHMENT TO ENDOTOXIN TREATED ROOT SURFACES

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MASTER OF SCIENCE, ORAL BIOLOGY

by

DAVID J. TRYLOVICH

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THE EFFECTS OF THE ND:YAG LASER ON IN VITRO FIBROBLAST ATTACHMENT TO ENDOTOXIN TREATED ROOT SURFACES

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University of Missouri-Kansas City, May, 1991

ABSTRACT

The purpose of this study was to evaluate the effects of the Nd:YAG laser on in vitro fibroblast attachment to endotoxin treated root surfaces and to describe any root surface alterations resulting from the use of the laser.

Thirty 4 mm x 4 mm cementum segments were obtained from unerupted third molars. Treatment groups were assigned as follows: 1) non-lased, non-diseased; 2) non-lased, diseased; and 3) lased, diseased. Each group contained ten root segments. The roots were considered diseased after soaking them in endotoxin (E. coli 055:B5 lipopolysaccharide [556 EU/ml]) for 72 hours. The lased, diseased root segments were treated with a neodymium: yttrium, aluminum, garnet (Nd:YAG) laser using a 320 micron contact optic fiber handpiece with an energy setting of 80 mJ at 10 pulses per second for one minute. The fiber was held perpendicular to the root surface. An attempt was made to cover the entire root surface equally during the one minute of exposure. The root segments were placed in fibroblast culture dishes (2.5×10^5 cells/ml)

for 40 hours and then prepared for SEM observation.

Attached fibroblasts were observed to be either round or flat in appearance. Round fibroblasts represented unhealthy fibroblasts due to the absence of well-developed lamellopoda. A statistically significant difference (ANOVA) was found in the number of round ($p < 0.001$) and flat ($p < 0.001$) fibroblasts among the various treatment groups. The Tukey studentized range method revealed a significant decrease ($p < 0.01$) in the number of flat fibroblasts in the lased, diseased versus the non-lased, non-diseased and non-lased, diseased. There was also a significantly increased ($p < 0.01$) number of round cells in the non-lased, diseased group as compared to the non-lased, non-diseased and lased, diseased root segments.

The lased root segments exhibited surface alterations which included charring, crater formation, cementum meltdown, and tracking. The organic matrix appeared to have been burnt off leaving behind a recrystallized substance with a lava-like appearance. It appeared that the laser altered the biocompatibility of the cementum producing a surface unfavorable for fibroblast attachment. This was confirmed in a subsequent pilot study using lased, non-diseased root segments.

A pilot study using photoacoustic spectroscopy on a

laser charred root surface revealed the presence of a charged ion of ammonium and an altered phosphate to carbonate ratio as compared to a non-lased root segment. The presence of this charged ion of ammonium may have played a role in inhibiting fibroblast attachment.

This abstract of about 260 words is approved as to form and content.

Charles M. Cobb, D.D.S., M.S., Ph.D.
Professor in charge of thesis

The undersigned, appointed by the Dean of the School of Graduate Studies, have examined a thesis entitled

THE EFFECTS OF THE ND:YAG LASER ON IN VITRO FIBROBLAST ATTACHMENT TO ENDOTOXIN TREATED ROOT SURFACES

presented by **DAVID JOSEPH TRYLOVICH**, a candidate for the degree **MASTER OF SCIENCE, ORAL BIOLOGY** and hereby certify that in their opinion it is worthy of acceptance.

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DEDICATION

This thesis is dedicated to:

My parents, Daniel and Bessie, for their constant love and support. Their educational guidance throughout my life has allowed me to reach this zenith in my career;

My wife, Monica, for her unending love, support, compromise, and understanding. I only hope to be as successful a periodontist as she is a wife and mother.

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INTRODUCTION

Gingivitis and periodontitis are inflammatory diseases of bacterial origin. Control of the inflammation is dependent upon the elimination of periodontopathic bacteria and their metabolic by-products (Waerhaug, 1978).

The cementum of roots exposed to a plaque infected periodontal pocket undergoes multiple alterations rendering them biologically unacceptable to fibroblasts and other cells. These changes include the adsorption of endotoxin, antigen-antibody complexes and products of microbial metabolism as well as changes in mineralization and hardness (Selvig, 1966; Ruben and Shapiro, 1978).

The classic treatment for periodontally diseased root surfaces continues to be scaling and root planing using hand instruments, ultrasonics, or a combination of both. The removal of root surface contaminants allows for the elimination of inflammation and reattachment of the adjacent tissue (Knowles, Burgett, Nissle, Shick, Morrison and Ramfjord, 1979).

Lasers may have the capability to treat the diseased root surface as either an alternate to or as an adjunctive therapy with hand and ultrasonic instrumentation. However, research must first determine that the laser causes no harm to the pulpal or hard tissues and that the therapeutic result is comparable to or better than that achieved using classical instrumentation.

Root Surface Contaminants

Endotoxin is the lipopolysaccharide (LPS) component of the cell membrane of gram-negative bacteria. When liberated in the host it is a multiply biologically active molecule. Hatfield and Baumhammers (1971) were the first to suggest an association between endotoxin and cementum that was characterized by cytotoxicity in vitro. Other investigators have confirmed the in vitro cytotoxicity of LPS (Aleo, DeRenzis, Farber and Varboncoeur, 1974; Aleo, DeRenzis and Farber, 1975). In addition, enhancement of other undesirable immune effects have been attributed to endotoxin (Morrison and Ryan, 1979). These effects include the stimulation of macrophages to release active inflammatory substances and hydrolytic enzymes, vasodilation with increased inflammatory cell infiltrate, LPS induced osteoclastic bone resorption, and activation of the classic and alternate pathways of the complement system (Daly, Seymour and Kieser, 1980).

