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

A M Savita[1], Nageswaran G R[1], Roopa Reddy[2]

[1]Department of Periodontics, Dayananda Sagar College of Dental Sciences, [2]Center for Incubation, Innovation, Research and Consultancy, Jyothy Institute of Technology, Bengaluru, Karnataka, India

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

Address for correspondence: Dr. Nageswaran G R, Department of Periodontics, Dayananda Sagar College of Dental Sciences, Shavige Malleshwara Hills, Kumaraswamy Layout, Bengaluru - 560 078, Karnataka, India.
E-mail: g.nageswaran61@gmail.com

Submitted: 11-Feb-2021, **Accepted:** 29-May-2021, **Published:** 31-Aug-2021

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

Access this article online

Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/0973-029X.325129

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: WKHLRPMedknow_reprints@wolterskluwer.com

How to cite this article: Savita AM, Nageswaran GR, Reddy R. 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. J Oral Maxillofac Pathol 2021;25:292-8.

reattachment and impairs the healing of the tissues.[4] Laser irradiation has been reported to show bactericidal and detoxification effects without leaving a smear layer.[5] Furthermore, laser irradiation has been shown to reduce pocket depths and tooth mobility.[6] 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.[7] 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].

Figure 1: Sectioning of the teeth

Figure 2: Flattening with Arkansas stone

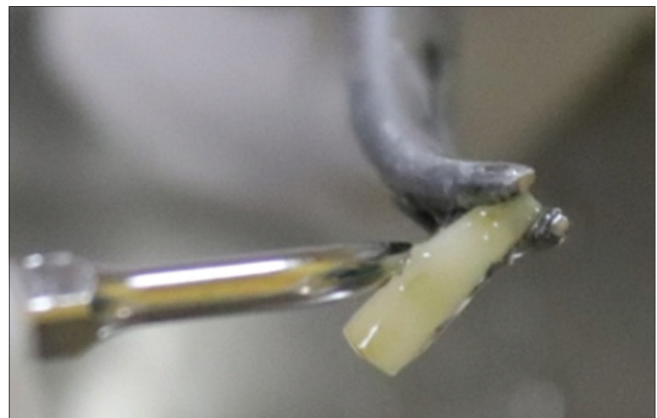


Figure 3: Scaling using piezoelectric ultrasonic scaler

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 1×10^5 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].[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 1×10^5 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

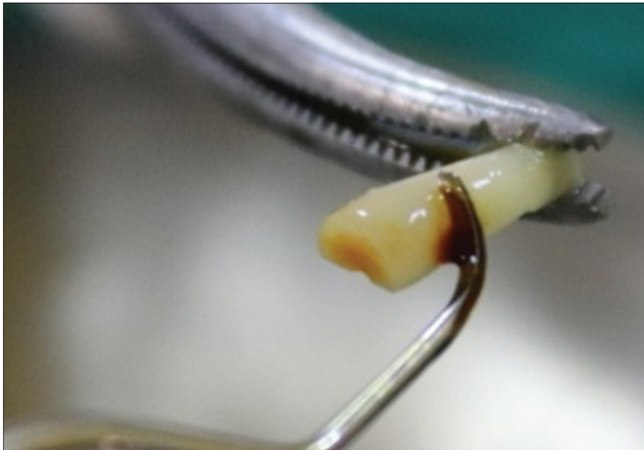


Figure 4: Root planing using Hu-Friedy curette(3/4)

