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Effect of GAALAS Laser Irradiation on the Proliferation Rate of Periodontal Ligament Fibroblasts -An *In* Vitro Study

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Abstract: Background: *Lasers depending on the wavelength, dose, and irradiation time are found to increase the proliferation rate of periodontal ligament fibroblasts and accelerates collagen synthesis thus have an effect on the wound healing in the cellular aspect.*

Objectives: *The present in vitro study was done to evaluate the rate of proliferation of human periodontal ligament fibroblasts by GaAlAs laser of (940 nm and power out put 400mw), at different irradiation time.*

Methods: *Primary cultures of hPDLc were obtained from healthy premolars. The study consisted of 48 samples which were assigned to five groups (4 test groups and 1 control). After seeding and incubation of periodontal ligament cells they were distributed in to five groups on 96-well tissue culture plates at a density of 10 x104 cells/cm2.Among these groups four groups were subjected to laser irradiation at different time period of 30, 60, 90 and 120 sec and one group was taken as the control. MTT assay was performed to determine the effect of these treatments on the proliferation rate of the cells on 24, 48 and 72 hrs.*

Results: *Statistically high significant rate seen when the irradiation time was increased from 30 sec to 120 sec at all hours of 24, 48 and 72. But when each group is compared on 1st, 2nd and 3rd day of incubation, 30 sec irradiated group showed a statistically significant increase in the rate of proliferation.(F value=12.95), (p=0.0001).All other groups of 60, 90, and 120 sec irradiation showed an increase in the mean proliferation rate but was not statistically significant.*

Conclusion: *Soft tissue diode laser (940nm, power output 400mw) causes an increase in the mean proliferation rate of human periodontal ligament fibroblasts in vitro. Further investigation are required with regard to the effect of the diode laser on cell attachment, migration and proliferation.*

Keywords: *Soft tissue diode laser,periodontal ligament fibroblasts*

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I. Introduction

Periodontitis is characterized by the destruction of the supporting structures of the teeth, including the periodontal ligament, bone and soft tissues[1]. The elusive goal in Periodontics is the regeneration of the tooth supporting structures. Periodontal regeneration involves several cell types: fibroblasts, cementoblasts, osteoblasts and endothelial cells. The fibroblast is the major cell type in the periodontal connective tissues as it plays a significant role in normal turnover, repair, and regeneration. The expression of osteoblast like properties by periodontal ligament cells, such as production of alkaline phosphatase, is of pivotal importance for the regeneration[5, 6]. Hence these cells are described as the "architect, builder, and caretaker of connective tissue"[7]. More recently, several studies have shown that laser therapy can enhance the fibroblast proliferation.

Among laser applications, low-level laser therapy (LLLT) has been widely applied as part of the treatment of oral diseases in dentistry.These soft lasers emit light of very low energy density and presumbly promote certain biochemical reactions with out inducing thermal effects. Two laser types are used in this field: helium neon lasers with a wavelength of 633 nm and semiconductors lasers emitting light in the range of 780-950 nm[11]. The effects that it can produce on the cell are related to photochemical reactions within cells, rather than thermal effects, although the mechanisms behind this are still unclear.A majority of animal experiments suggest that low-intensity lasers enhance wound healing by promoting cell proliferation and accelerating collagen synthesis. But, in some other studies no additional improvement with adjunctive application of laser was found in comparison to scaling and root planing[13].Problems encountered when interpreting the results of studies include the use of different kinds of lasers, time exposures, energy settings, laboratory animals, and cell types. However, it has been shown that depending on the wavelength, dose, and local conditions on soft tissues,

it is possible that LLLT effects on wound healing depend not only on the total dose of irradiation but also on the irradiation time and the irradiation mode.

Unfortunately, well controlled, in vivo, human studies of laser biostimulation are lacking. Well controlled research needs to be carried out in animal models that have similar features to man. In periodontal procedures GaAlAs is the commonly used soft tissue diode laser. Hence an attempt is made to evaluate the effect of GaAlAs diode (ezlase) laser of 940 nm wavelength and 400mW power output on the cultured periodontal ligament fibroblasts.

II. Materials And Methods

Specimen Samples are Cultured human periodontal ligament Fibroblasts. The study was done in Govt. Dental College, Thiruvananthapuram and Rajiv Gandhi Center for Biotechnology Jagathy, Thiruvananthapuram. Human periodontal ligament fibroblasts was obtained from the Maxillary and mandibular non carious and periodontally healthy premolar teeth atraumatically extracted for orthodontic correction.

