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Behavior of human periodontal ligament cells on CO_2 laser irradiated dentinal root surfaces: an *in vitro* study

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Objective: The aim of this study was to investigate the *in vitro* attachment behavior of human periodontal ligament fibroblasts on periodontally involved root surface after conditioning with CO_2 laser and to compare its efficacy with chemical conditioning agents, namely tetracycline hydrochloride, citric acid, hydrogen peroxide (H_2O_2) and EDTA, using scanning electron microscopy.

Methods: A total of 84 scaled and root-planed specimens from periodontally involved single-rooted human teeth showing hopeless prognosis were selected and assigned to two groups. One group was lased with a CO_2 laser (from 5 cm at 3 W for 0.8, 1.0 and 1.2 s), and the other group was treated with either tetracycline hydrochloride (2.5%), citric acid (saturated solution, pH 1), H_2O_2 (6%) or EDTA (5%; pH 7.4) for 3 min. The specimens were then seeded with human periodontal ligament fibroblasts, incubated for either 12 h or 24 h, and then the cell attachment behavior was observed.

Results: CO_2 laser irradiation for 1.0 s was found to be the most efficient, showing consistently good cell attachment with the highest mean value (15.00 ± 3.41 cells/10,000 μm^2 after incubation for 12 h and 29.17 ± 2.04 cells/10,000 μm^2 after 24 h), followed by irradiation for 0.8 s (13.11 ± 3.04 cells/10,000 μm^2 after incubation for 12 h and 22.91 ± 7.10 cells/10,000 μm^2 after 24 h). Charring was observed following irradiation for 1.2 s. Amongst chemical conditioning agents, citric acid was found to be the most efficient, with a mean cell attachment of 17.82 ± 2.16 cells/10,000 μm^2 after incubation for 12 h and 23.62 ± 1.94 cells/10,000 μm^2 after 24 h. EDTA and H_2O_2 did not do well in the study.

Conclusion: The results suggest that CO_2 laser irradiation for 1.0 s may promote comparatively better attachment of periodontal ligament fibroblast on dentinal root surfaces than the conventional chemical conditioning agents used in the study.

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The regeneration of the periodontal support lost due to periodontitis is the ultimate goal of periodontal therapy. In the last couple of decades a concerted effort has been made in the field of root conditioning to improve the outcome of regenerative periodontal therapies by favoring the attachment of the regenerated

periodontal structures. The periodontally involved teeth generally contain heavy bacterial contamination and/or their endotoxins (1, 2), which may jeopardize the outcome of periodontal regenerative procedures by preventing new connective tissue attachment (3–5). Disinfection and modification of the contaminated root surface in order to restore its biocompatibility and to favor the attachment of the regenerated periodontal structures becomes the necessity. Scaling and root planing alone cannot achieve this objective because bacterial toxins are not completely eliminated from the root surface (1, 2, 4). Also, mechanical instrumentation leaves a smear layer, which inhibits cell reattachment and can serve as a reservoir for microbial growth (6). Therefore, chemical conditioning of the roots was performed in order to remove the smear layer and to improve their biocompatibility. After the smear layer removal, the dentinal collagens are exposed and they are supposed to be a chemo-attractant for periodontal fibroblasts (7, 8). Citric acid and tetracycline hydrochloride are the most widely used substances for this purpose (9) and have shown a role in migration, growth and reattachment of fibroblast (10–13) and facilitate cementogenesis (14, 15). In addition to chemical conditioning, the applicability of CO_2 laser in the removal of the smear layer has been demonstrated by Misra *et al*. (16). Recently, Crespi *et al*. (17) suggested the role of a low power of CO_2 laser irradiation in the enhancement of attachment of L929, a mouse fibroblast, on the root surface of periodontally involved tooth, under *in vitro* conditions. However, the utility of CO_2 laser conditioning in clinical use is still unanswered due to poorly understood mechanism and lack of literature. Thus, the present study was conducted to investigate whether the CO_2 laser has any role in promoting periodontal ligament cell attachment on the dentinal root surface, and to compare its efficacy with chemical conditioning agents, under *in vitro* conditions.