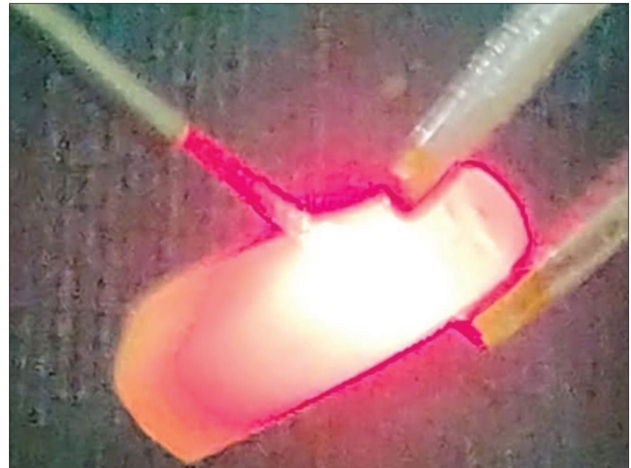


Figure 5: Diode laser exposure

Figure 6: Prepared sections

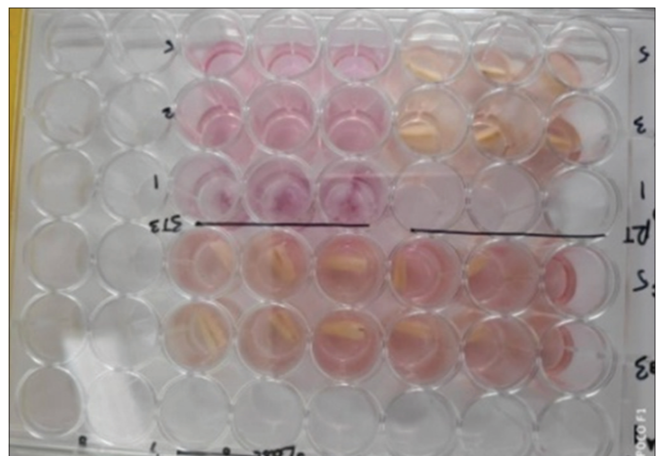


Figure 7: Specimens inoculated onto 48-well culture plates

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

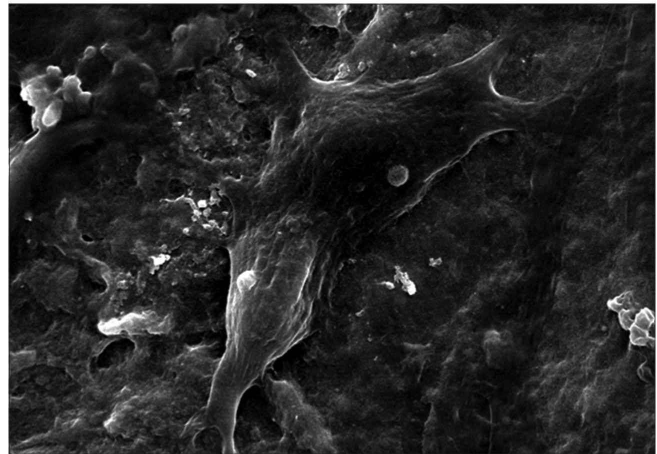


Figure 8: Scanning electron microscopy images showing fibroblast reattachment to the specimens

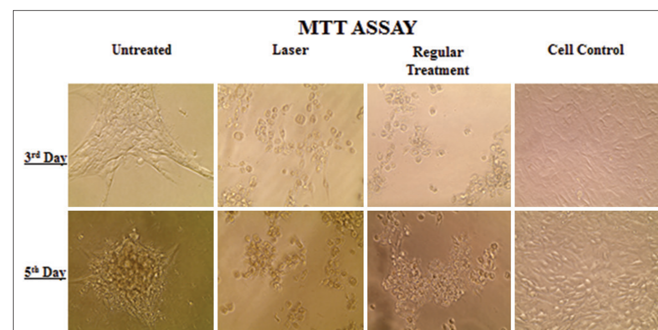
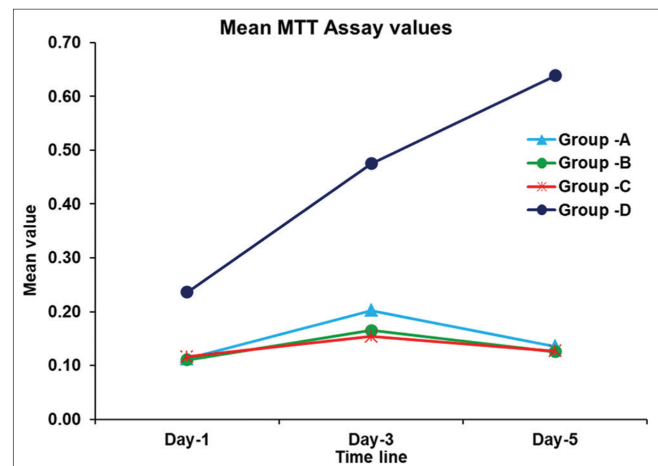


