An In Vitro Study of the Reaction of Periodontal and Gingival Fibroblasts to Low-level Laser Irradiation: A Pilot Study

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Purpose: To evaluate the effect of laser irradiation on cell proliferation of periodontal and gingival fibroblasts with a low energy level laser.

Materials and Methods: Periodontal and gingival fibroblast cultures were obtained from the tissue bank of the Universidad del Valle (Cali, Colombia). After 24 h of incubation, they were irradiated while in the logarithmic phase of growth, using a low energy level laser (Photon LASE As-Ga-Al), operated at 37 mW, 830 nm wavelength at an energy level of 3.75 J/cm^2. The rate of cell proliferation was evaluated in experimental and control cultures by the XTT test (Roche). The percentage of cytotoxicity was measured by the LDH test (Roche). The rate of proliferation was measured 24, 48, 72, 96, 120, and 144 h after irradiation. The cell cytotoxicity was measured 24 h after irradiation.

Results: The irradiation protocol used in this study did not cause cytotoxicity in any of the cell lines evaluated. The rate of gingival fibroblast proliferation was higher in the experimental culture than in the controls, although the difference was not statistically significant, while for periodontal fibroblasts the rate of cell proliferation was higher in the control group, but not significantly differently.

Conclusion: The cytoxicity test indicates that this laser treatment protocol is safe for both cell lines. There are no statistically significant differences in the rate of cell proliferation between or within groups.

Keywords: periodontal fibroblasts, gingival fibroblasts, low energy level laser, orthodontic tooth movement.

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Orthodontic dental movement is based upon the ability of the periodontal ligament and the alveolar bone to adapt themselves and be remodeled. The force applied to a tooth crown induces a chain of deformations in the surrounding tissues that ends as a cell deformation.

Previous studies[1-7] carried out on gingival fibroblasts, periodontal fibroblasts, and osteoblasts indicate that this kind of laser promotes cellular function and proliferation. It has been considered that laser irradiation acts on the cell at three essential levels: mitochondrial, nuclear and cell membrane, regulating permeability for ionic exchange.[8]

Previous studies in humans have shown the effectivity of laser to reduce pain, post-activation of the orthodontic archwires,[9-12] and inhibit the relapse of the gingival space after the orthodontic treatment.[14,15] In addition, animal models indicate that low level laser treatment stimulates bone resorption.[16,17]

There are different conclusions in previous in vitro studies. Coombe et al,[2] while studying the effects of laser irradiation on a line of osteoblastic cells, found

that activation or cell proliferation were not significantly changed when different energy levels were applied, but they observed an increase in the intracellular calcium concentration and concluded that these effects merited further studies before considering laser therapy as a potential way to accelerate orthodontic movement.

Studies in gingival fibroblasts, such as that by Almeida López et al[18] indicate that irradiation of this tissue with 2 J/cm^2 increases cell proliferation over that observed in the control. Marques and Pereira,[3] after irradiating gingival fibroblasts with a Ga-Al-As laser, reported ultrastructural changes that potentially affect the collagen metabolism. Pereira et al[4] measured the synthesis of pro-collagen after irradiation of cultures of gingival fibroblasts with a Ga-As laser, changing the energy from 3 to 5 J/cm^2 and analyzing the growth curves and the pro-collagen immunoprecipitation. They concluded that this laser protocol has an effect on proliferation without changing the synthesis of pro-collagen.

Kreisler[5] studied the potential stimulation by low-level laser irradiation on periodontal ligament fibroblasts. There is agreement about the fact that periodontal fibroblasts behave differently than gingival fibroblasts in culture, as is supported by Giaunnopolou.[19] Kreisler also found a considerable increase in the proliferation of irradiated fibroblasts compared to a control group, by measuring the fluorescence activity with an oxidation-reduction (REDOX) indicator.

Evaluations of changes after LED (light emitting diodes) irradiation and low-level laser have also been conducted on fibroblasts, showing that both stimulate cell proliferation.[20]

The previous studies were performed using different emission sources at arbitrary distances and even with manual non-standardized procedures. Timberlake[21] emphasized the relevance of standardizing parameters to control sources of variation when cultured cells are irradiated.