The adsorption of endotoxin to cementum appears to be a surface phenomenon with little or no actual penetration or chemical binding (Daly, et al. 1980). Nakib, Bissada, Simmelink and Goldstine (1982) soaked root surfaces in various concentrations of endotoxins for differing time periods. They showed that a similar pattern and intensity

of fluorescence was seen regardless of the incubation time or endotoxin concentration. Brushing for one minute with a soft toothbrush removed most of the adsorbed endotoxin, indicating a weak binding of the endotoxin to the root surface. They suggested that excessive root planing with the intention of removing endotoxin-containing cementum was not justified. Moore, Wilson and Kieser (1986) showed that 39% of the endotoxin could be removed by gently rinsing in water and that an additional 60% was removed using a slowly rotating bristle brush for one minute.

Antibody is also localized on the superficial surface of a tooth root exposed to a periodontal pocket (Bravman, Everhart and Stahl, 1979; Everhart, Dahab, Wolff and Stahl, 1982). The antibody is responding to unidentified antigens (possibly endotoxin which is known to act as an antigen). Antibody binding on the surface of the root results in antigen-antibody immune complexes which are capable of fixing complement. Complement fixation by the antigen-antibody complexes results in the activation (classic pathway) of the complement cascade with the generation of biologically active protein fragments. These active compounds exhibit multiple local inflammatory effects including chemotaxis, vascular changes, cytotoxicity, enhancing and amplifying effects and other potent sequelae (Attstrom and Lindhe, 1983). Since there is no penetration into the cemental structure, treatments

capable of mildly altering the root surface may be adequate to leave the root surface free of antigen-antibody complexes.

Nyman, Westfelt, Sarhed and Karring (1988) examined if removal of "diseased" cementum is required in the treatment of periodontal disease. A split-mouth design was used in eleven patients with moderate to advanced periodontitis. Full-thickness flaps were reflected and quadrants were either scaled and root planed (control), in order to remove all the cementum, or polished (experimental) with rubber cups, interdental rubber tips and polishing paste. Polished teeth had calculus removed with a curette with special precaution to avoid cementum removal. Maintenance was provided every 2 weeks for the first 3 months and then every 3 months until 24 months after treatment. Results showed that both treatments provided for the same degree of improvement of periodontal health.

The reattachment of the gingival tissues to a previously diseased root surface is the goal of regenerative periodontal therapy. For this to be accomplished the root surface must be rendered biologically compatible to the adjacent tissue. It is up to the clinician's interpretation of the research literature to decide upon whether periodontally involved teeth require conservative or aggresive treatment of the

"diseased" cementum.

Removal of Root Surface Contaminants

Hand and ultrasonic instrumentation represent the classic means to render the root surface biocompatible with the adjacent tissue (O'Leary, 1986). Aleo, et al. (1975) designed an in vitro model utilizing human gingival fibroblast cultures to determine root surface biocompatibility. Periodontally diseased teeth were either root planed, treated with hot phenol, or left as untreated controls. They were subsequently placed in fibroblast culture wells for 48 hours. The hot phenol endotoxin extraction technique has been used in laboratories to remove and quantitatively evaluate endotoxin content (Westphal and Jann, 1965). The results revealed that fibroblasts attached to the root planed and hot phenol treated teeth but not to the untreated diseased teeth. They concluded that to obtain clinical success the toxic materials must be completely removed from the diseased cementum or that the cementum itself must be removed.

Aleo, et al. (1975) described fibroblast attachment to root surfaces. Fibroblasts attached to non-periodontally diseased root surfaces were flat with well-defined points of attachment and numerous lamellopoda. Periodontally diseased root surfaces contained few attached fibroblasts. The few present were raised from

the root surface (round) and possessed few attachment processes.

Jones and O'Leary (1978) showed that scaling and root planing rendered periodontally diseased teeth practically free of endotoxin. Detectable levels of endotoxin from root planed teeth were comparable to unerupted third molar controls. Nishimine and O'Leary, (1979) compared scaling and root planing to ultrasonics for the removal of endotoxin from periodontally diseased teeth. Twenty to twenty-five teeth from each sample were pooled for the analysis of endotoxin. The average net endotoxin, using the Limulus lysate assay, showed the unerupted third molar controls (1.46 ng/ml) to be similar to the scaled and root planed teeth (2.09 ng/ml). In comparison, the ultrasonic teeth had 16.8 ng/ml versus 169.5 ng/ml for the diseased controls.

Thornton and Garnick (1982) and Hunter, O'Leary and Kafrawy (1984) demonstrated the ability of hand instruments and ultrasonics to remove root surface calculus equally. Breininger, O'Leary and Blumenshine (1987) stated that hand instruments and ultrasonics were remarkably effective in the bacterial debridement of subgingival root surfaces. Gellin, Miller, Javed, Engler and Mishkin (1986) compared the Titan-S sonic scaler with curettes for calculus removal and found no consistent difference between the two methods.

Use of Lasers in Dentistry

The word laser is an acronym for "Light Amplification by Stimulated Emission of Radiation" (Peck and Peck, 1967). Maiman (1960) reported the first operational laser in July, 1960. Since its discovery, uses for lasers have expanded to include applications in many fields, including medicine and dentistry.

