Comparative analysis of cytocompatible properties of the root surface exposed to diode laser (970 nm) as adjunct to scaling and root planing: An in vitro study

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Abstract

Aims: The aim of the current study was to examine the effects of diode laser (970 nm) when used as adjunct to mechanical debridement on reattachment of fibroblast cell cultures to the root surface.

Materials and Methods: Eighteen extracted single-rooted human teeth were used in the study. Briefly, the anatomical crowns were removed; roots were sectioned and preserved in antiseptic solution. The sections were divided into three groups: (i) scaling and root planing (SRP), (ii) SRP followed by diode laser exposure and (iii) untreated control group. All the sections were inoculated with NIH 3T3 mouse fibroblast cell lines and cell attachment was confirmed by scanning electron microscopy analysis and growth was quantitatively assessed by MTT assay after days 1, 3 and 5.

Results: On intragroup analysis, there was significant cell proliferation seen on day 1 and day 5. However, no significant differences were observed between the treatment groups at the various time points.

Conclusion: 970-nm diode laser exposure had a positive effect in enhancing the fibroblast reattachment to root surfaces. However, to ascertain the role of laser treatment in enhancing the rapid cell growth compared to SRP, further quantitative studies with more sample numbers are required.

Keywords: Cytocompatibility, diode laser, mouse fibroblasts, MTT assay, scanning electron microscopy analysis

INTRODUCTION

Periodontitis is defined as an infectious disease resulting in inflammation within the supporting structures of the teeth, progressive attachment loss and bone loss.[1] Periodontally compromised teeth tend to harbor bacteria and their endotoxins in the exposed areas of root surfaces that may interfere with the outcome of periodontal surgical procedures.[2] Mechanical debridement of contaminated root surface through scaling and root planing (SRP) alone may not provide a favorable environment for the healing of periodontal tissues.[3] Studies published in literature have demonstrated that mechanical instrumentation often leaves a smear layer that may interfere with cell
Fibroblast reattachment to laser‑treated root surfaces

Laser irradiation has been reported to show bactericidal and detoxification effects without leaving a smear layer. Furthermore, laser irradiation has been shown to reduce pocket depths and tooth mobility. Hence, laser-treated root surfaces may provide favorable conditions for connective tissue reattachment. Fekrazad et al. showed that after ErCr:YSGG laser exposure, mouse fibroblast cells showed favorable attachment to root surface at 3 and 5 days. To the best of our knowledge, there are no studies published in the literature assessing the role of diode laser in promoting fibroblast reattachment to root surfaces with significant results. In this study, we intend to investigate the reattachment of NIH 3T3 mouse fibroblast cells on root surfaces with adjunctive use of diode laser (970 nm) following mechanical debridement and compare the outcomes with those seen with mechanical debridement alone and with untreated surfaces.

**MATERIALS AND METHODS**

Single-rooted human teeth with different lengths and widths extracted due to periodontal disease were utilized for all the studies. Initially, a preliminary study was carried out using scanning electron microscopy (SEM) to ascertain the attachment of cells to the root surface. Six single-rooted human teeth with different lengths and widths extracted due to periodontal disease were utilized. Teeth which were carious, restored, hypoplastic and showing root resorption were discarded. The remaining teeth were cleared of blood, debris and tissue remnants and preserved in iodine solution. Anatomic crowns of these teeth were removed; roots were longitudinally sectioned using diamond-coated disks (Figure 1) and flattened using Arkansas stone (Figure 2). The specimens were repeatedly rinsed with iodine solution during the sectioning process to minimize microbial contamination. The specimens were randomized into three groups: Group A: untreated specimens, Group B: specimens subjected to diode laser exposure following mechanical debridement and Group C: specimens subjected to mechanical debridement alone.

Scaling was performed using piezoelectric ultrasonic tip under copious irrigation till all the embedded calculi were eliminated (Figure 3). Root planing was performed using area-specific curettes (no. 3/4) (Figure 4). Laser exposure was performed by positioning the optic fiber of the diode laser (970 nm) perpendicularly 1–2 mm above the surface of the specimen and directing the radiation at 0.6 W power, frequency of 1 Hz for 15 s, in noncontact mode (Figure 5).