The purpose of this in vitro experimental study was to evaluate the effect of laser irradiation on cell proliferation of periodontal and gingival fibroblasts by measuring the rate of cell proliferation for each cell line, and compare them to each other and with a control culture. The hypothesis tested was that As-Ga-Al laser irradiation of cultured cells has a cell-activating effect that enhances cell proliferation of gingival and periodontal fibroblasts, and that there is a statistically significant difference as compared to the control. The null hypothesis is that there is no significant difference between irradiated cultures and controls.

MATERIALS AND METHODS

This study did not require the participation of human subjects, as it was an experimental trial in vitro. Nevertheless, the protocol was approved by the Ethics Committee of the Faculty of Health at the Universidad del Valle.

Cell Cultures

Gingival and periodontal ligament fibroblasts were obtained from the culture bank of the Pharmacology Laboratory, Faculty of Health at the Universidad del Valle, and kept in culture media with 15% bovine serum in plates of 96 wells. The initial number of cells was 4000 per well.

Low-level Laser Irradiation

Cell cultures were distributed as follows: plate 1, 48 wells with gingival fibroblasts; plate 2, 48 wells with periodontal fibroblasts, and plate 3, 24 wells with gingival fibroblasts plus 24 wells with periodontal fibroblasts. The latter was the control plate, not receiving any laser irradiation.

The diameter of the wells was 6.39 mm and the area was 0.32 cm^2.

The irradiation was carried out using a container with oscillatory movement that is described by the following formula:

$\chi(t) = 7.332 \cos[360(0.496)t]$

with a range of 7.332 cm and a periodicity of 2.02 s.
The speed is obtained by the equation:

$v(t) = -7.274\ \pi\ \text{sen}[360(0.496)t)]$

and the interval $\chi = -2.34$ cm and $\chi = 2.34$ cm. A series of similar speed is achieved that yields an average speed of 22.25 cm/s (approaching a linear uniform movement).

The irradiation time per well and cycle was: $\tau = 0.0574$ s

obtained with: $\tau = \frac{2d}{\bar{\upsilon}}$

where d is the well diameter and $\bar{\upsilon}$ is the average speed.

The cultures were irradiated with the infrared emission laser Photon Lase III (As-Ga-Al) (DMC Equipamentos; São Carlos, Brazil) at a wavelength of 832.79 nm. To improve the beam field, a positive lens at a focal distance of 3 cm to the output was adapted to the laser unit 1.5 cm from the surface of the cell culture.

The power output was kept at 37 mW in continuous mode.

The fiber was positioned 1.5 cm perpendicular to the monolayer to provide equal exposition along the culture. Before the application, the energy was measured by an energy measuring device. The total irradiation time per well line was about 1140 s (19 min) and per well it was 32.45 s.

As a function of the irradiation time, the energy flow was 3.75 J/cm^2, calculated by:

$$F = \frac{P\tilde{t}}{A}$$

where A is the well area.

The irradiation was applied when the culture was in the logarithmic proliferation phase, after 24 h of incubation. Following the laser treatment, the plates were incubated for 24, 48, 72, 96, 120, and 148 h before measuring cell proliferation. Cell cytotoxicity was measured after 24 h.

Cell Proliferation Evaluation

The XTT (Molecular Biochemicals; Mannheim, Germany) employed to evaluate cell proliferation measures the metabolic activity of viable cells. It is a nonradioactive test and is fully worked out in a microplate. This kit is an alternative to the use of radioactive tracers such as [^{3}H]-thymidine, or the release of radioisotopes such as [^{51}Cr].

The test is based upon the reduction of the tetrazolium salt XTT by viable cells in presence of an electron donor reagent. The reduction changes the yellow XXT tetrazolium salt into orange formazan when the cells are metabolically active. Formazan is water soluble and it is easily quantified by the spectrophotometer (ELISA microplate reader; Stat fax 2100, Awareness Technology; Palm City, FL, USA).

The cells, grown in the 96-well plate, are incubated at 37°C in an atmosphere with 5% humidity and CO_2 with the XTT solution, for 4 to 164 h. Any increase in the number of viable cells causes a related increase in the activity of mitochondrial dehydrogenase in the sample that, in turn, is directly related to the amount of formazan produced, and the absorbance measured by the instrument.

Preparation of the solution

The XTT reagent and the electron donor reagent are heated in a water bath at 37°C. The two reagents are mixed together until obtaining a clear solution. For each 96-well microplate, 5 ml of XTT reagent are used plus 0.1 ml of the electron donor reagent.

