An In Vitro Study of the Reaction of Human Osteoblasts to Low-level Laser Irradiation

Angela Dominguez[a], Paola Castro[b], Mónica Morales[c]

[a] *Specialist in Orthodontics, CIEO Foundation, Bogotá, Colombia; Professor, Department of Orthodontics, Faculty of Dentistry, Universidad del Valle, Colombia.*
[b] *Specialist in Orthodontics, Universidad del Valle, Colombia.*
[c] *Third-year Orthodontics Program Resident, Universidad del Valle, Colombia.*

Purpose: To evaluate the effect of low-energy level laser irradiation on the proliferation of a culture of human osteoblasts, applying a method already standardized and tested.

Materials and Methods: The cell line (NHOst) cultures were distributed into two groups of wells: the experimental wells, containing cells in the proliferative phase, incubated for 48 h, then irradiated with laser photon LASE (As-Ga-Al) for 19 min. Further incubation previous to the cell proliferation test was carried out for 24, 48, 72, 96, 120, and 148 h. In the control group, 48 wells cultured with human osteoblasts were evenly distributed into 3 subgroups termed low (5% culture medium), medium (10% culture medium) and high (15% culture medium). The cell morphology was characterized by immunohistochemistry and the rate of cell proliferation was measured by the XTT test (Roche).

Results: After the incubation periods, the difference of the experimental group vs controls was always statistically significant.

Conclusion: Normal human osteoblast cells (NHOst) are sensitive to low-level laser irradiation, which increases bone remodeling in a way that could be clinically applied to accelerate orthodontic movement and enhance bone neoformation in the field of periodontal surgery.

Keywords: osteoblasts, low-intensity laser, orthodontic movement, bone remodeling.

J Oral Laser Applications 2009; 9: 21-28. *Submitted for publication: 31.01.09; accepted for publication: 03.03.09.*

It has been suggested that the application of low-intensity laser light accelerates cell changes responsible for orthodontic movement, and there are various reactions at the cell level that may be triggered which induce dental movement.[1]

Alhara et al[2] reported that during orthodontic dental movement, the OPG/RANKL/RANK (osteoprotegerin/receptor activator of NF-kappaB ligand/receptor activator of NF-kappa B) system in periodontal tissue is a very important factor determining the regulatory balance of alveolar bone resorption. The increased rate of orthodontic movement after low-intensity laser application might be related to its effect on the expression of RANKL and OPG in osteoblasts and periodontal ligament cells, as well as RANK during orthodontic movement. The expression of RANK molecules in pre-osteoclast cells was detected in early stages of irradiated cultures. This suggests that laser irradiation is able to induce differentiation and activation of osteoclasts via RANK.

Previous laboratory tests found a stimulatory effect of low-power laser irradiation on bone regeneration of the midpalatal suture during rapid maxillary expansion in rats.[3] Furthermore, Ozawa et al[4] proved that laser irradiation stimulates cell proliferation and differentiation of cells forming bone nodules, derived from an os-

teoblast lineage mainly in the relevant precursors, increasing the number of differentiated osteoblasts as well as bone neoformation. Additionally, Kawasaki and Shimizu[5] reported that low-level laser irradiation stimulates the rate of dental movement and osteoclast proliferation on the pressure side during experimental in vivo dental movement, due to the fact that bone remodeling is a physiological process involving both bone resorption by osteoclasts and bone matrix synthesis by osteoblasts.[6-8]

Studies performed on human subjects show the efficacy of laser in reducing pain after activation of the orthodontic archwires[9-11] and in reducing relapse.[12] Animal model studies indicate that low-energy laser beams stimulate bone resorption.[13,14]

While studying the effects of laser irradiation on a line of osteoblasts, Coombe et al[15] found that activation of cell proliferation was not dependent on the energy level, but they observed an increase in the intracellular calcium concentration and concluded that these effects deserved further study before considering laser therapy as a potential means of accelerating orthodontic movement.

Another study[16] in rat osteoblasts irradiated by laser of 780 nm wavelength found that phototherapy increases osteoblast proliferation independently of dexamethasone presence.