Storage And Transportation

The extracted teeth were placed immediately in a tube containing Hank's Balanced Salt Solutions (HBSS, Gibco New York USA) supplemented with antibiotics adjusted to pH 7.4. After extraction, contact of teeth with hand or any surface was avoided to prevent its contamination. The teeth samples were immediately transported to Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram.

Armamentarium For Pdl Cell Culture

Phosphate-buffered saline , 0.2% trypsin, Collagenase, Dulbecco's modified Eagle's medium (DMEM) , 25 cm^2 tissue culture flask (B.D. Falcon),Incubator maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2 (Sanyo, Japan)

Cell Culture

Under sterile conditions the tooth was washed with HBSS with antibiotics and then rewashed with 0.2% trypsin .Then 0.2% trypsin was added and kept for 2 hours and collected the cells in serum containing medium tube and then resuspended the cells in serum containing medium. The tubes were centrifuged (5000rpm/ 5min), the supernatant was discarded and cells were collected. The cells are allowed to multiply from the explants at 37°C in a humidified atmosphere of 95% air and 5% CO2. The cell cultures were observed periodically under microscope for visible growth and found to be mycoplasma free. Once the growth was achieved, the cells were trypsinized and transferred into 25 cm^2 tissue culture flask containing DMEM supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic antimycotic solution with penicillin G potassium (100µl), streptomycin sulfate (100 µg/ml) and amphotericin B (10µg/ml) at pH 7.4.

Culture flasks were maintained in an incubator with the same culture medium at 37°C in a humidified atmosphere of 95% air and 5% CO2 for 2-7 days. After incubation, the medium was replaced every alternate day until cell outgrowth created a subsequent monolayer. The cells were detached by trypsin / EDTA treatment and transferred to 25 cm^2 tissue culture flask for continued growth until the required number of cells was reached. Cells between the third and fifth passages were used in the experiment.

Seeding of cells for study

After the confluence was reached, cells were passaged using trypsin- EDTA (0.2%) and PBS treatment. Cells were collected and seeded in flat bottomed 96-well tissue culture plate with well diameter of 6mm at a density of 10 $x10^4$ cells/cm^2 in DMEM supplemented with 10% fetal bovine serum. These plates were then immediately transferred to Department of Periodontology where these groups were subjected to laser irradiation.

Sample Size

The study comprises of five groups. Cells at a density of 10 $x10^4$ cells/cm^2 is seeded in each 10 wells for each group. Total sample size was 48 samples. 10 samples (10 wells) in each of the four groups : group I, group II, group III, group IV: (10 × 4= 40). Corresponding two wells of each 4 group which were not subjected to laser treatment was taken as the control, group V: (4 × 2 =8). Total sample size =40+8=48

Study Group

The study comprises of five groups with laser irradiation at four different time periods and one group as control.
Group I – 30 sec laser irradiation
Group II – 60 sec laser irradiation
Group III – 90 sec laser irradiation
Group IV – 120 sec laser irradiation

Group V – No irradiation. For each group the corresponding two wells which were not subjected to laser treatment were taken as the control.
Each group was incubated at 24 hrs, 48 hrs and 72 hrs after laser irradiation
Proliferation assessed quantitatively by MTT assay.

Laser Dosimetry
The irradiation parameters of laser unit (*model no. 7400001, Biolase Technology Inc*) were as follows:
Wave length = 940 nm
Power output = 400 mW
Position of Laser Probe = Non-contact mode (Distance – 1 cm)
Time = 30, 60, 90 and 120 sec
Energy = 12 J, 24 J, 36 J, 48 J corresponding to the time of irradiation
The laser light was delivered with ezTips which was 400μm thick.