Cellular Cytoxicity Evaluation

The LDH (Roche) kit was employed. This is a colorimetric test to quantify cell death and lysis by the activity of the enzyme lactate dehydrogenase released from the cytoplasm of damaged cells in the supernatant. The test is designed for 96-well microplates and it is useful for in vitro detection of plasma membrane breakdown in different cell systems.

The cells must be removed by centrifugation at 250 G before measuring LDH. LDH is the enzyme which converts lactate into pyruvate, while the coenzyme NAD^+ is reduced to $NADH/H^+$. In a second step, H/H^+ from $NADH/H^+$ is transferred to the tetrazolium INT salt that is reduced to formazan. Any increase in death cells or cell membrane breakdown will increase the LDH enzymatic activity in the culture supernatant. This is directly related to the increased amount of formazan generated during a limited time. The amount of color produced is proportional to the number of lysed cells. Formazan is water soluble and presents a maximum absorption around 500 nm, while the INT tetrazolium salt has no absorption at this wavelength.

Preparation of the solution

The catalyst was reconstituted in 1 ml of water per 10 min and properly mixed. To calculate the percentage of cytotoxicity three controls are used:

1. Background control provides information about LDH activity in the test medium. This value of absorbance is subtracted from the other values.
2. Low level control provides information about LDH released from normal cells (spontaneously released LDH).
3. High level control provides information about maximum LDH activity of LDH released (maximum LDH released).

The calculated average of absorbance values, multiplied by 3 and subtracting the background absorbance, is introduced in the following equation.

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental – low control}}{\text{High Control – Low Control}} \times 100$$

Table 1 Results of absorbance per group and day

DAYS	1	2	3	4	5	6	7	Maximum change
Gingival controls	0.843	0.943	1.053	1.176	1.285	1.399	1.501	0.658
Median	0.858	0.966	1.096	1.232	1.358	1.478	1.586	0.728
	0.884	0.995	1.112	1.243	1.361	1.49	1.587	0.703
Mean	0.862	0.968	1.087	1.217	1.335	1.456	1.558	
SD	0.021	0.026	0.03	0.036	0.043	0.049	0.049	
Periodontal controls	0.593	0.702	0.831	0.973	1.102	1.241	1.355	0.762
Median	0.641	0.773	0.911	1.056	1.193	1.33	1.44	0.799
	0.904	1.042	1.184	1.334	1.484	1.627	1.714	0.81
Mean	0.713	0.839	0.975	1.121	1.26	1.399	1.503	
SD	0.1674	0.179	0.185	0.189	0.199	0.202	0.188	
Gingival irradiated	0.828	0.977	1.108	1.237	1.366	1.487	1.594	0.766
	0.875	0.987	1.126	1.287	1.422	1.544	1.643	0.768
	0.943	1.057	1.173	1.296	1.423	1.573	1.66	0.717
	1.002	1.128	1.241	1.366	1.492	1.607	1.701	0.699
Median	0.91	1.022	1.15	1.29	1.42	1.56	1.65	
Mean	0.912	1.037	1.162	1.297	1.426	1.553	1.65	
SD	0.076	0.07	0.059	0.053	0.046	0.051	0.044	
Periodontal irradiated	0.626	0.741	0.859	1.016	0.925	1.013	1.109	0.483
	0.645	0.771	0.913	1.068	1.01	1.041	1.14	0.495
	0.662	0.825	0.965	1.12	1.132	1.281	1.406	0.744
	0.692	0.835	1.012	1.198	1.365	1.53	1.642	0.95
Mean	0.656	0.793	0.937	1.1	1.108	1.216	1.324	
SD	0.028	0.044	0.066	0.077	0.191	0.241	0.25	
Median	0.65	0.8	0.94	1.1	1.07	1.16	1.27	

100 µl per well of medium was used. The cells were washed with the medium. The cell suspension was adjusted to a concentration of 2 x 10^6 cells/ml and titrated twice in a serial dilution in a micro plate. Using a multichannel micropipette, the following were added to each control: 200 µl of assay medium to the low control; 100 µl to the high control, plus 100 µl Triton X-100. The cells were then incubated at 37°C, 5% CO_2, 90% humidity. After centrifuging the microplate at 1500 rpm for 10 min, 100 µl of supernatant were carefully removed and transferred to the wells of a 96-well plain microplate.