The above-mentioned studies were performed using different emission sources, arbitrary distances, and even manual nonstandardized procedures. Timberlake[17] emphasized the importance of using homogeneous parameters to control the variables in studies of irradiated cell cultures. There are no reports found in the literature evaluating laser therapy on human osteoblasts, which are the cell line directly involved in orthodontic dental movement. To date, the studies have been conducted in rat cells[2] or cancer cells (osteosarcoma cell lines), which makes it difficult to extrapolate the results to healthy human tissues. In Coombe's study,[15] laser irradiation did not cause significant changes in protein expression or alkaline phosphatase activity; instead, a transitory positive change in intracellular calcium concentration was found after laser irradiation. Although the methodology was not standardized, the results from different studies usually report increased cell proliferation.[17-22]

The purpose of this in vitro study is to obtain better insight into the cellular events related to the morphological features and proliferation of human osteoblasts after low-level laser irradiation. Knowledge of these effects will provide scientific support to guide the clinical application of laser therapy.

This is the second of two articles from a research line devoted to the biology of dental movement. The first part was performed in gingival and periodontal fibroblasts at the Biology laboratory of the Universidad del Valle.[23]

MATERIALS AND METHODS

Source of Cell Cultures

Human Osteoblasts were obtained from Cambrex Bioscience (Charles City, IA, USA), and cultured in Osteoblast Growth Medium (Promocell Bioscience Alive; Heidelberg, Germany).

Cell Line Culture

The cell line was cultured as follows: In the first plate, number 1 (experimental group), 48 wells containing 10% culture medium were inoculated with human osteoblasts to receive low-level laser irradiation, and in plate number 2 (control group), 48 wells inoculated with human osteoblasts were evenly distributed into low control (5%), medium (10%), and high (15%) culture medium subgroups. The plates were purchased from Falcon Laboratories (Colorado Springs, CO, USA). The initial number of cells was adjusted to 4000 per well.

Low-level Laser Irradiation

The irradiation was performed via a carrier with oscillatory movement, described using a pre-set formula and tested in a previous study.[23]

The strains were irradiated with the infrared emission Photon LASE laser (As-Ga-Al, DMC Equipamentos; Sao Carlos, Brazil) at a wavelength of 832.79 nm. To improve the beam field control, a convergent lens was adapted to the laser with a focal distance of 5 cm, located 7 cm away from the output of the instrument and 7 cm above the cell culture surface. The energy output was kept constant at 36.73 mW in continuous mode. The fiber was positioned 70 mm above the monolayer, perpendicular to it, to assure an equal exposition throughout the whole culture. Before the application of laser irradiation, the energy output was measured using an energy measuring device. The total irradiation time per well line was about 1131 s (18.85 min). The flow of energy as a function of the irradiation time was 3.75 J/cm^2.

Irradiation was applied to the culture while in the logarithmic proliferation phase, attained after 48 h of incubation. After laser irradiation, the incubation times previous to testing cell proliferation using the XTT technique were 24, 48, 72, 96, 120, or 148 h.

The protocol was validated and previously described in an earlier article.[23]

Immunohistochemistry Tests
Enzymatic staining method

Some antibodies are used conjugated to enzymes. The method requires visualization of the enzyme through an optical microscope. The cell samples obtained from the culture were fixed by immersion in 10% formaldehyde buffer, and then washed for 5 min in 0.01-molar phosphate saline buffer (PBS). The endogenous peroxidase was blocked by the addition of 0.3% hydrogen peroxide for 5 min, followed by 30-min washing with PBS containing 5% normal serum and 1% bovine albumin.

Avidin-biotin complex (ABC) method

This indirect method is very sensitive. After three incubation periods and staining the enzyme, the primary antibody was incubated one night at 4°C in a wet chamber, washed in PBS, and incubated again with the secondary antibody, adding 2 μg/ml biotin and a 50X dilution of the ABC complex, in a wet chamber for 30 min at room temperature.