Materials and methods

Specimen preparation

A total of 42 single-rooted teeth with hopeless periodontal prognosis showing no caries and/or filling material with no hypoplastic defects were used in the present investigation. The specimen preparation was done by following the method of Crespi *et al*. (17) with some modifications. In brief, extracted teeth were cleansed by irrigating with sterile phosphate-buffered saline to remove blood and debris. The root surfaces were then scaled with an ultrasonic scaler and root planed using hand curettes to obtain a smooth hard surface. The test area on each tooth was the proximal surface. Specimens approximately 1 mm thick and measuring 5 mm × 5 mm were sliced from the proximal region 3 mm apical to the cervical line using a water-cooled high speed bur. A total of 84 sections were obtained. The specimens were then washed and cleaned with normal saline. Thereafter, the tooth sections were kept in absolute ethanol overnight for complete dehydration.

Experimental design

Samples were distributed into five groups, A to E. Group A, containing 36 samples, was further divided into three subgroups, A1, A2 and A3 (12 specimens each), and was irradiated using a CO_2 laser (Satelac Dental CO_2 laser Model Lasersat, Meriganc, France) with a helium–neon laser guide, from a distance of 5 cm at an angle of 90° at 3 W for different time periods, i.e. for 0.8, 1.0 and 1.2 s, respectively. The light focused on the specimen had a beam diameter of 3 mm. Specimens from groups B, C, D and E were treated with tetracycline hydrochloride (2.5%), citric acid, saturated solution of pH I, H_2O_2 (6%) and EDTA (5%; pH 7.4), respectively. In each specimen, a notch was created on one side using a water-cooled high speed bur. In group A, an area approximately 1 mm from the notch was irradiated with laser and served as the experimental site; the peripheral area served as the control. In groups B, C, D and E, the half specimen towards the notch served as the experimental area and the other half served as the control. Experimental solutions at their respective concentrations were rubbed vigorously for 3 min on the prepared specimens using cotton pellets. Pellets were changed every 30 s. Immediately after chemical treatment, the specimens were rinsed thoroughly with normal saline and dried in sterile air.

Cell culture

The primary cultures of human periodontal ligament fibroblasts were obtained by following the methods of Rompen *et al*. (18) with minor modifications. Briefly, the teeth were washed in minimal essential medium containing antibiotic–antimycotic, then adherent soft tissues were removed from the crown and the coronal one-third of the root and discarded. The crown and coronal one-third of the root were then placed in 5.25% NaClO for 2 min to reduce bacterial contamination, as well as to kill any remaining gingival epithelial cells. The middle third of the root was then scraped to obtain periodontal ligament tissue specimens. The tissue specimens were placed in a sterile Petri dish containing a thin layer of minimal essential medium with 10% fetal bovine serum. The periodontal ligament tissue was disaggregated using 0.2% collagenase and 0.125% trypsin for 30 min at 37°C and the cells were collected by centrifugation at 100 *g* for 5 min. The pellet of packed cells was then re-suspended in the six-well culture plates in complete minimal essential medium and incubated at 37°C with an atmosphere of 95% air–5% CO_2 for the attachment. Growth was permitted to continue until the cells attained a confluent monolayer, at which time they were trypsinized (trypsin 0.05%–EDTA 0.53 mM) and passaged into T-25 culture flasks to expand the cell population (first cell passage). The cells of third and fourth passages were trypsinized and pooled for experimentation (to control the cell variability). Cell

number for experimentation was determined using an Electronic Coulter Counter (Model Zf, Coulter Electronics, Hialeah, FL, USA). The number of viable cells in each batch was measured by the trypan blue dye exclusion test before each experiment, and batches showing cell viability of more than 95% were used for the experiment.

Cell attachment

The confluent monolayer of cells was harvested by trypsinization and the cell suspension was centrifuged at 100 *g* for 10 min and the pellet was resuspended in aliquots minimum essential medium. The cellular density of viable cells was adjusted to 1×10^5 cells/ml of medium. One milliliter of cell suspension was distributed in each well of 24-well culture plates containing the treated dental specimens and then incubated in a 5% CO_2–95% air atmosphere. One group was processed for scanning electron microscopy after incubation for 12 h, and the other group was processed after incubation for 24 h. The untreated samples were processed under identical conditions and served as control.