The laser has been advocated to have potential in practically all fields of dentistry (Myers, 1991). The current potential capabilities in dentistry include: vaporization of caries; dentin removal; sterilization of tooth surfaces; etching of enamel; removal of extrinsic stains; preparation of pits and fissures for sealants; soft tissue procedures such as gingivectomies and frenectomies; and gingival curettage (Dunlap, 1988). Due to the laser's ability to sterilize, vaporize and ablate, it has been advocated for treatment of periodontally diseased teeth by removing or altering root surface adsorbed endotoxin, calculus, plaque, and other surface contaminants (Myers, 1989; Myers, 1991).

There are several types of lasers available today including the ruby, Nd:YAG, (neodymium: yttrium, aluminum, and garnet), erbium (Er):YAG, argon, carbon dioxide, diode, excimer, helium-neon, and others. All of these have potential applications in dentistry. The most popular laser in dentistry today is the Nd:YAG.

Lasers work by taking an incoherent light source and converting it into monochromatic, coherent and collimated radiation in an optical resonator. An energy source (flashlamp) is reflected off a substance to generate photons. These photons are reflected off opposing mirrors until a collimated beam of light is produced (Frentzen and Koort, 1990). The laser is named according to the substance from which photons are generated. The Nd:YAG laser generates its photons from neodymium grown on a crystal rod of yttrium, aluminum and garnet (Myers, 1989).

Pulpal Effects of the Laser

A primary concern of most dentists is the laser's potentially damaging effects on the pulpal and hard tissues. Using animal models, several authors have examined the effects of both the ruby and carbon dioxide laser on pulpal tissue. Stern, Renger and Howell (1969) evaluated the effects of a Korad K-2 QP pulsed ruby laser on ten chimpanzee teeth *in vivo*. Laser energies ranged from 60 to 250 joules/cm^2. Minimal pulpal changes were observed. The authors felt that these changes would be reversible. Adrian, Bernier and Sprague (1971) used a ruby laser to determine the threshold pulpal response in dogs. Responses occurred between 1,880 and 2,330 joules/cm^2. Pulpal changes included hemorrhage, coagulation necrosis of the odontoblasts, edema, and some interstitial polymorphonuclear leukocytes. Melcer,

Chaumette, Melcer, Zeboulon, Hasson, Merard, Pinaudeau, Dejardin and Weill (1985) examined the effects of the carbon dioxide laser on 2 adult Macaca mulatta monkeys and 4 beagle dogs 1 month after lasing the pulpal walls of cavity preparations. The laser was capable of inducing a reactional dentinogenesis with an energy of 15 joules emitted in eight 0.6 second pulses of 3 watts. This appeared comparable to what is expected after a slight inflammatory process.

The use of pulsed lasers with a short pulse duration significantly reduces the amount of heat conduction as compared to continuous wave lasers. The development of the pulsed Nd:YAG, Er:YAG, Excimer, and CO2 lasers has allowed for the ability to lase teeth with decreased thermal effects upon the pulp. Pulsed UV excimer lasers presently have the shortest wavelength (190-351 nm) and exhibit the least amount of thermal damage (Frentzen and Koort, 1990).

Hard Tissue Effects of the Laser

The thermal effects of the laser on hard tissues are determined by the energy absorbed. The laser's wavelength, pulse length, peak power, and power density are important determinants of its surface altering capacity. The energy absorbed is also affected by the chemical composition of the surface and its optical properties: reflectivity, opacity (or translucency), and

color (Peck and Peck, 1967).

Several authors have examined the effects of various lasers on enamel and dentin surfaces and have observed structural, compositional and phase changes. Lobene and Fine (1966) described the enamel surface as appearing glazed, opaque and hard after pulsed ruby laser radiation. However, the surface was brittle and fusion of enamel was not accomplished. Craters were produced at the point of laser impact. Peck and Peck (1967) also used a pulsed ruby laser and observed cratering of the enamel (0.1 to 1.1 mm) and dentin (less that 0.1 mm). Higher energies resulted in deeper craters. Observation by polarized light suggested the presence of laser-induced crystallographic changes in the enamel bordering the craters. This suggests the possible alteration of the orientation or composition of the hydroxyapatite crystals. The laser produced three distinct zones of dentinal destruction: 1) a completely destroyed central zone; 2) a surrounding zone of partial destruction; and 3) an outlying scattered zone of dark speckling (possible areas of irregular dentinal mineralization or interglobular dentin). Vahl (1968) examined the effect of the ruby laser on carious and sound dentin. Scanning electron microscope (SEM) photos showed crater formation with drops of melted dental hard tissue apparently sprayed from the crater. Melted drops, occasionally, had the appearance of

burst shells. The author felt that the dentin was melted by the laser bombardment and that the melted substance was ejected by the pressure of the evaporated material.

Scheinin and Kantola (1969) used a CO^2 laser on enamel and, depending upon the energy used, observed effects ranging from minimal surface alterations to deep craters penetrating into dentin. The borders around the craters were elevated and were felt to represent condensed material from melted and vaporized dental enamel. Fracturing of the enamel was seen and thought to be due to the rapid expansion of water and/or enamel fluid as a result of the high energy laser beam. The organic component of the enamel was combusted to varying extents with carbonized residue being observed at the periphery of the craters. The crater surface itself showed heavy carbonization. Lobene, Bhussry and Fine (1968) using a CO^2 laser observed incineration of enamel interrod substance and suggestive incineration of odontoblastic processes.