Staining

Horse radish peroxidase (HRP) was stained by a histochemical reaction with 3,3´-diaminobenzidin tetrachloride, hydrogen peroxide, and diaminobenzidine (DAB). Alkaline phosphatase reacts with naphtol AS-MX phosphate plus Fast Red yielding a brilliant red, insoluble product called formazan. Finally, the preparation was contrasted with hematoxilin and mounted in Permount.

Cell Proliferation Evaluation

The XTT (Roche; St Louis, MO, USA) colorimetric method was used in a format of 96-well microplates, which measures cell viability and proliferation in cell culture samples adhered or in suspension. The incubation of cells with XTT was followed by spectrophotometric detection of the color product.

The XTT method is a nonradioactive method developed to use in microplate readers. The cells grown in a 96-well plate are incubated at 37°C in an environment with 5% humidity and CO_2, plus the XTT solution, for 4 to 148 h. After the incubation period, the concentration of the orange reaction product, formazan, is evaluated by spectrophotometry. Any increase in the number of viable cells is directly correlated to the amount of formazan produced and therefore to the absorbance measured by an ELISA microplate reader.

RESULTS

Cell Proliferation

In the control subgroups (low, medium, and high concentration of culture medium), a progressive increase was detected in the absorbance measured from day 1 to day 6, that is, it was a time dependent increment (Table 1 and Fig 1). In the experimental group, the tendency up to day 5 was an increment as in the control groups, but for day 6, a reduction was observed in the absorbance.

The Bonferroni test (Table 2 and Fig 2) was applied at a significance level $p < 0.001$ due to the high number of possible comparisons between averages. ANOVA (not shown) demonstrated that in general, the variances were homogeneous, but they were higher in the experimental group than in the controls. On the first day, there was a significant difference between the average for the high control subgroup and the experimental group, indicating a possible immediate deleterious effect of the irradiation, reaching values even lower than those for the low control subgroup.

On the second day, these differences disappeared, and the groups became more homogeneous. On the third day, the experimental group presented significant differences above the low and medium control subgroups, and above the high control subgroup, but this was not statistically significant. The same tendency is found for the fourth day's data. On the fifth day, the differences between the medium control subgroup and experimental group are no longer present, as the medium control reached its highest value on this day. Nonetheless, the difference between low control and experimental groups was constant during the whole period studied. The sixth day's data indicate that the experimental group's growth was similar to the

Table 1 Results: Absorbance per group and days

LEVEL	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Low	(n = 24)					
Average	0,6296	0,832	0,978	1,153	1,197	1,216
Std.Dev.	0,03825	0,05594	0,0771	0,3752	0,3552	0,2019
Medium	(n = 24)					
Average	0,648	0,937	1,109	1,213	1,378	1,686
Std.Dev.	0,04665	0,10924	0,1442	0,1633	0,3668	0,6281
High	(n = 24)					
Average	0,67929	1,173	1,38	1,591	1,988	2,534
Std.Dev.	0,05667	0,1725	0,21	0,355	0,5852	0,485
Experimental	(n = 32)					
Average	0,593	0,96	1,568	1,837	1,929	1,746
Std.Dev.	0,0809	0,4598	0,7135	0,8157	0,7048	0,644