To measure LDH activity in the supernatant, 100 µl of reagent were added and the mixture was incubated for 30 min between 15°C and 25°C, keeping the microplates in darkness. The absorbance was measured at 492 nm using an ELISA reader.

Statistical Analysis

Descriptive statistics (average, median, standard deviation, and range) were obtained for each cell line. The Mann-Whitney U-test was applied to compare the cell groups. The significance level was set at $p < 0.05$.

RESULTS

Cell Cytotoxicity Evaluation

Negative values for percentage of cytotoxicity were obtained: -1.24% for irradiated gingival fibroblasts and -4.75% for irradiated periodontal fibroblasts. This result is interpreted as a lack of cytotoxicity against the tested cells of the low-level laser irradiation protocol used.

Table 2 Gingival fibroblasts, control and irradiated results (OD units)

	Gingival cells	
Descriptive statistics	Control	Experimental
Average	1.21	1.29
Median	1.24	1.30
SD	0.036	0.057
Minimum	0.72	0.78
Maximum	1.67	1.74

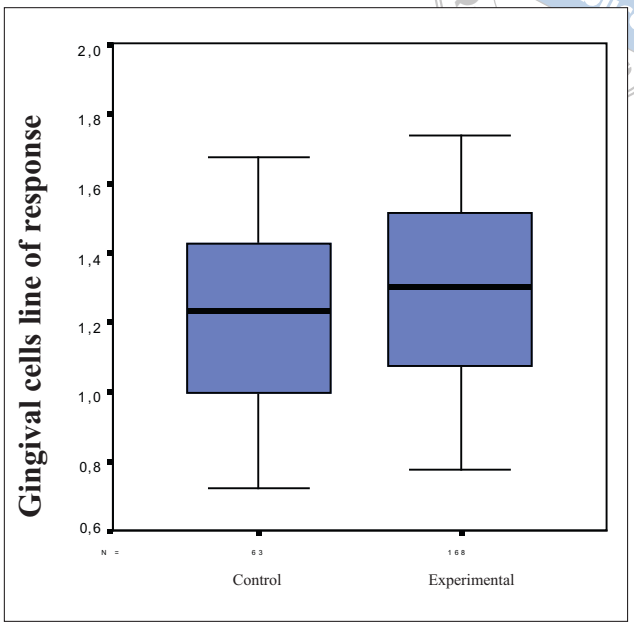

Fig 1 (right) Box diagram for gingival cells. Gingival cells exposed to laser irradiation do not behave in a significantly different way as compared to the control.

Table 3 Average cell proliferation in control and treated gingival fibroblasts, according to time in days

	Gingival cells	
Day	Control	Irradiated
1	0.862	0.912
2	0.968	1.037
3	1.087	1.162
4	1.217	1.297
5	1.335	1.426
6	1.456	1.553
7	1.558	1.650

N = 63 168

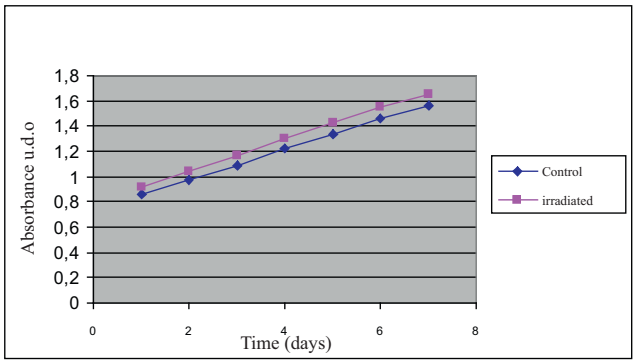

Fig 2 Gingival cell proliferation as a function of time.

Cell Proliferation Evaluation

Table 1 summarizes the descriptive statistics of absorbance including mean, median, standard deviation, and maximum difference per group and day of evaluation, expressed as absorbance in optical density (OD) units.

Gingival fibroblasts

The mean ± standard deviation (SD) optical density units were 1.21 ± 0.036 for the gingival control fibroblasts and 1.29 ± 0.057 for irradiated gingival fibroblasts (Table 2, Fig 1). There is a positive correlation between time and rate of cell proliferation both in control and treated fibroblasts (Table 3, Fig 2).

Periodontal fibroblasts

The mean ± SD in optical density units for control periodontal fibroblasts was 1.11 ± 0.18 and 1.02 ± 0.31 OD units for irradiated periodontal fibroblasts. The difference is not statistically significant, but a mild tendency was observed towards reduced proliferation in the irradiated cells (Table 4, Fig 3).