Keller and Hibst (1990) and Hibst and Keller (1989) used an Er:YAG laser on dentin and enamel surfaces of human teeth. They hypothesized that vaporization of water in dentin produces an increase in internal pressure and a subsequent microexplosion. They observed deeper crater depths in dentin versus enamel for the same energy level, as did Hame, Voss, Johnson, Papoiannou and Grundfest

(1988) and Hame, Voss, Papioannou, Grundfest and Johnson (1990) using an excimer laser on bovine maxillary incisors. They felt the increased water content of dentin as compared to enamel was responsible for the increased crater depth.

Myers (1990) used a Nd:YAG laser on enamel and dentin. The pulse width was in the 150 microsecond range. Enamel was lased at 10 pulses per second with an energy level of 80 millijoules (mJ). Scanning electron microscopy showed enamel and dentin cratering with resolidified spheres of hydroxyapatite. The carbonized layer formed from lasing organic and inorganic material could easily be removed with a spoon excavator. Enamel cratering occurred to a depth of between 14 and 40 microns. Dentin cratering was seen with each pulse creating a crater of 40-60 microns. No subsurface cracking or fissuring was observed. This indicated the absence of thermal damage. A change in the dentinal tubules could be seen beneath the carbonized layer for approximately 40 microns. The tubular structure was apparently normal below this layer. Hess (1990) examined the morphologic changes of enamel after Nd:YAG laser exposure. SEM observation revealed a pockmarked surface with indistinct crater formation when 30 mJ of energy was used. Increasing to 75 mJ resulted in distinct cratering 230-250 um in diameter. Mounding of solidified enamel was

observed at the periphery of the craters. The residual surface had a lava-like appearance.

Human tooth enamel melts when heated above 1,280°C (Corcia and Moody, 1974). The laser is capable of causing surface temperatures of greater than 1400°C; thus, compositional, structural and phase changes are to be expected (Fowler and Kuroda, 1986). Kantola (1972) and Kantola, Laine and Tarna (1973) lased enamel with a CO2 laser and observed an increased calcium and phosphorus content and the formation of alpha calcium orthophosphate. They observed that the laser burned off the organic matrix resulting in recrystallization of inorganic material. Newesely (1977) showed that at temperatures above 1,450°C thermal decomposition of hydroxyapatite resulted in the formation of alpha-tricalcium phosphate and tetracalcium phosphate. Kuroda and Fowler (1984) lased human enamel with a CO^2 laser and observed enamel melting with crater formation. The solidified melt was composed of minor phases of alpha-tricalcium phosphate, tetracalcium phosphate and a major phase of modified apatite. Keller and Hibst (1990), using x-ray diffraction observed an increase in carbonate-containing apatite after Er:YAG lasing of the (dentin or enamel) surface.

Effect of the Laser on Plaque Formation

Iwase, Saito, Nara and Morioka (1989) examined the effect of the Helium-Neon gas laser on the growth of

dental plaque in hamsters. The right mandibular molars were lased for 2 minutes per day, 5 days per week for 4 weeks. The laser produced an inhibitory effect on dental plaque formation in 19 out of 20 cases. The authors were unclear as to the mechanism of plaque inhibition, but suggested that the laser beam may affect the viability of bacterial cells.

Summary

Use of the Nd:YAG laser on contaminated root surfaces may have a role during nonsurgical or surgical periodontal procedures. Removal of many of the surface alterations occurring in the cementum of a periodontally diseased tooth root may be within the capabilities of the laser's ability to vaporize and ablate. Thus, use of the instrument, particularly in areas of difficult access, may ensure thoroughness of root decontamination.

If the laser is capable of destroying the bacteria and removing the endotoxin present on diseased root surfaces, then reattachment of previously diseased gingival tissue to the treated, biocompatible root surface may be enhanced.

Problem Statement

The purpose of this study was to evaluate the effects of the Nd:YAG laser on *in vitro* fibroblast attachment to endotoxin treated root surfaces and to describe any root surface alterations resulting from the use of the laser.

MATERIALS AND METHODS

Sample Collection

Thirty unerupted third molars were collected from the offices of local oral surgeons. A siliconized bottle filled with 50 ml of triple distilled water and 1 ml of a solution containing 10,000 units penicillin, 10 mg streptomycin, and 25 ug amphotericin B per ml in 0.9% sodium chloride was provided for specimen collection. Thirty root segment squares, approximately 4 mm by 4 mm, were cut from the root surfaces of the collected teeth. The segments were randomly assigned to one of three groups: 1) control: non-lased, non-diseased; 2) non-lased, diseased; and 3) lased, diseased. The root segments were coded on the pulpal side using a 1/4 round bur to indicate their treatment group.

Endotoxin Preparation

A commercially available endotoxin[1] (Escherichia coli 055:B5 lipopolysaccharide) was prepared by adding 16,700 endotoxin units (EU) to 30 ml of endotoxin free water. The solution was vortex mixed for 5 minutes as directed. This yielded a concentration of approximately 556 EU/ml.

Sample Preparation

Twenty-five of the root segments were mounted in baseplate wax so that only the cemental surfaces were

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[1]Endotoxin Standard, 210-SE, Sigma Chemical Company, St. Louis, MO.

exposed. Five of these were control teeth which were mounted to determine if the wax mounting process had any detrimental effects on fibroblast attachment. The remaining 5 controls were not mounted in wax. All of the root segments were subsequently exposed to an abrasive sodium bicarbonate aerosol spray (Prophy-jet[1]) for 10 seconds followed by a 10 second water rinse. The aerosol spray ensured the removal of any residual root surface contaminants. Twenty teeth assigned to the diseased group were placed in 2 Petri dishes each containing 10 ml of E. coli endotoxin solution. Ten teeth in the non-diseased group were placed in a similar Petri dish with 10 ml of the specimen collection solution. Specimens were allowed to remain in the Petri dishes for approximately 72 hours. All root segments were then removed from their respective solutions and allowed to air dry under a vacuum hood for approximately one hour. After air drying the baseplate wax was removed from all of the root segments.