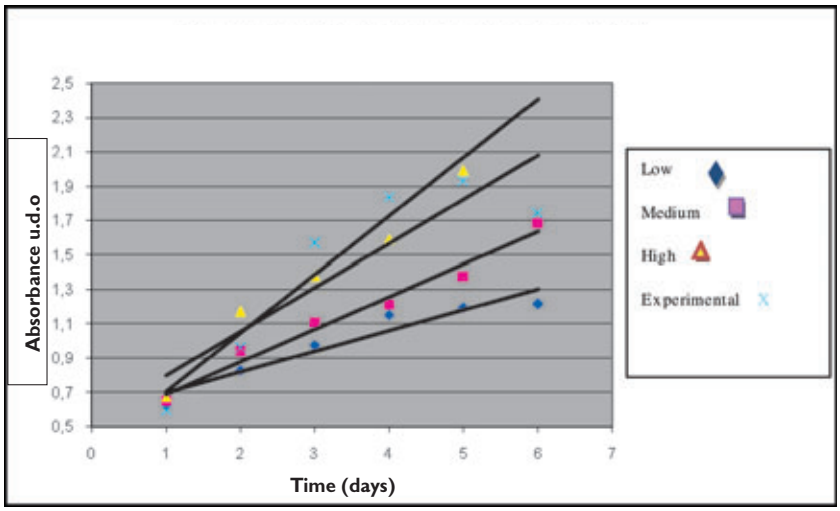

Fig 1 Progressive increase in the absorbance measured from day 1 to day 6.

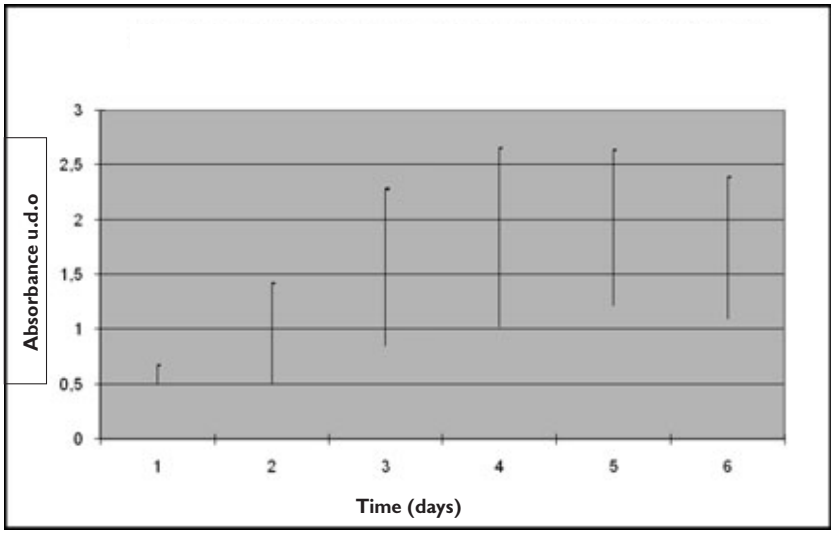

Fig 2 Bonferroni test.

Table 2 Statistical anylysis of results – Bonferroni t test

BONFERRONI t TEST (Level of significance, p < 0,001)			
AVERAGE COMPARED		p	Significance
Day 1	Low vs Experimental	0,04527	
(n = 24)	Medium vs Experimental	0,0046	
	High vs Experimental	4,00E-05	Significativa
Day 2	Low vs Experimental	0,1006	
	Medium vs Experimental	0,5795	
	High vs Experimental	0,07	
Day 3	Low vs Experimental	1,00E-06	Significativa
	Medium vs Experimental	2,00E-05	Significativa
	High vs Experimental	0,00397	
Day 4	Low vs Experimental	0,00012	Significativa
	Medium vs Experimental	0,00018	Significativa
	High vs Experimental	0,0941	
Day 5	Low vs Experimental	0,00012	Significativa
	Medium vs Experimental	0,00569	
	High vs Experimental	0,2678	
Day 6	Low vs Experimental	7,50E-05	Significativa
	Medium vs Experimental	0,507	
	High vs Experimental	2,00E-05	Significativa
Experimental	Day 1 vs day 2	9,00E-06	Significativa
(n = 32)	Day 1 vs day 3	1,40E-10	Significativa
	Day 1 vs day 4	7,00E-13	Significativa
	Day 1 vs day 5	1,20E-15	Significativa
	Day 1 vs day 6	1,90E-15	Significativa
	Day 2 vs day 3	2,00E-07	Significativa
	Day 2 vs day 4	2,00E-07	Significativa
	Day 2 vs day 5	2,00E-07	Significativa
	Day 2 vs day 6	2,00E-07	Significativa
	Day 3 vs day 4	8,70E-07	Significativa
	Day 3 vs day 5	3,00E-08	Significativa
	Day 3 vs day 6	2,00E-07	Significativa
	Day 4 vs day 5	8,59E-01	
	Day 4 vs day 6	6,11E-01	
	Day 5 vs day 6	4,52E-01	
	Low vs medium level	1,50E-01	
	Low vs high level	3,90E-16	Significativa
	Medium vs high level	3,90E-06	Significativa

medium control data, with the difference between the low control and the high control still present. That is, the experimental group was on day 6 lower than the high control, but higher than the low control.