Sample preparation for scanning electron microscopy

The fixed samples were dehydrated in a graded series of aqueous ethanol (30%, 50% 70%, 90%, 95%, 100% and 100%) for 20 min at each concentration. The specimens were then immersed in isoamyl acetate and dried at critical point with liquid CO_2 in a critical point dryer. The specimens were then mounted on the scanning electron microscopy coating unit (Polaron Sc Model no. 7646 Sputter Coater) and coated with approximately 20 nm gold palladium alloy under a vacuum pressure of 10^{-3} torr, 1.0 kV voltage, and 20 mA current for 20 min. All the specimens were examined under a scanning electron microscope (Model No. Leo-430, Leo EM Ltd., Cambridge, UK) at 15 kV accelerating voltage and at 3×10^{-11} A probe current. Six photographs addressing six different areas of dentin surfaces of each specimen were taken. Then the cells attached in the area of 100 μm × 100 μm in each photograph were calculated using a grid made on transparent sheets and analyzed for the general surface topography, density of the fibroblast cells attached on the surface, structural differentiation in the fibroblast and removal of smear layer.

Statistical analysis

One-way analysis of variance was carried out to find out the statistical significance of mean values in different treatment groups for 12 h and 24 h separately. Prior to analysis, homogeneity of variance between the treatment groups was ascertained. Inter-group comparisons were carried out by calculating least significant differences at 5% level of significance. Intra-group comparisons in laser-treated samples were made using Student's *t*-test and *p*-value < 0.05 is considered as the minimum significant level.

Results

Attachment kinetics

The periodontal ligament cell attachment in the experimental sites treated with laser groups was lagging behind with the citric acid-treated samples after 12 h of incubation period. However, this difference was statistically insignificant, except in the case of subgroup A3 ($p < 0.001$) (Table 1). In contrast, after 24 h of incubation, subgroup A2 performed well enough, which could be demonstrated by assessing the significant difference level of $p < 0.05$ (citric acid), $p < 0.01$ (tetracycline hydrochloride), $p < 0.001$ (H_2O_2) and $p < 0.001$ (EDTA) (Table 2). However, the cell attach-

Table 1. Mean human periodontal ligament fibroblast attachment per 10,000 μm^2 on chemical conditioned and laser treated root surfaces after incubation for 12 h: a comparative account

		Laser-treated groups/A		
		Subgroup A1	Subgroup A2	Subgroup A3
		0.8 s	1.0 s	1.2 s
Chemical agent/groups	Mean ± SE	13.11 ± 3.04	15.00 ± 3.41	4.99 ± 0.52
Tetracycline/B	14.99 ± 3.63	NS ↓	NS ↑	$p < 0.02$ ↓
Citric acid/C	17.82 ± 2.16	$p < 0.1$ ↓	NS ↓	$p < 0.001$ ↓
H_2O_2/D	6.03 ± 0.44	$p < 0.1$ ↑	$p < 0.1$ ↑	NS ↓
EDTA/E	5.43 ± 0.83	$p < 0.05$ ↑	$p < 0.02$ ↑	NS ↓
Control	0.23 ± 0.04	$p < 0.001$ ↑	$p < 0.001$ ↑	$p < 0.001$ ↑

Exposure time for chemical conditioning, 3 min.
NS, non-significant; SE, standard error.

Table 2. Mean human periodontal ligament fibroblast attachment per 10,000 μm^2 on chemical conditioned and laser treated root surfaces after incubation for 24 h: a comparative account

		Laser treated groups/A		
		Subgroup A1	Subgroup A2	Subgroup A3
		0.8 s	1.0 s	1.2 s
Chemical agent/groups	Mean ± SE	22.19 ± 7.1	29.17 ± 2.04	5.90 ± 0.73
Tetracycline/B	19.85 ± 1.82	NS ↑	$p < 0.01$ ↑	$p < 0.002$ ↓
Citric acid/C	23.62 ± 1.94	NS	$p < 0.05$ ↑	$p < 0.001$ ↓
H_2O_2/D	8.90 ± 2.63	$p < 0.05$ ↑	$p < 0.001$ ↑	NS ↓
EDTA/E	10.30 ± 1.54	$p < 0.1$ ↑	$p < 0.001$ ↑	$p < 0.05$ ↓
Control	0.20 ± 0.03	$p < 0.001$ ↑	$p < 0.001$ ↑	$p < 0.001$ ↑