Laser Treatment

Ten root segments comprising the experimental group were lased with an Nd:YAG laser[2] using a 320 micron contact optic fiber with an average energy reading of 80 mJ at 10 pulses per second for one minute each. The

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[1]Prophy-Jet 30, Dentsply International, York, PA.

[2]American Dental Laser Inc., Birmingham, MI.

average energy reading was obtained prior to lasing each root segment using a Model EM22 Energy Meter[1]. The fiber was held perpendicular to the root segment and moved in a back and forth motion in an attempt to expose the entire root segment equally.

Fibroblast Culture Preparation and Incubation

All 30 root segments were placed in 3 Petri dishes keeping the three treatment groups separate. Human gingival fibroblasts in the sixth passage were counted using a hemocytometer. Approximately 2.5×10^5 cells per milliliter were added to each of the dishes. The fibroblasts were incubated for 40 hours at 37°C, 100% humidity, with 5% CO_2. The cells were then gently agitated and placed in fixative. Root segments were prepared for SEM observation using a series of alcohol dilutions followed by hexamethydisilazane. The segments were then mounted on aluminum studs and sputter-coated with gold/palladium. A Phillips 515 SEM was used to examine the specimens. All photographs were taken at a magnification of 170x at 15 Kv.

Scanning Electron Microscopic Observation

Specimens were coded to ensure that the examiner (CMC) operating the scanning electron microscope and counting the fibroblasts was blind as to the treatment

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[1]Sunrise Technologies, Inc., Fremont, CA.

group of the segment. During SEM observation, an imaginary diagonal line was drawn from the upper left corner through the center to the lower right corner of the root segments. One photomicrograph was then randomly taken in each of the three areas (upper left, center, and lower right). This methodology aided in consistency of sampling areas between root segments.

Data Collection and Analysis

The number of attached flat fibroblasts and the number of attached round fibroblasts observed in each SEM micrograph was recorded separately. When specimens showed proliferative attachment of fibroblasts, resulting in a confluent monolayer, the fibroblasts were counted in a small subset area and calculated for the entire area of the micrograph. A maximum number was set at 80 fibroblasts. The micrographs were re-examined in ten days when the fibroblasts were re-counted. Intraexaminer correlation was determined. The data were analyzed using a one-factor analysis of variance (ANOVA) test for both round and flat fibroblasts and the Tukey studentized range method.

RESULTS

Intraexaminer reliability between the first and second counting exhibited a very high correlation ($r = 0.99$).

Attached fibroblasts were observed to be either round (Fig. 1, 2) or flat (Fig. 3) in appearance. Lamellopoda of round fibroblasts were either spindly or absent; this type of attachment was considered to be unhealthy. Flat fibroblasts exhibited well-developed lamellopoda which represented a healthy attachment to the cementum. The fibroblasts occasionally coalesced to form a confluent monolayer (Fig. 4). There was a low correlation ($r = 0.30$) between the number of round and flat cells among the thirty specimens; this allowed for them to be examined as separate dependent variables.

The non-lased, non-diseased root segments were originally separated into five wax-mounted and five non-wax-mounted root segments. This was done to determine if the wax-mounting process had any effect upon the attachment of fibroblasts to the cementum. T-tests revealed that there was no significant difference ($p < 0.80$) in the number of round and flat fibroblasts between the two groups. The results from the ten segments were then combined to represent the non-lased, non-diseased group.

As shown in Table 1 and Table 2, the number of

fibroblasts attached to the root segments in the various treatment groups was found to be significantly different (ANOVA) for both round ($p < 0.001$) and flat ($p < 0.001$) cells. Further analysis using the Tukey studentized range method (Table 3) revealed a significantly increased ($p < 0.01$) number of round cells in the non-lased, diseased group as compared to the non-lased, non-diseased and lased, diseased. There was also a significant decrease ($p < 0.01$) in the number of flat fibroblasts in the lased, diseased versus the non-lased, non-diseased and non-lased, diseased.

SEM analysis of the non-lased, non-diseased group revealed a mean of 33 flat and 3 round fibroblasts per examined field. The fibroblasts characteristically appeared healthy as determined by their flat appearance with well-developed lamellopoda.

Non-lased, diseased root segments contained a mean of 29 flat and 7 round fibroblasts. The difference in flat fibroblasts between the non-lased, non-diseased and non-lased, diseased was not statistically significant.

Lased, diseased root segments were observed to contain an average of 2 round and 0 flat fibroblasts. Flat fibroblasts were observed on only one lased specimen despite the presence of root surface alterations (Fig. 5). Fibroblast attachment was minimal regardless of the degree of alteration.

Scanning electron microscopy of the lased root segments showed various degrees of cementum surface alterations (Fig. 6, 7). These alterations included charring, crater formation (Fig. 8, 9), cementum meltdown with subsequent recrystallization of inorganic material (Fig. 10), and tracking (Fig. 11).