In general terms, the differences between days are always significant, which is interpreted as a significant rate of growth in both cultures. Between levels of the control groups, the only significant difference was between low and high levels.

Taken together, the results suggest that growth in the high control was higher per time unit, while the low control presented the lowest rate of growth. The experimental and the medium control had a similar rate of growth, but it was higher in the experimental

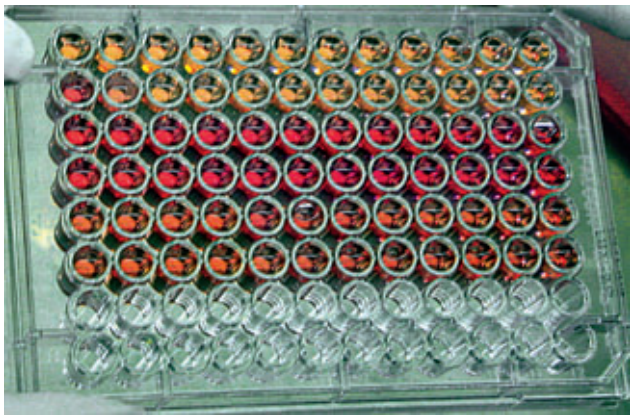

Fig 3 Microplate for the control groups. Notice the distribution of wells according to the subgroups (control low, medium, and high).

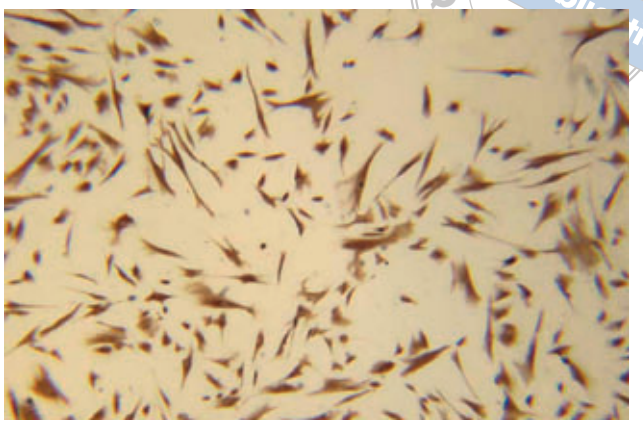

Fig 4 Good cell morphology and presence of nuclei or cell proliferation centers.

group. The peak level of growth in the low control was attained between days 1 and 2, while for the medium and high controls, the peak was reached between days 5 and 6. In the experimental group, the peak value of cell proliferation was attained between days 2 and 3, but the sixth day reduction lowers it to the level of the third day. The results are rather variable for the days 3 to 5.

Immunohistological Results

Cell proliferation – antiprotein antibodies

Under a light microscope, four plates were observed with different kinds of staining. The validated method uses anti-mitotic protein antibody (Ki-67, Dako; Cambridge, UK) that warrants the presence of the same for the whole mitotic process (G1, S, G2 mitotic phases). The qualitative interpretation of the microscopic observations is that there was an abundant growth of the osteoblast monolayer, with good cell morphology and presence of nuclei or cell proliferation centers (Fig 4).

DISCUSSION

There are a number of experimental in vitro studies about the biostimulation mechanisms of laser irradiation in bone tissue cells, such as osteoblasts and osteoclasts,[1,8-11,15,16,21] but they do not provide strong evidence for the therapeutic application of laser in orthodontics. Therefore, this system is being used as a therapeutic tool without sound scientific evidence of the effects on cells involved in dental movement. The present results suggest that low-level laser irradiation of human osteoblasts stimulates cell proliferation for up to 6 days, as indicated by the increased absorbance shown in the XTT test. This increment is significantly higher than that observed in nonirradiated control groups.