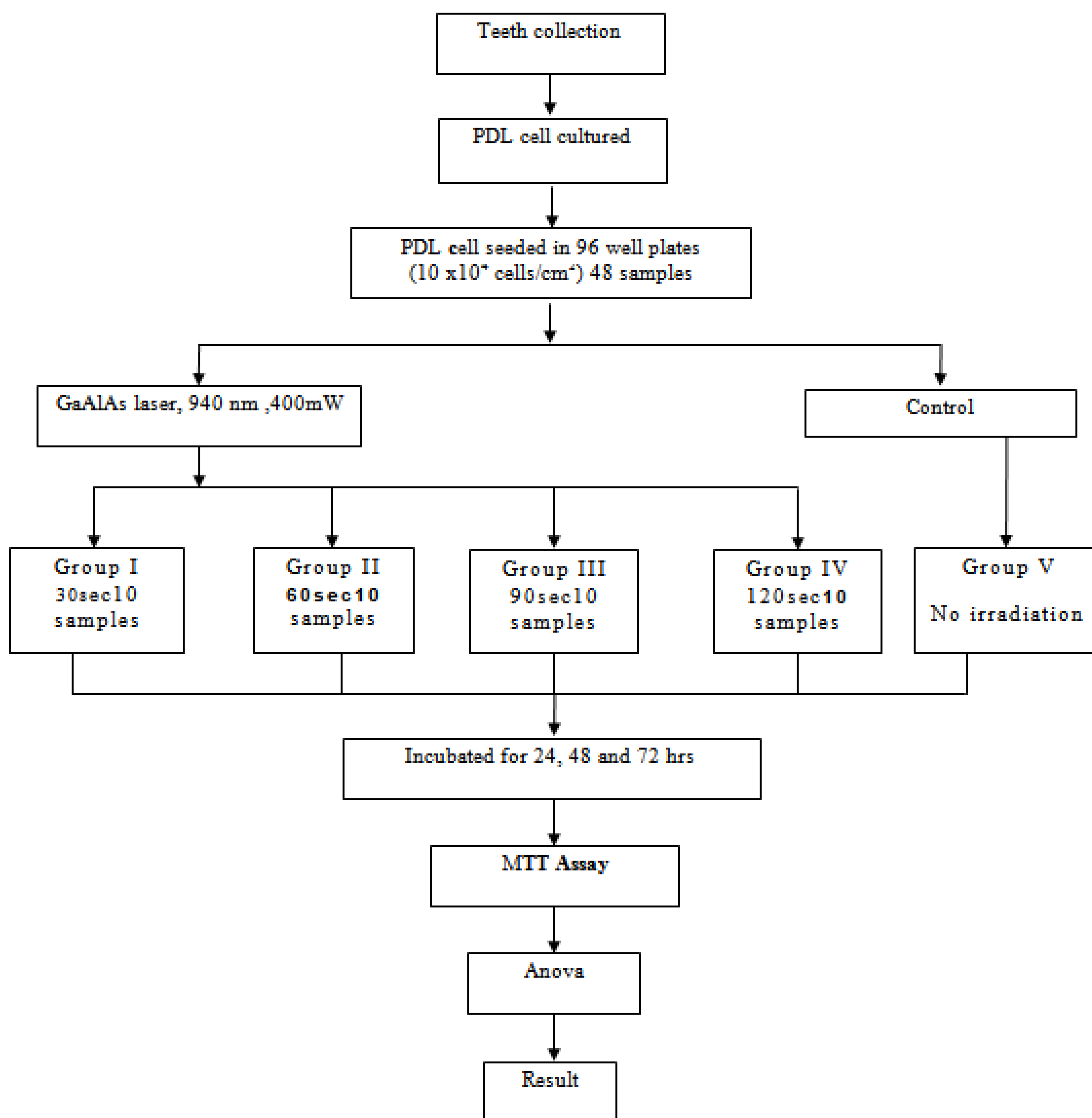
Procedure
All groups except control were subjected to treatment by GaAlAs laser. The Laser light with the wavelength of 940 nm and 400mW power was used in a non-contact mode. The distance between the fibre and the sample was 1cm. During the delivery of the laser light to this group, other wells were kept covered with aluminium foil. As a function of irradiation time the energy was 12 J, 24 J, 36 J, 48 J respectively.
After laser irradiation the plates were transferred immediately to Rajiv Gandhi Center for Biotechnology Jagathy, Thiruvananthapuram for the cell proliferation assay. All groups were incubated for 24, 48, and 72 hours.

Cell Proliferation Assay: MTT Assay
MTT assay was done to evaluate the rate of proliferation of cell. The chief advantage of this assay is that it requires fewer cells than standard assays. It is a fast, simple, cheap, accurate and a reliable method. In particular, the MTT method proved to be useful to estimate cell densities in small culture volumes and was more accurate and reliable than haemocytometer counting.
A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl)-2H-tetrazolium (MTT) assay was performed to assess the cell proliferation activity. The MTT assay is the colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. A main application of this assay is to assess the viability (cell counting) and the proliferation of cells .