Figure 9: Bright-field images of the cells growing on the culture plate where different tooth samples were placed for MTT assay (images from the 3rd to the 5th day)



Graph 1: Rate of cell proliferation from day 1 (baseline) to day 5 (120 h)

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 ($P = 0.001$).

Table 1: Repeated measures ANOVA to compare the mean MTT values within the groups at different time points

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

SD: Standard deviation

Table 2: Tukey's *post hoc* results to compare the outcomes within the groups between different time points

Group	MTT assay	*P*
Group A	Day 1 versus day 3	<0.001
	Day 1 versus day 5	0.015
	Day 3 versus day 5	<0.001
Group B	Day 1 versus day 3	<0.001
	Day 1 versus day 5	0.006
	Day 3 versus day 5	<0.001
Group C	Day 1 versus day 3	<0.001
	Day 1 versus day 5	0.061
	Day 3 versus day 5	<0.001
Group D	Day 1 versus day 3	<0.001
	Day 1 versus day 5	<0.001
	Day 3 versus day 5	<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]

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: Day 1	Group A	12	0.11217	0.007133	<0.001
	Group B	9	0.11078	0.005890	
	Group C	9	0.11611	0.009413	
	Group D	9	0.23644	0.007502	
MTT assay: Day 3	Group A	12	0.20217	0.047124	<0.001
	Group B	9	0.16544	0.013758	
	Group C	9	0.15411	0.009048	
	Group D	9	0.47489	0.026512	
MTT assay: Day 5	Group A	12	0.13550	0.028443	<0.001
	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)

MTT assay	Group	Mean difference	*P*
MTT assay: Day 1	Group A		
	Group B	0.001389	0.975
	Group C	−0.003944	0.641
	Group D	−0.124278	<0.001
	Group B		
	Group C	−0.005333	0.449
	Group D	−0.125667	<0.001
	Group C		
MTT assay: Day 3	Group D	−0.120333	<0.001
	Group A		
	Group B	0.036722	0.045
	Group C	0.048056	0.005
	Group D	−0.272722	<0.001
	Group B		
	Group C	0.011333	0.857
	Group D	−0.309444	<0.001
	Group C		
MTT assay: Day 5	Group D	−0.320778	<0.001
	Group A		
	Group B	0.009722	0.849
	Group C	0.008833	0.882
	Group D	−0.502944	<0.001
	Group B		
	Group C	−0.000889	0.999
	Group D	−0.512667	<0.001
	Group C		
	Group D	−0.511778	<0.001

Misra *et al*. and Sriram *et al*. reported complete removal of smear layer after CO_2 laser exposure.[13,14] Crespi *et al*. reported that adjunctive use of CO_2 laser to SRP promoted fibroblast attachment to root surfaces.[15] Negi *et al*. reported the fibroblast attachment to root surfaces after neodymium:yttrium-aluminum-garnet exposure.[16] Fekrazad *et al*. and Liu *et al*. reported that ErCr:YSGG laser exposure adjunct to SRP provided superior fibroblast reattachment than SRP alone.[7,17]

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. 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. However, further long-term studies of *in vitro, ex vivo* and animal model designs can be conducted to arrive at a robust conclusion.