The observed reduction in cell proliferation on the sixth day is likely due to the contact inhibition characteristic of primary cultures of normal human osteoblasts (NHOs), which clearly distinguishes them from tumor cell lines that, after occupying the growth area, still proliferate and increase the number of cells per area unit .

Current research with low-level laser in animal models and controlled clinical trials have yielded contradictory results,[11,12] partly due to the technical difficulties of measuring variables such as pain and tissue repair; these technical problems are reduced in cell biology studies. The detailed mechanism of laser-cell interactions is still a matter of research, and information about the effect of low-level laser irradiation on cell proliferation and cytotoxicity is far from complete in the available literature.[8] There are different methods to evaluate cell proliferation, including staining spectrophotometric methods and radioactive trace of thymidine and proline incorporation in cells. In this study, the XTT test (Roche) was used, which is a nonradioactive, simple, low-cost alternative to quickly evaluate a great number of samples.

In general, the evaluation showed significant differences in cell proliferation during 7 days of evaluation of

culture growth. The peak of growth for the controls is not exactly the same as the experimental peak of growth: for the low control it was reached in 1 to 2 days, while for the medium and high controls it occurred on days 5 or 6, and in the experimental group on days 2 or 3. In the control groups, the time-related increase in the absorbance is progressive from days 1 to 6, while for the experimental group, the incremental tendency was observed until the fifth day, and then it was reversed, probably due to contact inhibition which is characteristic of NHOs.

As opposed to Coombe's study,[15] which used an osteosarcoma line of osteoblasts irradiated with low-level laser light, in this study, the rate of proliferation was always significantly increased. Coombe reports that the cell reaction to laser irradiation was not characterized by an increment of proliferation, but by an increment of temperature and intracellular calcium concentration. These effects should be further investigated before applying the laser therapy as an accelerating stimulus for dental movement.

At the level of significance indicated by the Bonferroni test for multiple average comparison ($p < 0.001$), assuming homogeneous variances, the results were always higher for the experimental group vs the controls. The difference between days of incubation was also significant, and the time to peak was different, as already mentioned.

The energy level used in this study (3.75 J/cm^2) was selected taking into account that previous studies[24,25] indicate that energy levels of 2 to 8 J/cm^2 are useful to increase cell proliferation, independent of the energy level in this range.

It is also acknowledged that laser effects are wavelength and dose dependent. The molecular absorption of laser light is required to achieve any cellular effect. The single dose of irradiation used in this study was selected because in a previous study,[26] it was reported to cause results similar to those yielded by multiple doses of irradiation. The stimulating effect of laser occurs during early stages of precursor proliferation, but not in late stages of proliferation. The results of this study suggest that low-level laser therapy has a positive effect on the mechanisms involved in dental movement, such as increases in the osteoblast proliferation, but without a significant effect on fibroblasts as was shown in a previous study.[23]

The increased proliferation of osteoclasts, not tested in this study, might be related to the cascade of events required to induce dental movement, and this fact could involve osteoclast differentiation via RANK/RANKL.

It is important to study the laser irradiation effects on other cell lines involved in dental movement, in order to obtain further insight into the mechanisms of accelerated orthodontic dental movement, mediated by laser irradiation.

CONCLUSION

Normal human osteoblast cells (NHOs) are sensitive to low-level laser irradiation with Photon LASE (As-Ga-Al) in early stages of culture, causing an enhancement of cell proliferation from the first day, until the time of contact inhibition.