Exposure time for chemical conditioning, 3 min.
NS, non-significant; SE, standard error.

ment in subgroup A3 was minimum under both the incubation periods (4.99 ± 0.52/10,000 μm^2 at 12 h and 5.90 ± 0.73/10,000 μm^2 at 24 h). As compared to samples treated with H_2O_2 and EDTA, A2 clearly indicates a highly statistically significant increase (up to $p < 0.001$) in the cell attachment (Tables 1 and 2). The rate of periodontal ligament cell attachment on chemical conditioned and lased root surfaces proved to be highly significantly increased ($p < 0.001$), as compared to untreated control, under both the incubation periods (Tables 1 and 2). Among the chemical conditioning agents used in the study, citric acid was found to be the most efficient and ranked first (17.82 ± 2.16/10,000 μm^2 at 12 h; 23.62 ± 1.94/10,000 μm^2 at 24 h), followed by tetracycline hydrochloride (14.99 ± 3.63/10,000 μm^2 at 12 h; 19.86 ± 1.82/10,000 μm^2 at 24 h). After 12 h of incubation, EDTA was found to be the least efficient among the chemical agents used, with the lowest attachment of cells on the surface treated with it (5.43 ± 0.83/10,000 μm^2); however, after 24 h of incubation, H_2O_2 showed the lowest attachment (8.90 ± 2.6/10,000 μm^2) (Tables 1 and 2). Statistically, citric acid and tetracycline hydrochloride were found to be almost equally effective in the increase in cell attachment under both the incubation periods. The cell attachment was found to be significantly high, i.e. $p < 0.05$ and $p < 0.001$, in tetracycline hydrochloride treated surfaces in comparison to H_2O_2 and EDTA, respectively, after 24 h of incubation. This difference was found to be maximum between citric acid vs. H_2O_2 and citric acid vs. EDTA, i.e. $p < 0.001$. The difference between H_2O_2 vs. EDTA was statistically insignificant for 12-h and 24-h incubation periods (Table 3). The growth difference between A1 vs. A2 was statistically insignificant under both incubation periods. However, this difference in the attachment reached a significance level of $p < 0.05$ for A1 vs. A3 under both the incubation periods and, interestingly, this difference reached a significance level of $p < 0.001$ between A2 vs. A3 when the incubation period was 24 h (Table 3).

Table 3. Statistical comparison of mean attached fibroblasts per 10,000 mm^2 amongst the chemical-conditioned and laser-treated section after incubation for 12 h and 24 h

Group	Incubation period	*p*-value
B vs. C	12 h	NS
B vs. C	24 h	< 0.1
B vs. D	12 h	< 0.1
B vs. D	24 h	< 0.01
B vs. E	12 h	< 0.05
B vs. E	24 h	< 0.01
C vs. D	12 h	< 0.05
C vs. D	24 h	< 0.001
C vs. E	12 h	< 0.001
C vs. E	24 h	< 0.001
D vs. E	12 h	NS
D vs. E	24 h	NS
A1 vs. A2	12 h	NS
A1 vs. A2	24 h	NS
A1 vs. A3	12 h	< 0.05
A1 vs. A3	24 h	< 0.05
A2 vs. A3	12 h	< 0.01
A2 vs. A3	24 h	< 0.001

B, tetracycline; C, citric acid; D, H_2O_2; E, EDTA; A1, laser 0.8 s; A2, laser 1.0 s; A3, laser 1.2 s.
NS, non-significant.