Immediately after the intervention, each specimen was rinsed thoroughly with iodine solution and dried in sterile air (Figure 6). The specimens were thoroughly washed with sterile phosphate-buffered saline (PBS) buffer to remove traces of iodine solution and autoclaved for *in vitro* cell culture experiments.
Scanning electron microscopy analysis

Initial studies were carried out using SEM to ascertain the attachment of cells to the root surface. Briefly, equally cut tooth samples with different treatment groups were placed in a 12-well cell culture plate, and 1 ml of NIH 3T3 cell stock containing $1 \times 10^5$ cells/ml was inoculated on the tooth specimens [Figure 7]. The plates were incubated at 37°C in 5% CO$_2$ incubator for hours. After incubation, the tooth samples were subjected to series of fixation steps before imaging. The samples were incubated with 2% glutaraldehyde overnight at 4°C. This was followed by sequential fixing in different concentrations of ethanol – 50% for 1.5 h at −20°C, 70% for 1.5 h at −20°C, 85% for 1.5 h at −4°C and 95% for 1.5 h at −4°C. The samples were sputtered with gold particles using an ion sputter (Hitachi Ion Sputter MC 1000) for 120 s, and the current was maintained at 1.8 mA. Cell attachment on the tooth surface was captured using Hitachi SU3500 SEM, and the image was captured at 15 kV [Figures 8].

Cytocompatibility assay (MTT assay)

Cell proliferation on the root surfaces subjected to different treatment procedures was quantitatively assessed by MTT assay. Briefly, 18 periodontally compromised, single-rooted teeth were selected after fulfilling the inclusion and exclusion criteria. Tooth preparation, randomizing into different groups and the interventions were performed as described earlier. The sterile tooth samples were placed in 48-well cell culture plates and inoculated with 300 µl of NIH 3T3 mouse fibroblast cells from the stock concentration of $1 \times 10^5$ cells/ml. The plates were incubated at 37°C in CO$_2$ incubator for 1, 3 and 5 days. After each incubation time, the specimens were washed with PBS to remove any dead cells. The cells used in the assay were NIH 3T3 mouse fibroblast cells. In addition to the three study groups of specimens, the fourth group representing a positive control was set up by inoculating the cells directly onto the cell culture plate. After each incubation time, the specimens were transferred to fresh cell culture plate and washed with PBS to remove any dead cells. This was followed by the addition of 300 µl of MTT (1 mg/ml) solution and incubating the plate for 3 h for the purple formazan crystals to appear. The MTT
solution is carefully aspirated and purple formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and 100 µl of DMSO was added to dissolve the formazan crystals.[9] The color change was measured at 570 nm using microplate reader (Multiskan GO, Thermo Fisher Scientific), and the values are expressed as percentage of viable cells at specified incubation times.

Cytocompatibility was evaluated through MTT assay. In addition to the three groups of specimens, the fourth group representing a positive control was set up by inoculating the cells directly onto the cell culture plate. After the desired time points (days 1, 3 and 5), the sections were incubated with 300 µl of MTT reagent for almost 3 h. The specimens were incubated in 96-well microplate readers, and absorbance was measured at 570 nm [Figure 9].

Statistical analysis
Repeated measures ANOVA was used to compare the mean MTT values in each group between individual time points. One-way ANOVA was used to compare MTT assay values between the groups at each time point. Tukey’s test was used for post hoc analysis for both intragroup and intergroup comparisons.

RESULTS
Initially, 12 specimens were randomized to three different study groups – untreated, diode laser treated (970 nm) and SRP. After the necessary interventions, they were subjected to SEM analysis to confirm the fibroblast attachment. Robust cell attachment was confirmed through SEM in all the randomized groups included in the study. In order to understand the role of different treatment procedures in promoting better cell attachment and cell growth, time-dependent quantitative assay was performed, wherein 36 specimens were subjected to similar interventions. After performing the interventions, specimens were randomized into three different groups, namely Groups A, B and C, and quantitatively analyzed by MTT assay. Growth rate of the cells over a period of 5 days on the cell culture plate without the specimens served as a positive control and labeled as Group D.