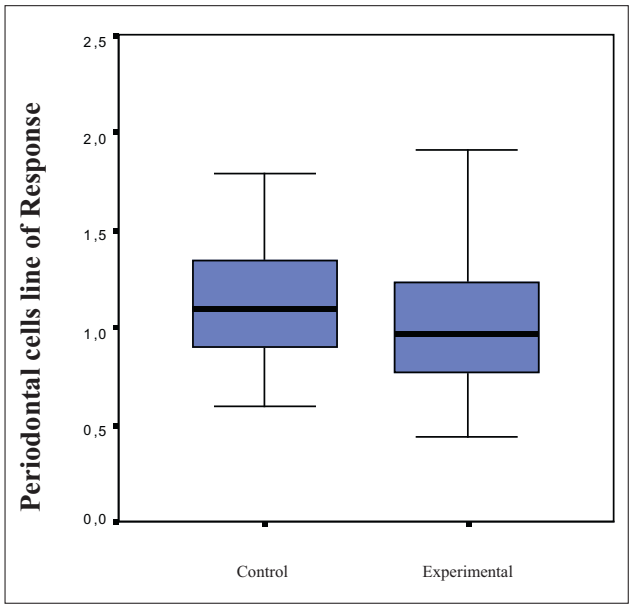

Table 4 Descriptive statistics for periodontal cells

	Periodontal cells	
Descriptive statistics	Control	Experimental
Average	1.11	1.02
Median	1.10	0.96
SD	0.18	0.12
Mínimum	0.59	0.43
Maximum	1.79	1.91

Fig 3 (left) Box diagram for periodontal cells.

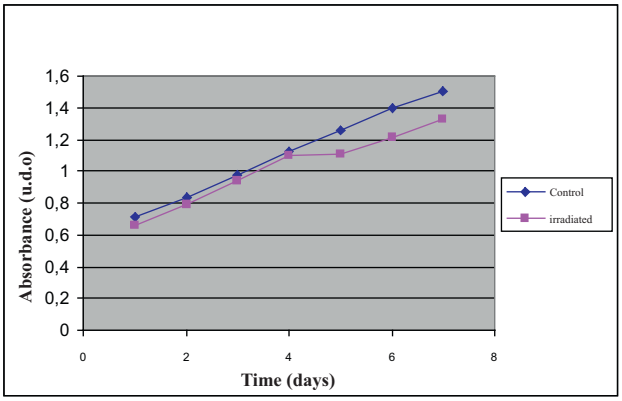

Fig 4 Periodontal fibroblast proliferation as a function of time.

Table 5 Average proliferation of periodontal fibroblasts, control and irradiated according to time

	Periodontal cells	
Day	Control	Irradiated
1	0.713	0.656
2	0.839	0.793
3	0.975	0.937
4	1.121	0.100
5	1.260	1.108
6	1.399	1.216
7	1.503	1.324

Taking into account the time changes, a great variability was found in the response of periodontal fibroblasts as compared to that observed in the corresponding control group (Table 5, Fig 4).

Relationship between irradiated gingival fibroblasts and irradiated periodontal fibroblasts

There is a slight difference between the average response of irradiated periodontal and gingival fibroblasts: 1.02 ± 0.12 vs 1.29 ± 0.057, respectively. The variability of response was higher in irradiated periodontal fibroblasts than in gingival fibroblasts, ranging from 0.43 to 1.91 and from 0.77 to 1.73, respectively (Table 6, Fig 5).

The comparison of the medians of the group of irradiated gingival fibroblasts (1.30 ± 0.057) and the respective control (1.24 ± 0.036) gives a p value of 0.2 (not significant). The difference between irradiated periodontal fibroblasts (0.96 ± 0.128) and the respective control (1.10 ± 0.18) was also not statistically significant (p = 0.2). The comparison of medians for gingival control and periodontal control yielded no significant difference (p = 0.7), nor did irradiated gingival vs irradiated periodontal fibroblasts (p = 0.343) (Tables 7 to 11, Fig 6).