Morphological studies

The untreated controls specimens exhibited an amorphous, irregular surface with numerous irregular mounds and occasional attachment of periodontal ligament cells with small blebbing or protuberance-like morphology. A surface smear layer prominently appeared on the whole root surface (Fig. 1). The number of cells attached and morphology were markedly altered when cultured human periodontal ligament cells were grown on root surfaces treated with EDTA, H_2O_2 and laser for 1.2 s (A3). At the same time, removal of the smear layer from the root surface was inefficient. The cells appeared to be retracted from the root surface. The cytoplasm of these cells showed holes, giving them a dead appearance, even at the incubation period of 12 h (Fig. 2). After the 24 h incubation period with the same treatment groups, although the cell attachment number increased overall, clear-cut cell shrinkage and dryness with filopodial and lamellipodial extensions penetrating into dentinal openings were clearly seen. An increase in cellular debris was also noted (Fig. 3). In the sample A3, charring appeared clearly, which markedly hindered the periodontal ligament attachment to the surface. In contrast, cells grown on the root surfaces treated with tetracycline hydrochloride, citric acid, A1 and A2 displayed a smooth homogeneous removal of the smear layer surface with a confluent monolayer of healthy cells, well and tightly attached to the surface (Fig. 4). With the increased incubation period, i.e. 24 h, the differentiation of attached cells became clearer, with an increase in the number of attached cells. The typical structural morphology of fibroblasts was clearly apparent, with relatively debris-free conditions (Fig. 5).

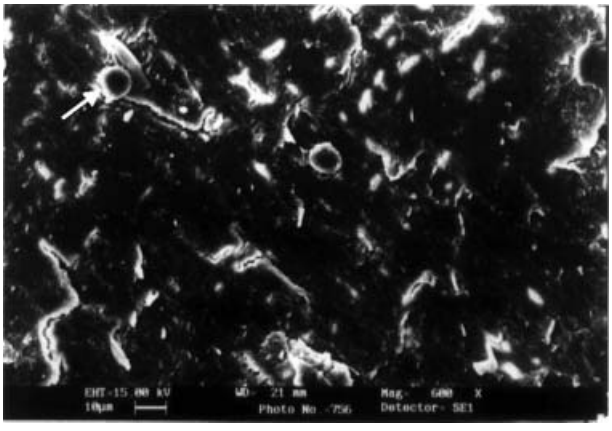

Fig. 1. Untreated (Control) surface (original magnification 600 ×).

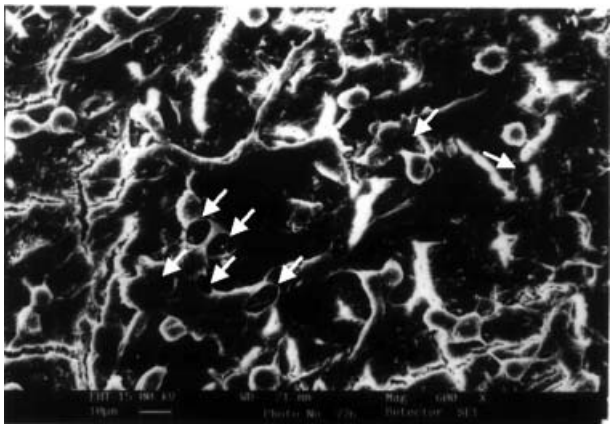

Fig. 2. Photograph of Group D. The similar cellular attachment and spreading were also observed in Group E and subgroup A3 after 12 h of incubation (original magnification 600 ×).

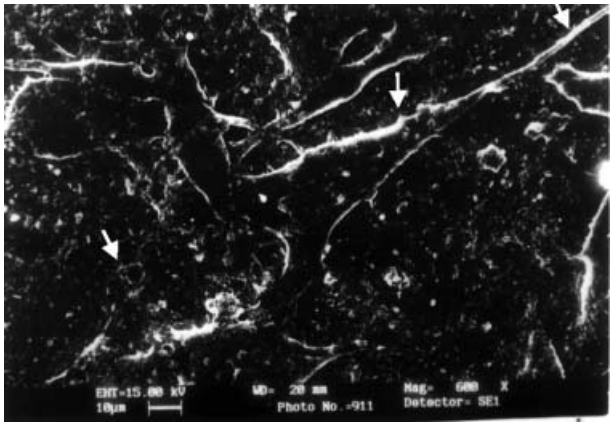

Fig. 3. Photograph of Group D. The similar cellular attachment and spreading were also observed in Group E and subgroup A3 after 24 h of incubation (original magnification 600 ×).