Interestingly, on intragroup comparison, uniform monolayer growth of the cells along with good proliferation rate was observed in all the four groups (P < 0.001) [Table 1 and Graph 1]. Tukey’s post hoc analysis revealed that in Groups A, B and C, significant cell proliferation was noticed between days 1 and 3 and days 3 and 5, whereas in Group D, significant cell proliferation was observed at all intervals (P < 0.001) [Table 2].

On intergroup comparison, it was observed that the positive controls expressed a gradual increase in the cell numbers over a period of time in all the three groups (P = 0.001) [Table 3]. Post hoc analysis by Tukey’s test revealed that at all intervals,
Group D showed significant results with respect to cell proliferation ($P = 0.001$) [Table 4].

- Day 1: Comparing the outcomes of Group A with Group B ($P = 0.975$) and Group C ($P = 0.641$) did not yield significant results. However, comparison between Group A and Group D ($P = 0.001$) revealed significant outcomes. Comparison between Group B with Group C ($P = 0.449$) did not reveal any significant results, but comparison between Group B and Group D ($P = 0.001$) showed significant results. Statistical significance was observed between Group C and Group D ($P = 0.001$).

- Day 3: Comparing the outcomes of Group A with Groups B ($P = 0.045$) and C ($P = 0.060$) did not yield any significant outcomes. However, significant results were observed between Group A and Group D ($P = 0.001$). No statistical significance was observed between Group B and Group C ($P = 0.857$), whereas significant outcomes were observed between Group B and Group D ($P = 0.001$). Statistical significance was observed between Group C and Group D ($P = 0.001$).

- Day 5: Comparing the outcomes of Group A with Groups B ($P = 0.849$) and C ($P = 0.882$) did not reveal any significant results. Statistical significance was observed between Group A and Group D ($P = 0.001$). Comparison of outcomes between Group B and Group C ($P = 0.999$) did not significant results. However, significant results were observed between Group B and Group D ($P = 0.001$). Statistical significance was observed between Group C and Group D ($P = 0.001$).

### DISCUSSION

Periodontitis is a chronic disease characterized by the interaction between pathogenic microbes and the host inflammatory response, which results in progressive soft- and hard-tissue destruction and ultimately leading to tooth loss. Mechanical debridement by SRP is one of the most commonly performed procedures for the treatment of periodontal diseases. However, this procedure often leaves a film of smear layer on the root surfaces which are produced by collagen denaturation and may interfere with cell attachment.[10] Laser irradiation is shown to possess bactericidal and detoxification effects. Studies have been published in the literature wherein the efficacy of other laser types such as CO$_2$ and ErCr:YSGG in smear layer removal has been evaluated. To the best of our knowledge, there are no studies published regarding the efficacy of diode laser in smear layer removal. Hence, we carried out the study with the objective of assessing the efficacy of diode laser (970 nm) exposure when used as an adjunct to SRP in promoting fibroblast reattachment. SEM analysis was done as a preliminary study to ascertain cell reattachment on the treated root surfaces and later time-dependent cell growth was quantitatively assessed by MTT assay.

From this study, intragroup comparison showed significant results in all the four groups from day 1 to day 5 ($P = 0.001$). On intergroup comparison, there were significant results observed when Groups A, B and C were compared with Group D ($P = 0.001$) with increased cell proliferation observed in Group D. However, no statistically significant difference was observed when the results were compared between the three study groups. The results of our study were similar to those reported by Aghayan et al. who reported that diode laser (940 nm) exposure after ultrasonic scaling resulted in better fibroblast attachment than ultrasonic scaling alone.[11]

Previous studies published in literatures have reported on outcomes of fibroblast reattachment on root surfaces after smear layer removal by citric acid conditioning, ErCr:YSGG exposure and CO$_2$ exposure. Rompen et al. in their study reported reattachment of fibroblasts to root surface after smear layer removal by citric acid or minocycline.[12]
Savita, et al.: Fibroblast reattachment to laser-treated root surfaces