Acknowledgment

We would like to thank Mr. K. Boopathi, Senior Technical Officer, ICMR- NIE, Chennai, for his valuable contribution with the statistical analysis.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Newman MG, Carranza FA, Takei H, Klokkevold PR. Carranzas Clinical Periodontology. 10th ed. Amsterdam: Elsevier Health Sciences; 2006.
2. Adriaens PA, De Boever JA, Loesche WJ. Bacterial invasion in root cementum and radicular dentin of periodontally diseased teeth in humans. A reservoir of periodontopathic bacteria. J Periodontol 1988;59:222-30.
3. O'Leary TJ, Kafrawy AH. Total cementum removal: A realistic objective? J Periodontol 1983;54:221-6.
4. Schwarz F, Aoki A, Sculean A, Georg T, Scherbaum W, Becker J. *In vivo*

effects of an Er:YAG laser, an ultrasonic system and scaling and root planing on the biocompatibility of periodontally diseased root surfaces in cultures of human PDL fibroblasts. Lasers Surg Med 2003;33:140-7.

5. Kreisler M, Al Haj H, D'Hoedt B. Clinical efficacy of semiconductor laser application as an adjunct to conventional scaling and root planing. Laser Surg Med 2005;37:350-5.
6. Mirzaei A, Gholami L. Effects of 980nm diode laser irradiation on gingival fibroblasts and periodontal ligament stem cells. Acta Sci Micro 2020;3:50-4.
7. Fekrazad R, Lotfi G, Harandi M, Ayremlou S, Kalhori KA. Evaluation of fibroblast attachment in root conditioning with Er, Cr:YSGG laser versus EDTA: A SEM study. Microsc Res Tech 2015;78:317-22.
8. Nataraj D, Reddy R, Reddy N. Crosslinking electrospun poly (vinyl) alcohol fibers with citric acid to impart aqueous stability for medical applications. Eur Polym J 2020;124:109484.
9. Battampara P, Nimisha Sathish T, Reddy R, Guna V, Nagananda GS, Reddy N, *et al*. Properties of chitin and chitosan extracted from silkworm pupae and egg shells. Int J Biol Macromol 2020;161:1296-304.
10. Bowen RL, Eick JD, Henderson DA, Anderson DW. Smear layer: Removal and bonding considerations. Oper Dent Suppl 1984;3:30-4.
11. Aghayan S, Ebrahimi H, Jafari ES, Hashemi SS, Sheidaeen M. Effect of 940 nm diode laser as adjunct treatment to mechanical instrumentation on root surface gingival fibroblast adhesion in periodontally compromised extracted teeth: An *in vitro* study. J Dent Mater Tech 2020;9:123-9.
12. Rompen EH, Goffinet GH, Nusgens B. Human periodontal ligament fibroblast behavior on chemically conditioned dentine: An *in vitro* study. J Periodontol 1999;70:1144-52.
13. Misra V, Mehrotra K, Dixit J, Maitra S. Effect of a carbon dioxide laser on periodontally involved root surfaces. J Periodontol 1999;70:1046-52.
14. Sriram T, Mythili R, Gokul K, Kumar SS, Anuradha B. Efficacy of CO_2 laser on removal of smear layer – An *in-vitro* study. Int J Curr Res Rev 2012;4:101-10.
15. Crespi R, Barone A, Covani U, Ciaglia RN, Romanos GE. Effects of CO_2 laser treatment on fibroblast attachment to root surfaces. A scanning electron microscopy analysis. J Periodontol 2002;73:1308-12.
16. Negi S, Krishnamurthy M, Ganji KK, Pendor SS. Modulatory effects by neodymiumdoped yttrium aluminum garnet laser on fibroblast attachment to single rooted tooth surfaces following ultrasonic scaling and root planing: An *in vitro* study. J Indian Soc Periodontol 2015;19:25-31.
17. Liu J, Zhou Z, Zhang S. Effects of Er:YAG laser on the attachment of human periodontal ligament fibroblasts to denuded root surfaces simulating delayed replantation cases: An *in vitro* study. Photobiomodul Photomed Laser Surg 2020;38:145-50.