Calculation of MTT assay
At 24 hrs for each group of I,II,III,IV 10 values are obtained. (For 10 wells)
For each well the % of cell viability is calculated by the formula:
% of cell viability=test value / control value × 100.
Mean of these 10 values for each group was calculated.
Similar calculation was done for all plates at 48 and 72 hrs. Then statistical analysis was done.

Statistical Analysis
Data were analyzed using computer software, Statistical Package for Social Sciences (SPSS) version 10. Data are expressed in its mean and standard deviation. Analysis of variance (One Way ANOVA) was performed as parametric test to compare different groups. Tukeys test was employed as post hoc analysis to elucidate comparisons among different groups. For all statistical evaluations, a two-tailed probability of value, < 0.05 was considered significant.

FLOW CHART SHOWING STUDY DESIGN



III. Results

DATA ANALYSIS

The mean and S.D values of MTT assay results at different time periods in different hours.

TABLE – 1

Observation	Group	Mean	S.D	F value	p value
At 24 hrs	30 sec	76.584	2.972		
	60 sec	92.017	5.149	61.150	0.0001
	90 sec	103.445	7.143		
	120 sec	109.085	6.947		
At 48 hrs	30 sec	78.294	10.615		
	60 sec	95.714	3.932	25.082	0.0001
	90 sec	106.974	10.222		
	120 sec	114.751	12.912		
At 72 hrs	30 sec	91.742	6.181		
	60 sec	98.242	7.273	8.317	0.0001
	90 sec	111.092	13.561		
	120 sec	117.648	19.881		

Table 1 shows the mean and S.D values of MTT assay results at different time periods of 30, 60,90 and 120 sec in different hours of 24,48 and 72 hrs. At 24 hrs there is statistically significant increase in the rate of proliferation in all groups. (F value=61.2), (p=0.0001).Statistically significant increase in the rate of proliferation in all groups is seen at 48 hrs of incubation. (F value=25.1), (p=0.0001). The rate of proliferation in all groups at 72 hrs of incubation was shown to be statistically significant. (F value=8.32), (p=0.0001).This shows that groups I(30 sec) II(60 sec), III(90 sec), IV(120 sec) shows an increase in the mean rate of proliferation of periodontal ligament fibroblasts on 1st,$2^{nd}$and 3rd day after incubation. When irradiation time is increased it is seen that there is an incremental increase in the proliferation rate from 30 sec to 120 sec. At all hrs of incubation there is a highly significant increase in the proliferation rate from group I (30 sec)to group IV(120 sec) irradiation. Thus PDLFs proliferation increased in time dependent manner up to 72 hrs.

TABLE – 2 Mean and S.D of assay results at different hrs in different secs.

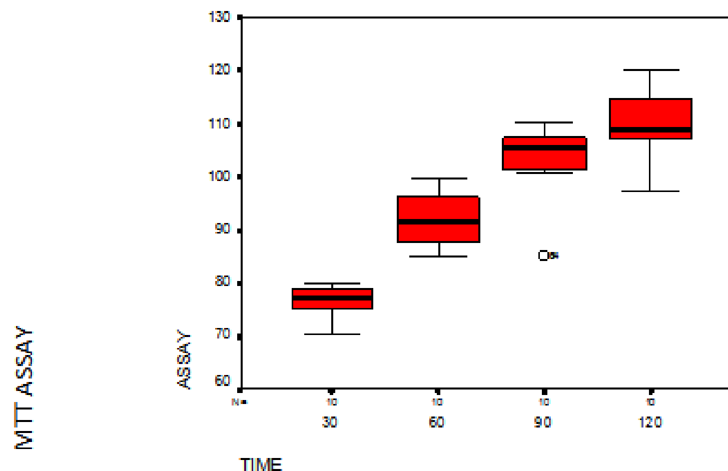
Observation	Group	Mean	S.D	F value	p value
At 30 sec	24 hrs	76.584	2.972		
	48 hrs	78.294	10.615	12.947	0.0001
	72 hrs	91.742	6.181		
At 60 sec	24 hrs	92.017	5.149		
	48 hrs	95.714	3.932	3.10	0.061
	72 hrs	98.242	7.273		
At 90 sec	24 hrs	103.445	7.143		
	48 hrs	106.974	10.222	1.295	0.029
	72 hrs	111.092	13.561		
At 120 sec	24 hrs	109.085	6.947		
	48 hrs	114.751	12.912	0.933	0.406
	72 hrs	117.648	19.881		