TABLE 1

ONE-WAY ANALYSIS OF VARIANCE OF ROUND FIBROBLASTS PER MICROGRAPH

Source	df	Sum of Squares	Mean Square	F Value	Tail Probability
Treatment	2	391.47	195.73	7.65	0.0009
Error	87	2226.93	25.60		

TABLE 2

ONE-WAY ANALYSIS OF VARIANCE OF FLAT FIBROBLASTS PER MICROGRAPH

Source	df	Sum of Squares	Mean Square	F Value	Tail Probability
Treatment	2	18775.49	9387.74	17.79	0.0000
Error	87	45914.73	527.76		

TABLE 3

MEAN (STANDARD DEVIATION) OF
ATTACHED FIBROBLASTS BY TREATMENT

TREATMENT	ROUND	FLAT
NON-LASED, NON-DISEASED	2.67 (3.36) *	32.50 (25.28) *
NON-LASED, DISEASED	6.80 (7.88) *	28.93 (30.70) *
LASED, DISEASED	2.13 (1.87)	0.23 (1.28)

* TUKEY STUDENTIZED RANGE METHOD ($p < 0.01$).

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Fig. 1. Round fibroblasts (typical). Round fibroblasts were considered unhealthy due to the lack of well-developed lamellopoda. SEM original magnification of 3540x.

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Fig. 2. Round fibroblast with damaged cellular membrane (arrow). SEM original magnification of 326x. High power magnification of fibroblast is approximately 1863x.

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Fig. 3. Flat fibroblasts (typical). Notice conformation to root surface with well-developed lamellopoda. SEM original magnification of 2500x.

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Fig. 4. Confluent monolayer of fibroblasts. Fibroblasts almost completely obscure the root surface. SEM original magnification of 170x.

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Fig. 5. Lased specimen with flat fibroblasts. Flat fibroblasts (arrows) are present despite obvious tracking and crater formation. SEM original magnification of 170x.

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Fig. 6. Lased specimen with minimal surface alteration. Arrows indicate round fibroblasts. SEM original magnification of 170x.

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Fig. 7. Lased specimen with gross alteration of cementum surface. No fibroblasts were observed. SEM original magnification of 170x.

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Fig. 8. Crater formation on a lased specimen. Both small (small arrow) and large (large arrow) crater formation is evident. SEM original magnification of 170x.

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Fig. 9. High power magnification of a crater. An unhealthy fibroblast is observed within it. SEM original magnification of 312x.

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Fig. 10. Resolidified spheres of inorganic crystalline material on cementum surface. Surface has a lava-like appearance. SEM original magnification of 1150x.

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Fig. 11. Cementum tracking (typical). A smear layer appears to have been formed on either side of the track (small arrows). SEM original magnification of 326x.

DISCUSSION

The purpose of this study was to evaluate the effects of the Nd:YAG laser on *in vitro* fibroblast attachment to endotoxin treated root surfaces and to describe any root surface alterations resulting from the use of the laser.

Three treatment groups were established: 1) non-lased, non-diseased; 2) non-lased, diseased; and 3) lased, diseased. The anticipated results were that maximum fibroblast attachment would occur on the non-lased, non-diseased group and minimal attachment on the non-lased, diseased group. No prior studies have examined the use of lasers on root surfaces so the fate of these fibroblasts was unknown.

SEM observation clearly revealed a difference in fibroblast attachment to the cementum surface as being either flat (healthy) or round (unhealthy). Results revealed that the lased, diseased root segments had significantly decreased ($p < 0.01$) numbers of flat fibroblasts versus the non-lased, non-diseased and non-lased, diseased root segments. The absence of flat fibroblasts in the laser treated root segments was a consistent finding. It appeared that the laser altered the biocompatibility of the cementum surface so as to make it unfavorable for fibroblast attachment.

Though the examiner was blind as to the treatment group from which the specimen originated it became very

easy to identify the laser treated specimens. These specimens exhibited surface changes which included charring, crater formation, cementum meltdown, and tracking. The organic matrix appeared to have been burned off leaving behind a recrystallized substance with a lava-like appearance.

A pilot study was performed (Appendix 1) to examine fibroblast attachment to lased, non-diseased cementum surfaces. In this pilot project, cementum surfaces from unerupted third molars were lased for varying periods of time. Fibroblast attachment to these surfaces was then measured and compared to the extent of lasing of the cementum surface. Root segments lased short of any visible charring allowed for attachment of fibroblasts, but to a significantly lesser extent ($p < 0.01$) than the non-lased segments. Fibroblast attachment to specimens lased short of visible charring was significantly greater ($p < 0.05$) than the lased, charred segments. One specimen was grossly charred and then exposed to the Prophy-jet which easily removed the charred surface. This segment also allowed for fibroblast attachment but again to a significantly lesser degree ($p < 0.01$) than the non-lased segments. This suggests that the inhibiting factor is a surface contaminant which requires thorough removal to once again make the root surface biocompatible. However, it has not been determined if the lased surface can be

rendered as biocompatible as a non-lased, non-diseased root surface.

A second pilot study using photoacoustic spectroscopy (Appendix 2) revealed that a root surface that was evenly charred with the laser had an altered phosphate to carbonate ratio and the presence of a charged ion of ammonium as compared to a non-lased root surface. The presence of the charged ion of ammonium may have played a role in inhibiting fibroblast attachment.

The findings, in this study, and subsequent pilot studies suggests that the laser alters the cementum surface making it unfavorable for fibroblast attachment. The root surface alterations, altered phosphate to carbonate ratio and presence of a charged ion of ammonium may all play a role in the decreased attachment of fibroblasts. Fibroblast attachment tended to increase when the root segment was exposed to an abrasive spray (Prophy-jet). This indicates that root preparation (scaling and root planing) is required after laser treatment of the cementum surface in hopes of making the root surface biocompatible with the adjacent gingival tissue.