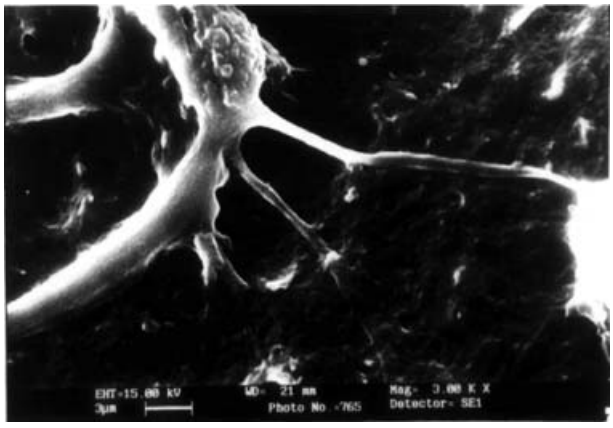

Fig. 4. Photograph of Group B. The similar cellular attachment and spreading were also observed in Group C and subgroup A2 after 12 h of incubation (original magnification 3000 ×).

Discussion

A lased surface at 3 W, for 1 s from a distance of 5 cm (A2) was found to be the best performer amongst the entire treatment groups used in this study. This might be attributed to the well-exposed dentinal tubules with clear orifice and complete absence of a granular smear layer. Similar results for removal of the smear layer have also been shown by previous workers (16, 19). However, a better fibroblast attachment by citric acid was observed in the first 12 h of the incubation period. This can be supported by the earlier studies of Labahn *et al*. (20), in which they suggested that citric acid is more potent to cause extensive changes on the dentin surface than tetracycline hydrochloride and stimulates the initial attachment of fibroblasts comparatively faster under *in vitro* conditions. Although the topical application of tetracycline hydrochloride and citric acid have several advantages for root conditioning, inhibitory effects on periodontal connective tissue cells have also been reported by a few workers (21, 22). This might be one of the reasons why a statistically significant defeat of tetracycline hydrochloride and citric acid in comparison to A2 after the extended incubation period of 24 h was observed in the present study.

Even after an almost equal level of smear layer removal by all the three conditioning agents, i.e. A2, tetracycline hydrochloride and citric acid, the specimen surfaces treated with A2 achieved better attachment, which might be due to the comparatively less wider opening of dentinal tubules, as wider openings will reduce the attachment surface area. The funnel-shaped widening of dentinal tubules after citric acid conditioning has already been reported, whereas there were no such changes in dentinal structures with the exposure of laser irradiation for a time period of 1 s (16). Low pH of the saturated solutions of citric acid and tetracycline hydrochloride has also been suggested as one of the reasons for reduced cell attachment and perhaps is responsible for the unpredictable results for the reattachment, by denaturing the organic matrix of the root as

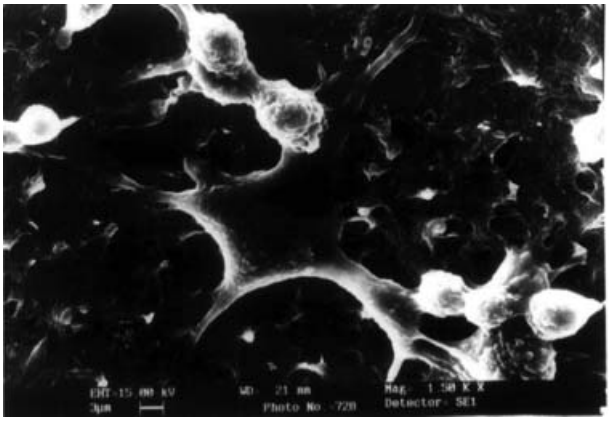

Fig. 5. Photograph of Group B. The similar cellular attachment and spreading were also observed in Group C and subgroup A2 after 24 h of incubation (original magnification 1500×).

well as by demineralizing the surface (23). It has also been suggested that etching with acids can interfere with periodontal healing by their necrotizing effect on the progenitor cells of the surrounding periodontal tissues (24). Therefore, it may be anticipated that the cumulative effect of all these factors might show up after a period of 12 h. Thus, laser took a lead in the extended incubation period of 24 h.