Table 2 shows the mean and S.D of assay results at different hours of 24,48 and 72 hrs in different groups I(30 sec) II,(60 sec), III,(90 sec), IV,(120 sec). A comparison is made among all groups at 24, 48 and 72 hrs. In group I (30 sec) the mean value at 24 hrs was 76.58±2.97, at 48 hrs 78.29±10.62, at 72 hrs 91.74±6.18. Highly significant PDL fibroblasts proliferation was observed for this group. (F value=12.95), (p=0.0001). In group II (60 sec) at 24 hrs the mean value was 92.02±5.15 at 48 hrs 95.71±3.93 at 72 hrs 98.24±7.27 (F value=3.10), (p=0.061). There is gradual increase in the rate of proliferation at 72 hrs, but no significant difference was seen. In group III (90 sec) at 24 hrs the mean value was 103.44±7.14, at 48 hrs 106.97±10.22, at 72 hrs 111.09±13.56. (F value=1.295), (p=0.29). An increase in the rate of proliferation was seen with no statistical significant difference. In group IV (120sec) at 24 hrs the mean value was 109.08±6.95, at 48 hrs 114.75±12.91, at 72 hrs 117.65±19.88. (F value=0.933), (p=0.406). An increase in the rate of proliferation with no statistical significant difference was seen.

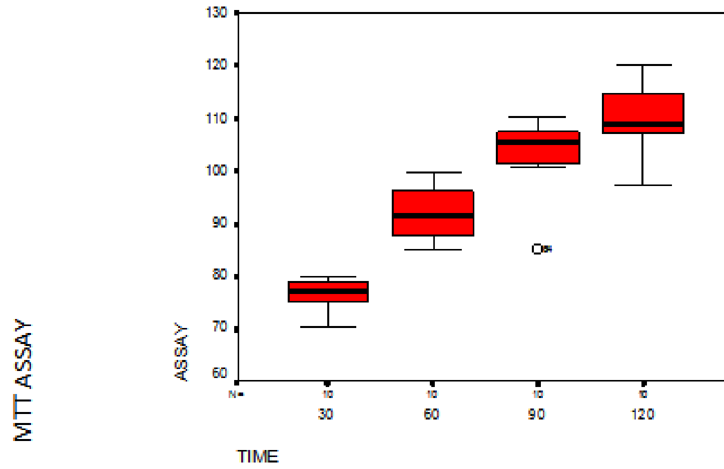
Though there is increase in the mean proliferation rate seen in all irradiated groups, statistical analysis (ANOVA) has shown a significant increase only in the group I (30 sec) irradiated group when compared on 1st,2nd and3^{rd} day.

Analysis of variance (One Way ANOVA) comparing mean values of MTT assay at different time periods in different hours.

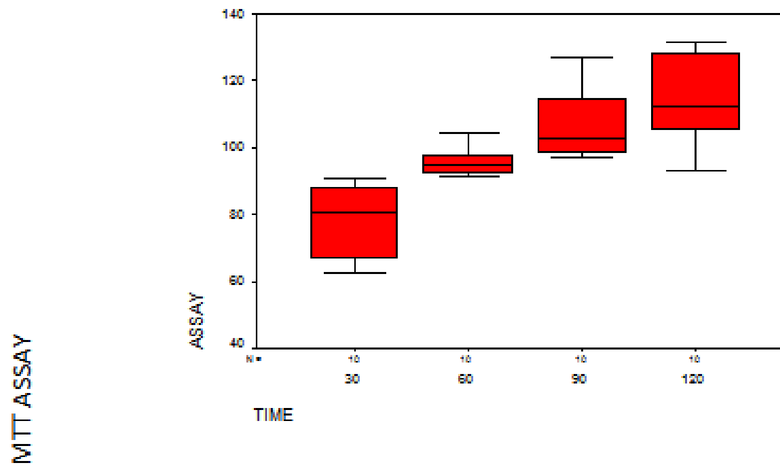
At 24 hrs comparison of 30, 60, 90, and 120 sec groups