The non-lased, diseased group had a significantly increased ($p < 0.01$) number of round fibroblasts versus the other two groups. This was expected due to adsorbed endotoxin contamination. Lucas, Chen and Aleo (1979)

observed that as endotoxin concentration increased so did the lysosomal enzyme acid phosphatase. This increase in lysosomal enzyme content may result in cellular damage or death (Fig. 2). The authors were unsure as to whether the endotoxin acted directly on the lysosomes or if the increased enzyme levels were due to increased cell damage or death.

No significant differences were found in the number of flat cells in the non-lased, non-diseased versus the non-lased, diseased. Both groups had specimens exhibiting fibroblast attachment ranging from no attached cells to a confluent monolayer.

Several authors have investigated the attachment of fibroblasts to periodontally diseased root surfaces using in vitro studies. Aleo, et al. (1975) observed minimal fibroblast attachment to diseased root surfaces. Wirthlin and Hancock (1980) saw a sparse distribution of fibroblast attachment to cementum in diseased, untreated teeth. Both flat and round cells were observed. Gilman and Maxey (1986) did not observe fibroblast growth on calculus-laden root segments. In contrast, Adelson, Hanks, Ramfjord and Caffesse (1980) observed that healthy fibroblasts were attached to dentin, calculus, diseased cementum, and enamel. A wide variation in cell growth was seen from specimen to specimen. Their results indicated that the fibroblasts did not preferentially attach to the

instrumented versus the non-instrumented periodontally diseased root surfaces. Fardal, Aubin, Lowenberg and Freeman (1986) observed that periodontally diseased teeth incubated with human gingival fibroblasts showed equal attachment regardless of whether they were instrumented, non-instrumented or non-diseased. They also observed attachment of fibroblasts to calculus. The lack of a significant difference in the number of flat cells in the non-lased, non-diseased and non-lased, diseased root segments was unexpected. However, it appears from the above-mentioned articles that fibroblast attachment to periodontally diseased root surfaces is not uncommon.

The teeth used, in this study, were not obtained from patients with naturally occurring periodontal disease. Healthy, unerupted third molar root segments were considered diseased after soaking them in endotoxin for 72 hours. Nakib, et al. (1982) immersed healthy teeth in E. coli 011:B4 endotoxin at concentrations of either 150 ug/ml or 500 ug/ml for 2, 4, 8, or 12 weeks and then examined them using indirect immunofluorescence. The fluorescent pattern and intensity appeared similar regardless of incubation time or concentration of endotoxin employed. The 72 hours of soaking time, in this study, may not have been sufficiently long enough for the endotoxin to adsorb to the cemental surface. In addition, the root segments had originated from unerupted third

molars whose cementum may not have shown any of the changes associated with disease (Selvig, 1966) which may have made them less likely to adsorb endotoxin.

Commercially obtained endotoxin may not significantly affect the attachment of fibroblasts but may only affect their growth (Bergman and Nilsson, 1963; DeRenzis, 1981; and Olson, Adams and Layman, 1985) or both growth and viability (Aleo, et al. 1974; Singer and Dutton, 1979). DeRenzis (1981) cultured fibroblasts in the presence of endotoxin and observed that endotoxin did not affect the viability of the fibroblasts nor did it interfere with the ability of them to reach a monolayer in Petri dishes.

No prior studies evaluating fibroblast attachment to healthy, unerupted third molars exposed to endotoxin have been published at this time. The results obtained, in this study, raise questions as to whether the teeth were actually diseased at the onset of the investigation and if so then what role does endotoxin have on fibroblast attachment.

One experimental error that potentially affected all groups was non-uniformity of specimen thickness. Individual thicknesses ranged from one to three millimeters which resulted in several slanted specimens (Fig. 12). Upon placement of fibroblast culture media into the Petri dishes it became apparent that portions of some specimens were barely covered by media. Fibroblasts

tended to slide to the lowest aspect of the specimen as was later observed upon SEM observation. Adelson, et al. (1980) observed that fibroblasts did not migrate up inclined planes. Fernyhough and Page (1983) saw that fibroblasts preferentially attached to cracks, crevices and flat surfaces versus vertical surfaces. This problem, however, appeared to be equally present in all groups.

The potential use of lasers in treating periodontal disease has recently been advocated with hopes that the laser will be capable of sterilizing the diseased root surface, periodontal pocket microflora and adjacent pocket epithelium (Myers, 1989). The optimal result in treating root surfaces is to achieve reattachment by epithelial or, ideally, a connective tissue attachment to the previously diseased root surface. Based on the results from this study, the laser alters the biocompatibility of the root surface and thus, potentially renders it less likely to achieve tissue attachment. It has not yet been determined if the lased surface can be rendered as biocompatible as a non-lased, non-diseased root surface.

Scaled and root planed periodontally diseased teeth have been shown to behave similar to non-diseased controls using in vitro fibroblast culture studies (Aleo, et al. 1975; Fernyhough and Page, 1983). Hand and ultrasonic instrumentation continues to be the treatment of choice for treating periodontally diseased root surfaces. The

use of the lasers must be reserved until well-designed research studies reveal that it performs equal to or better than conventional instrumentation. This study suggests that the laser alters the biocompatibility of the root surface making it less likely to achieve tissue attachment.

The American Academy of Periodontology (AAP) published a statement on the use of lasers in periodontics dated November 21, 1990. They cautioned that the laser has received FDA approval for use on soft tissue surgical procedures only and that it has not been cleared for use on hard tissues or the periodontium. The Academy is not aware of any published studies which compare the use of the laser to conventional forms of instrumentation. Their recommendation is to heed the recommendation of the FDA (Becker, 1990 and 1991).