EDTA overall could not perform well in the present study, as it caused a high level of surface cracking with several pits formation and very feeble removal of smear layer and poor opening of dentinal tubules. However, a few workers have suggested EDTA as a better conditioning agent than citric acid and tetracycline hydrochloride (16, 25, 26). H_2O_2 was observed to be the least efficient, as attachment was affected severely due to a critical damage in the root surface, with accumulation of large amount of debris and the presence of an irregular smear layer, even after the conditioning. This finding was in accordance to the results of a previous study with the same concentration of H_2O_2 (16).

Morphological assessment by scanning electron microscopy also demonstrated that A2 was the most efficient amongst all the treatment groups used in the present study. With the increase in the incubation period, i.e. 24 h, both cell number as well as differentiation in healthy attached cells became clearer and a confluent monolayer of well-differentiated attached cells was formed on the smooth root surface. This is perhaps due to the smooth homogenous removal of the smear layer, which makes the surface more biocompatible and attractive to periodontal ligament fibroblasts. These modifications could be either a direct consequence of root conditioning, through which some of the extracellular matrix constituents acting on the attachment mechanism of periodontal ligament cells became more exposed, or an indirect effect by the increased fixation of biochemical factors on the demineralized root surface. The demineralization causes exposure of the organic matrix of the dentin so there is a direct contact of cells with a continuous layer of oriented type I collagen fibers. Fibrillar collagen is indeed known to promote the migration of fibroblasts and to favor cell attachment through membrane-associated integrals. De Long *et al.* (27) demonstrated similar results for the demineralized root surface with citric acid and tetracycline hydrochloride. However, Babay (28) demonstrated a decreased spreading of the cells on demineralized dentin surface.

The poor performance by A3 was probably due to the pronounced visible surface charring and the failure in exposing any marked dentinal tubules, with prominent surface cracking, pitting and crater formation on the surface of the specimens. A globular and porous-like appearance, i.e. the formation of re-solidified lava in some instances, and occasional striations resulting from instrumentation, with uneven and irregular surface texture and rough topography in a few specimens, are also perhaps the reasons for such poor performance. The presence of a smear layer confirmed by the presence of granular structures was also an added factor for adverse effects in certain areas in the specimens. Similar findings due to random distribution of pit and crater formation and melting of the roots mineral phase, with subsequent re-solidification as porous globules, were observed when ND-YAG laser irradiation was used at low energy levels with extended period of time (29). Misra *et al.* (16) reported charring in the samples with an increase in the exposure time, i.e. 1.2 s and 1.4 s under a constant energy level of 3 W and a constant distance of 5 cm. These findings are suggestive that the exposure time may be one of the important factors to cause charring and carbonization on the root surface. However, other factors, such as distance between specimen and laser point, penetration power of the laser, pathological condition of the tooth, etc., may influence the output of the laser irradiation therapy.

The occasional attachment of periodontal ligament fibroblast was observed on the whole root surface in the untreated control samples due to the presence of a prominent loosely textured smear layer, which masks the exposure of chemo-attractant collagen. Reports are available with similar cell behavior, where unconditioned dentin surface was compared with citric acid conditioning (7, 23), presence of surface smear layer subsequent to instrumentation and absence of dentinal tubule openings on the surface, and the occasional presence of striations on the surface of specimens (17, 28, 30, 31). However, the observations of Rompen *et al.* (18) showed contradictory results and demonstrated that untreated dentin supports the viability of attached human periodontal ligament cells with good proliferative potential and biosynthetic activity, although their rate of proliferation and protein synthesis activity was significantly lower than

that of cells attached to a plastic surface.

Conclusion

The present study has suggested that the CO_2 laser irradiation for 1 s at an energy level of 3 W from a distance of 5 cm is able to remove the smear layer completely and enhances the attachment of cultured human periodontal ligament fibroblasts on the root surfaces of periodontally involved tooth under *in vitro* conditions. However, periodontal clinical studies are needed before reaching to any firm conclusion.

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