At 48 hrs comparison of 30, 60, 90, and 120 sec groups

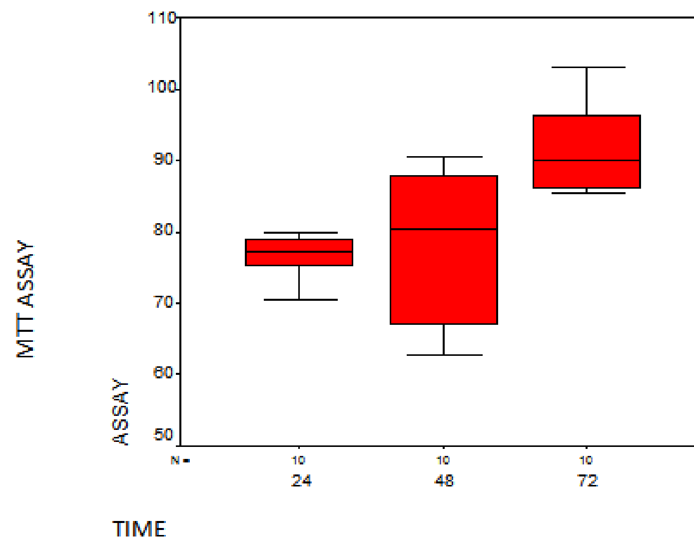


At 72 hrs comparison of 30, 60, 90, and 120 sec groups

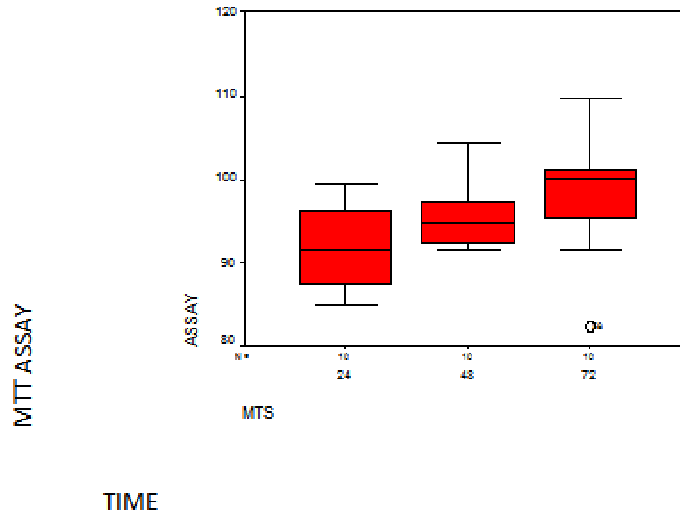


Analysis of variance (One Way ANOVA) comparing mean values of MTT assay at different hours in different secs.

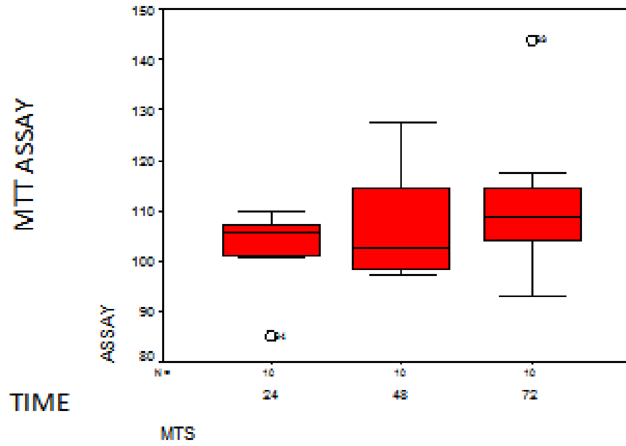
Group I (30 sec) compared at 24,48 and 72 hrs.



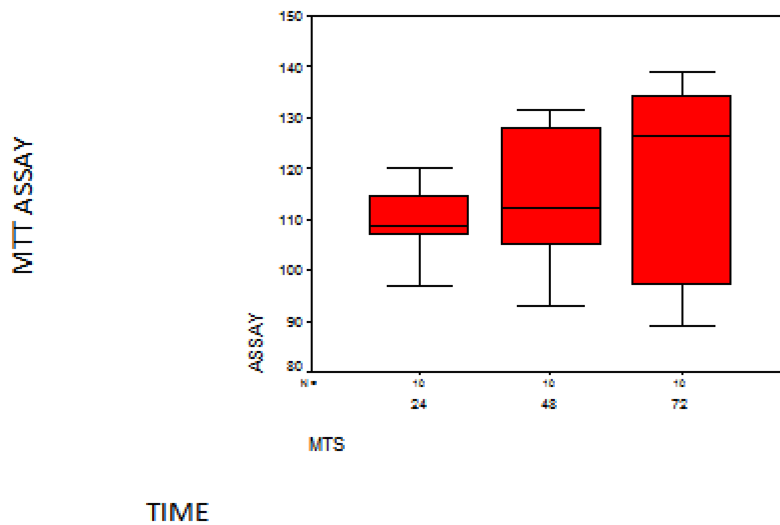
Group II (60 sec) compared at 24,48 and 72 hrs.