Table 6 Behavior of irradiated gingival cells vs irradiated periodontal cells

Descriptive statistics	Gingival irradiated	Periodontal Irradiated
Average	1.29	1.02
Median	1.304	0.964
SD	0.057	0.128
Minimum	0.77	0.43
Maximum	1.736	1.91

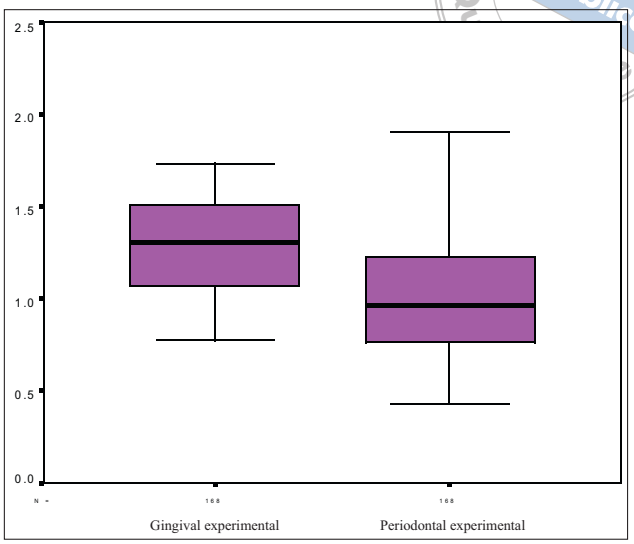

Fig 5 (right) Box diagram for irradiated gingival fibroblasts vs irradiated periodontal fibroblasts. The response as rate of cell proliferation in gingival fibroblast is more homogeneous than that of irradiated periodontal fibroblasts.

Table 7 Comparison of medians of control vs irradiated gingival fibroblasts (significant at $p < 0.05$)

Cells	Median
Gingival control	1.24
Gingival irradiated	1.30
p	0.2

Table 8 Comparison of control vs irradiated periodontal fibroblasts (significant at $p < 0.05$)

Cells	Median
Periodontal control	1.10
Periodontal irradiated	0.96
p	0.2

Table 9 Comparison of controls, gingival vs periodontal (significant at $p < 0.05$)

Cells	Median
Gingival control	1.24
Periodontal control	1.10
p	0.7

Table 10 Comparison of irradiated gingival fibroblasts vs irradiated periodontal fibroblasts (significant at $p < 0.05$)

Cells	Median
Gingival irradiated	1.30
Periodontal irradiated	0.96
p	0.343

Table 11 Average proliferation rate per group and time

Days	Control Gingival	Irradiated Gingival	Control Periodontal	Irradiated Periodontal
1	0.86	0.89	0.71	0.64
2	0.97	1.01	0.84	0.79
3	1.09	1.14	0.98	0.94
4	1.22	1.28	1.12	1.11
5	1.33	1.41	1.26	1.14
6	1.46	1.53	1.40	1.29
7	1.56	1.63	1.50	1.40

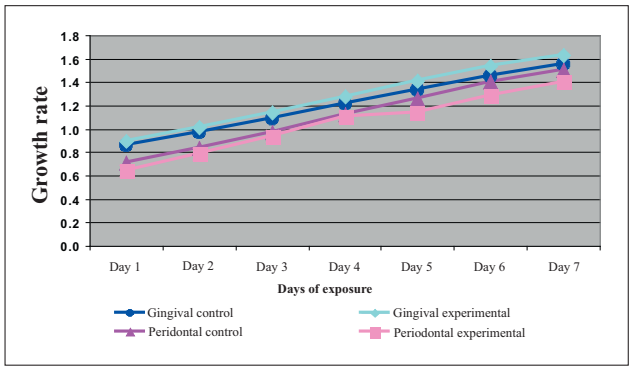

Fig 6 Average cell proliferation per group of cells, as a function of time.

DISCUSSION

The results of the cell cytotoxicity test show that low-level laser therapy, particularly with this irradiation protocol, was not deleterious for the cell lineages evaluated. This is demonstrated by the lack of release of lactate dehydrogenase, secondary to any cell membrane damage. This suggests that the irradiation protocol described in this study can be employed for further research in vitro using cell cultures, as it is certain that it does not cause cell lysis. In the literature reviewed to support this study, we found no reports of laser effects including cell cytoxicity evaluation.