The inconsistencies in the results were possibly due to prolonged storage of samples in antiseptic solution. Even after repeated washing of the specimens several times in sterile PBS, the yellow stain of the antiseptic was still persistent. It is possible that the iodine in the preservative might have interfered in normal cell growth and exhibited cytotoxicity which has limited cell growth. Another factor could be the variations in dimensions and nonuniformity of the different teeth specimens. Adherent cells such as NIH 3T3 cells require proper and uniform substrate for the cells to attach and grow. Any inclination on the tooth surface would have been difficult for cells to attach and proliferate and hence limit the growth rate. Given the nonuniformity of the tooth surfaces, the probability of the cells getting attached to tooth surfaces could be minimal thus reflecting as poor cell attachment and growth.

CONCLUSION

The outcomes of our study suggested that root surface exposure to diode laser irradiation may have an impact on reattachment of fibroblasts to root surfaces. There were certain challenges in conducting this study. These include maintaining uniform dimensions of the specimens throughout the course of the study, maintaining an aseptic environment and beginning with the culturing and processing steps as early as possible without long interval between the sectioning and transporting to the laboratory. The findings of this study are relatively encouraging. Further, long-term studies of in vitro, ex vivo and animal model designs can be conducted to arrive at a robust conclusion.

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Conflicts of interest

There are no conflicts of interest.

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Table 3: Comparison of outcomes between the groups at various time points (day 1, 3 and 5)

| Assay          | Group  | n   | Mean     | SD       | P     |
|---------------|--------|-----|----------|----------|-------|
| MTT assay:    | Group A| 12  | 0.11217  | 0.007133 | <0.001|
| Day 1         | Group B| 9   | 0.11078  | 0.005890 |       |
|               | Group C| 9   | 0.11611  | 0.009413 |       |
|               | Group D| 9   | 0.23644  | 0.007502 |       |
| MTT assay:    | Group A| 12  | 0.20217  | 0.047124 | <0.001|
| Day 3         | Group B| 9   | 0.16544  | 0.013758 |       |
|               | Group C| 9   | 0.15411  | 0.009048 |       |
|               | Group D| 9   | 0.47489  | 0.026512 |       |
| MTT assay:    | Group A| 12  | 0.13550  | 0.028443 | <0.001|
| Day 5         | Group B| 9   | 0.12578  | 0.015031 |       |
|               | Group C| 9   | 0.12667  | 0.014526 |       |
|               | Group D| 9   | 0.63844  | 0.041089 |       |

SD: Standard deviation

Table 4: Tukey’s post hoc results for outcomes between the groups at various time points (day 1, 3 and 5)

| Assay          | Group         | Mean difference | P     |
|---------------|---------------|-----------------|-------|
| MTT assay:    | Group A       |                 |       |
| Day 1         | Group B       | 0.001389        | 0.975 |
|               | Group C       | −0.003944       | 0.641 |
|               | Group D       | −0.124278       | <0.001|
| MTT assay:    | Group A       |                 |       |
| Day 3         | Group B       | 0.036722        | 0.045 |
|               | Group C       | 0.048056        | 0.005 |
|               | Group D       | −0.272722       | <0.001|
| MTT assay:    | Group A       |                 |       |
| Day 5         | Group B       | 0.011333        | 0.857 |
|               | Group C       | −0.309444       | <0.001|
|               | Group D       | −0.320778       | <0.001|

The proliferation of cells in the positive control exhibited a linear increase in cell number overtime while inconsistencies were observed in the cell growth in the three study groups.

Misra et al. and Sriram et al. reported complete removal of smear layer after CO₂ laser exposure. Crespi et al. reported that adjunctive use of CO₂ laser to SRP promoted fibroblast attachment to root surfaces. Negi et al. reported the fibroblast attachment to root surfaces after neodymium:yttrium-aluminum-garnet exposure. Fekrazad et al. and Liu et al. reported that ErCr:YSGG laser exposure adjunct to SRP provided superior fibroblast reattachment than SRP alone.

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