Group III (90 sec) compared at 24,48 and 72 hrs.



Group IV (120 sec) compared at 24, 48 and 72 hrs.



IV. Discussion

The present in vitro study was done to evaluate the effect of GaAlAs semiconductor diode laser (Ezlase) in the proliferation rate of periodontal ligament fibroblast cells. A wavelength of 940 nm and power output of 400 mW was used. After laser irradiation of the four groups of cultured periodontal ligament fibroblasts for 30, 60, 90 and 120 sec, each plates were incubated for 24, 48 and 72 hrs. Cell proliferation was assessed by MTT assay at each hour. The study was designed as an in vitro study because the use of in vitro models proves to be very helpful in assessing newer therapeutic adjuncts. They allow more controlled conditions than it is possible in vivo experiments and also to perform measurements that would otherwise not possible or ethical in living subjects. This allows determining their efficacy before they can be introduced into in vivo set-up. Also, clinical trials pose number of limitations like patient recruitment problems, patient compliance, lack of correlation of diagnosis versus patient response and observable clinical symptoms, cost and large reported placebo effects[120]. Human periodontal ligament cells were cultured and utilized in this study**.** It is currently thought that these periodontal progenitor cells reside in the periodontal ligament remaining around the tooth as they have the capacity to function as osteoblasts and cementoblasts. They are considered as the multi potent cells[121]**.** The expression of osteoblast – like properties such as the production of alkaline 67 hosphatise is of pivotal importance for the regeneration of periodontal tissues[4]. Several investigators have successfully cultivated the cells of periodontal ligament earlier and the culturing is easy as compared to other cells like osteoblasts and cementoblasts [122,123].

Today in medicine and dentistry, diode lasers have been used predominantly [124]. These lasers are compact, low cost devices which have very high electrical and optical efficiencies. They can produce higher power outputs in a continual wave mode, and are reliable. These lasers are currently used for pocket curettage because of their flexible fiber delivery system, which is suitable for pocket insertion. The diode laser (Ezlase) with a wave length of 940 nm was used in this study. This is in accordance with the study by Elke M. Vinck[77]who showed that there is an increased fibroblast proliferation with a LED light source emitting wavelengths 950 nm. Also procollagen production in human skin fibroblast was found to be increased by 904 nm GaAlAs laser by Lam et al 1986[126]. When El sayed , Dayson et al[127] compared different wavelength in in vivo studies they found that 940 and 950 nm wavelength produced an increase in mast cell number and degranulation in wounds in rats.

The power of the GaAlAs lasers used in this study was 400mW.The biomodulatory effect of LLL i.e., power ≤ 500 mW, on cell functions like change in cell numbers and the rate of collagen synthesis of human fibroblasts have been reported previously[68,114-118]. This is consistent with the study of Kreisler et al [11] in which when power is increased from 0.5–2.5 W cell numbers of human gingival fibroblast was found to be decreased. In another study when 500 mW was used by Eun-Jeong et al [85] proliferation of cultured PDLFs was seen. A power of 10mW was also fond to enhance the proliferation of PDLFs by Kreisler et al [76].

Another example of the importance of this parameter selection was reported by Kreisler et al [73] i.e., little to no damage on the root surface is seen at a power output of ≤1W, whereas power selections of 1.5, 2.0, and 2.5 W produced varying degrees of carbonization and heat-induced surface cracking following in vitro irradiation of tooth root specimens with the GaAlAs diode. A pilot study has been performed with different power out put. Of these, the power output of 400mW gave an enhancing effect on the proliferation rate of PDL cells. So the power of the study was fixed to 400mW.

The duration of irradiation in the present study was 30, 60, 90 and 120 sec and incubation time was 24, 48 and 72 hrs. This is in accordance with the previous studies of Henrik Loevschall [69] who found that LLL irradiation for 0, 1, 3, 10, 32, 100, 316, or 1,000 seconds induced increased DNA synthesis of human oral mucosa fibroblasts. Kreisler et al [76] also observed that 75–300 s of irradiation increased the proliferation activity of human periodontal ligament fibroblasts on 1st 2nd and 3rd day. Another study of Eun-Jeong et al [85] showed the same result that 10, 20 and 30 sec irradiation for the first 3 days of incubation caused an increase in the proliferation rate of PDLFs. So an attempt is made to know whether different duration of irradiation and time period is having an effect on fibroblast proliferation.