Previous studies using low-level laser in animal models and clinically controlled trials provide contradictory results.[16, 17] The inherent difficulties in measuring pain and tissue repair variables emphasize the need for research about this therapy in cell biology. The exact mechanism of the laser/cell interaction is still unclear, and specific data about the effects of low-level laser irradiation upon cell proliferation and cell cytotoxicity are clearly needed.[5]

There are different methods described to evaluate cell proliferation, including methods applying spectrophotometric assays and radioactive techniques based on thymidine and proline incorporation into the tissue. In this study, the XTT (Roche) kit was selected to measure the rate of proliferation by a colorimetric technique, because, compared to the radioactive tests, it has the advantages of simplicity, low cost, and rapid evaluation of a large number of samples.

In general terms, the evaluated variables did not show significant differences in the rate of cell proliferation during the seven days evaluated. The results are different from those reported by Kreisler et al,[5] who found an increase in the rate of cell proliferation in periodontal ligament fibroblasts after low-level laser irradiation, while in the present study there was a tendency toward reduction of the activity in periodontal fibroblasts as compared to the control, although the difference was not statistically significant The difference in results may be due to the use of different irradiation protocols; Kreisler et al[5] used 809 nm wavelength, a power of 10 mW, and exposition times of 75, 150, and 300 s, with energy levels of 1.96 and 7.84 J/cm^2, while in this study the wavelength was 832 nm, the energy level was 3.75 J/cm^2, the power was 37 mW, and the time 32.4 s. In this study the energy level was fixed at 3.75 J/cm^2, because it has been shown that energy levels between 2 and 8 J/cm^2 increase the rate of cell proliferation over the controls, without a further increase when the energy level is above this range.[4,5] Besides, the studies with gingival fibroblasts indicate that the optimum energy level for stimulation of these cells is 3.37 J/cm^2.[24]

The response of periodontal ligament fibroblasts to low-level laser in vitro was first described by Shimizu in 1995[25] using 830 nm wavelength from a GaAlAs laser, as manifested by the inhibition of PGE_2 and I1b. It is accepted that laser effects are wavelength and dose dependent. Molecular absorption of the laser light is required to produce any cellular effect. The effect of this absorption on a fibroblast layer is particularly related to cytochrome oxidase that has an absorption spectrum in the range of 800 to 830 nm.[26]

As in the present study, Coombe et al[2] also did not find a statistically significant difference when they compared the cell proliferation in the experimental group vs the control in osteoblastic cells from osteosarcoma, irradiated with GaAlAs laser at a wavelength of 830 nm, 90 mW power and energy levels between 0.3 and 4 J. Although the cell lines evaluated are different, the

results agree that this therapy stimulates the proliferation of cells related to dental movement.

In this study, we used only one irradiation dose, as previous studies[27] indicated that a single dose in an early state of the culture yields results similar to those with multiple irradiation doses. This is related to the fact that stimulatory action of the laser occurs during early periods, when immature precursors are proliferating, but not in late states.

The cell proliferation did not increase in a significant way under these experimental conditions, but it is not possible to rule out that low-level laser therapy could favor the mechanisms involved in dental movement, as it is reported in the literature that collagen production is not dependent on the increase in cell population.[28] It is necessary to further investigate the effects of low-level irradiation on the morphological, metabolic, and functional characteristics of fibroblasts.

Due to the small size of the sample in this study and the size difference between the experimental and control group, it was not possible to use parametric tests, or non-parametric analysis of variance such as the two-way ANOVA (Friedman test) or the non-parametric one-way ANOVA (H Kruskal-Wallis). The use of the Mann-Whitney U-test is indicated when dealing with small samples, and the description is better accomplished by the medians, rather than the mean and the standard deviation. Although the difference was not found to be significant, a higher inhibitory effect on periodontal fibroblasts was observed, which show the lowest levels of absorbance at any time interval. The irradiated gingival fibroblasts presented higher levels, above the control, but again without reaching a significant difference.

CONCLUSIONS

1. The irradiation protocol here described is not cytotoxic against any of the cell lines evaluated.
2. There is no statistical difference in the rate of cell proliferation between the control and the irradiated cells, either between the irradiated groups or the controls.
3. There is an increase in the rate of cell proliferation in every group, relative to the time of evaluation.
4. The irradiation protocol described in this study can be employed for the next two in vitro studies, using cell cultures of human osteoblasts and osteoclasts.

This pilot study established a protocol to irradiate other cells involved in orthodontic tooth movement, such as normal human osteoblasts and osteoclasts, in the attempt to understand metabolic, morphological, and functional effects on the cellular level.

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