Valid criticisms of this study could include: 1) an in vitro study; 2) used healthy, unerupted third molars; 3) root segments were lased at a 90 degree angle in a dry environment; 4) assumed diseased status of the endotoxin soaked root segments; and 5) slanted root segments may have resulted in altered fibroblast attachment.

Areas of further research should include performing an <u>in vitro</u> study to determine if root planing the cementum surface after laser treatment will allow for similar fibroblast attachment as non-lased, root planed

controls. In vivo studies are needed to determine if altered tissue attachment and root surface alterations are similarly observed.

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Fig. 12. Slanted specimen (typical). SEM original magnification of 20x.

CONCLUSIONS

1. Lased, diseased root segments had significantly decreased numbers of flat (healthy) fibroblasts versus the non-lased, non-diseased and non-lased, diseased root segments.

2. Non-lased, diseased root segments had significantly increased numbers of round (unhealthy) fibroblasts versus non-lased, non-diseased and lased, diseased root segments.

3. Lased root segments showed obvious surface changes which included charring, crater formation, cementum meltdown, and tracking.

APPENDICES

APPENDIX 1

A pilot study was conducted to determine *in vitro* fibroblast attachment to lased, non-diseased root surfaces. Unerupted third molars were sectioned to provide eight 4 mm by 4 mm roct segments. Four treatment groups were established: 1) non-lased; 2) lightly lased; 3) heavily lased; and 4) heavily lased and then polished with an air-water-powder abrasive spray (Prophy-jet) for 10 seconds. The lased teeth were exposed to an average energy of 80 mJ at 10 pulses per second. Laser treatment was discontinued at the first sign of charring (37 and 60 seconds) for the 2 specimens in the lightly lased group. The heavily lased root segments were exposed for 2 minutes each.

Fibroblast culture preparation, SEM observation, and data collection were the same as in the previous study. Two root segments were placed in each of 4 fibroblast culture dishes. They were paired as follows: 1) non-lased with lightly lased; 2) non-lased with heavily lased; 3) non-lased with heavily lased/Prophy-jet; and 4) lightly lased with heavily lased.

Results revealed a statistically significant difference ($p < 0.001$) in the number of flat cells only. Mean cell attachment per examined field was as follows: 1) non-lased = 73; 2) lightly lased = 32; 3) heavily lased = 9; and 4) heavily lased/Prophy-jet = 25. Tukey

studentized range method revealed that the non-lased root segments had significantly more ($p < 0.01$) flat fibroblasts than the other three treatment groups. There was also a statistically significant difference ($p < 0.05$) between the heavily lased and lightly lased groups. Heavy lasing followed by treatment with the Prophy-jet resulted in a mean increase in fibroblast attachment from 9 cells to 25 cells but the difference was not statistically significant.

These results suggest that the laser alters the biocompatibility of non-diseased root surfaces making them less acceptable for fibroblast attachment. Exposing the heavily lased surface to an abrasive spray (Prophy-jet) helped increase the number of attached fibroblasts, but numbers of cells were still significantly lower than the non-lased root segments.

APPENDIX 2

A pilot study using photoacoustic Fourier transform infrared (FT-IR) spectroscopy was used to determine if laser treatment results in any molecular group alterations of the cementum surface. In this study, photoacoustic FT-IR spectroscopy allowed for a qualitative analysis of the molecular groups present on the cementum surface.

Six millimeter diameter cementum circles were cut from the root surfaces of two previously unerupted third molars. Both specimens were exposed to an air-water-powder abrasive spray (Prophy-jet) for 10 seconds to remove any tissue remnants. The specimens were then rinsed in tap water and brushed gently to remove any residual Prophy-jet powder. The experimental sample was lased with the Nd:YAG laser using a 320 micron contact optic fiber with an average energy of 80 mJ of energy at 10 pulses per second until the entire surface was evenly charred. Total lasing time was approximately two minutes. The other specimen served as the non-lased control. The specimens were placed in a desiccator for one week prior to spectroscopic analysis.

Photoacoustic FT-IR spectra were recorded on an analect RFX 75 Fourier transform infrared spectrometer, equipped with a photoacoustic cell. All spectra were recorded at a resolution of 8 cm^{-1}, with a scan speed of 0.5 cm^{-1}/sec. An improved signal to noise ratio was

obtained by collecting 1000 scans for each sample. The sample single beam spectra were ratioed against a carbon black reference.

Results revealed that the lased sample had an altered carbonate to phosphate ratio. A distinct absorption band was detected at wavenumber 2021 cm^{-1}. This band corresponds to that of a charged ion of ammonium (Fig. 13).

It appears that the laser caused a change in the molecular groups present on the cementum surface. Previous studies have detected changes in phosphate and carbonate ratios (Kantola, 1972; Kantola, et al., 1973; Kuroda and Fowler, 1984; and Keller and Hibst, 1990), however, there have been no reports on the detection of a charged ion of ammonium. This group may result from protein degradation and may have played a role in the inhibition of fibroblast attachment seen in the previous study.

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Fig. 13. Photoacoustic FT-IR spectroscopy graph. The lased specimen is the lower graph. Note peak at 2021 cm^{-1} (thick arrow). This peak corresponds to a charged ion of ammonium. The lased specimen also showed an increase in the carbonate (c-arrow) and phosphate (p-arrow) peaks.

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