To evaluate the rate of proliferation and cell viability, MTT assay was done. The chief advantage of this assay is that it requires fewer cells than standard cytotoxicity assays. It is a fast, simple, cheap, accurate and a reliable method. In particular, the MTT method proved to be useful to estimate cell densities in small culture volumes and was more accurate than haemocytometer counting[128]. For data analysis an ELISA plate reader linked with a computer is used which allows rapid and automated data processing.

Results of the present study showed that there is mean increase in the rate of proliferation of periodontal ligament fibroblast when they are irradiated at 30, 60, 90 and 120 sec. This shows that when the irradiation time was increased from 30 to 120 sec there was statistically significant increase in the proliferation rate at all hrs of 24,48 and 72. This is in accordance with the study by Eun-Jeong et al [85] who showed that a GaAlAs diode laser with a wavelength 810 nm and power output 500 mW caused an increase in the proliferation rate of PDLFs when irradiated at 10, 20 and 30 sec for the first 3 days of incubation. Another study

by shelly et al [80] also showed the similar finding 3 days after 904 GaAs laser irradiation. When laser irradiation was done with 660 nm, Cecilia et al [72] found a stimulation of fibroblasts for the first 4 days. The reason may be the biostimulation effects of low-level laser with molecular absorption of laser light causing an increase in cellular metabolism characterized by stimulation of photoreceptors in the mitochondrial respiratory chain, changes in cellular ATP levels, release of growth factors, and collagen synthesis [129].

But the present result is inconsistent with the previous study of Kreisler M et al [76] in which they used GaAlAs laser with a wavelength of 809nm and duration of 75-300 sec and PDLFs proliferation was found to be increased significantly for the first two days and in the 3rd day it was reduced. Another study by Anthony Pogrel [70] showed no biostimulatory effect on fibroblasts when GaAlAs laser with a 5-100 mW and 10-120 sec was used. The reasons for these conflicting results may be the inconsistency in the differences in power output, and irradiation time. However further laboratory research work is required to understand the mechanism leading to the stimulation of periodontal fibroblasts in these laser parameters.

From our study the lowest irradiation time of 30 sec was found to be showing a significant increase in the proliferation when all the three days were compared. This is consistent with the study of Almeida-Lopes et al[74] who showed that the time of exposure to laser radiation interferes with the repair process, shorter the time of exposure to the laser, the greater the proliferation of fibroblasts. Kreisler et al also consider the time of exposure to laser radiation important for the stimulation of fibroblast proliferation, which is even more relevant than the actual laser power.

Therapeutic methods to produce predictable regeneration of periodontium lost as a result of periodontal disease are still being investigated as a main goal in periodontal therapy. The use of a laser has been increasingly suggested as a more selective, more efficient, less difficult instrumentation and atraumatic technique to promote periodontal healing. But only a limited number of studies have evaluated the biostimulatory effect of low level laser therapy and its clinical efficacy. Thus, additional well controlled studies are needed to carry out in in vivo models to determine the ideal parameters to obtain a good environment for maximum healing during periodontal treatment. Moreover, clinical studies are needed to evaluate whether the application of low-level laser might be beneficial in regenerative periodontal therapy.

V. Conclusion

(iii) Soft tissue diode laser causes an increase in the mean proliferation rate of human PDL fibroblasts when they are irradiated at 30, 60, 90 and 120 sec with a wavelength of 940 nm and power output of 400mW in a non contact mode.
(ii) Statistically high significant rate seen when the irradiation time was increased from 30 to 60,90 and 120 sec at all hours of 24, 48 and 72.
(iii) Group I (30 sec) showed a statistically significant increase in the rate of proliferation when compared at 24,48 and 72 hrs. (F value=12.95), (p=0.0001).All other groups of 60, 90, and 120 sec irradiation showed an increase in the mean proliferation rate but was not statistically significant.

Thus we concluded that soft tissue diode laser (λ=940nm, power output 400mW) causes an increase in the mean proliferation rate of human periodontal ligament fibroblasts.

The application of lasers as an adjunct to conventional periodontal therapy is becoming more and more prevalent in dentistry. A beneficial effect from adjunctive laser application with regard to the formation of new connective tissue attachment remains questionable. Further in vitro investigation, especially with regard to the effect of the diode laser on cell attachment, migration and proliferation is needed. This can subsequently improve healing after periodontal reconstructive surgery.

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