REFERENCES

1. Herbert K. Effect of laser light at 820 nm on adenosine nucleotide levels in human lymphocytes. Lasers Life Sci 1989;3:37-45.
2. Aihara N, Yamaguchi M, Kasai K. Low–energy irradiation stimulates formation of osteoclast-like cells via RANK expression in Vitro. Lasers Med Sci 2006;21:24-33.
3. Saito S, Shimizu N. Stimulatory effects of low-power laser irradiation on bone regeneration in midpalatal suture during expansion in the rat. Am J Orthod Dentofac Orthop 1997;111:525-532.
4. Ozawa Yntal tooth movement in rats. Lasers Surg Med 2000; 26:282-291.
5. Kawasaki K, Shimizu N. Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. Lasers Surg Med 2000;26:282-291.
6. Rodan GA. Coupling of bone resorption and formation during bone remodeling. In: Marcus R, Feldman D, Kelsey J (eds). Osteoporosis. Academic: New York, 1996:289-299.
7. Lemaire V, Tobin FL, Greller LD, Cho CR, Suva LJ. Modeling the interactions between osteoblast and osteoclast activities in bone remodeling. J Theor Biol 2004;229:293-309.
8. Cruz DR, Kohara EK, Ribeiro MS, Wetter NU. Effects of low-intensity laser therapy on the orthodontic movement velocity of human teeth: a preliminary study. Lasers Surg Med 2004;35:117-120.
9. Lim HM. A clinical investigation of the efficacy of low level laser therapy in reducing orthodontic postadjustment pain. Am J Orthod Dentofacial Orthop 1995;108:614-622.
10. Harazaky M. Soft laser irradiation effects on pain reduction in orthodontic treatment. Bull Tokyo Dent Coll 1997;38:291-295.
11. Abello H. Marcela Efecto del láser blando (ir) en la velocidad de alineación del tratamiento ortodóncico. Tesis de postgrado Fundación C.I.E.O 1996.
12. Meguro D. Laser irradiation inhibition of open gingival embrasure space after orthodontic treatment. Aust Orthod J 2002;18:47-52.
13. Kreisler M. Sun X Effects of low energy laser on tooth movement and remodeling of alveolar bone in rabbits. Hua Xi Kou Qiang 2001;19:290-293.
14. Kawasaki K, Shimizu N. Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. Lasers surg Med 2000;26:282-291.

15. Coombe AR. The effects of low level laser irradiation on osteoblastic cells. Clin Orthod Res 2001;4:3-14.
16. Neusa A. Fujihara, Karen R. N. Hiraki, Marcia M. Marques. Irradiation at 780nm increases proliferation rate of osteoblasts independently of dexamethasone presence. Lasers Surg Med 2006; 38:332-336.
17. Timberlake GT. An inexpensive, automated instrument for laser irradiation of cultured cells. Photomed Laser Surg 2004;22:233-239.
18. Lopes A. Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and influence. Lasers Surg Med 2001;29:179-184.
19. Marques M, Pereira A. Effect of low-power laser irradiation on protein synthesis and ultrastructure of human gingival fibroblasts. Lasers Surg Med 2004;34:260-265.
20. Pereira AN. Effect of low-power laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts. Lasers Surg Med 2002;31:263-267.
21. Kreisler M. Effect of low-level GaAlAs laser irradiation on the proliferation of human periodontal ligament fibroblasts: an in vitro study. J Clin Peridontol 2003;30:353-358.
22. Vinck EM .Increased fibroblast proliferation induced by light emitting diode and low power laser irradiation. Lasers Med Sci 2003;18:95-99.
23. Domínguez A, Clarkson A, López R. An in vitro study of the reaction of periodontal and gingival fibroblasts to low- level laser irradiation: A pilot study. J Oral Laser Applic 2008;8:235-244.
24. Lemaire V, Tobin FL, Greller LD, Cho CR, Suva LJ. Modeling the interactions between osteoblast and osteoclast activities in bone remodeling. J Theor Biol 2004;229:293-309.
25. Cruz DR, Kohara EK, Ribeiro MS, Wetter NU. Effects of low-intensity laser therapy on the orthodontic movement velocity of human teeth: a preliminary study. Lasers Surg Med 2004;35:117-120.
26. Lopes A. Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and fluence. Lasers Surg Med 2001;29:179-184.

ACKNOWLEDGMENTS

Thank you, Dr. Oscar Gutiérrez, for your management and cooperation with this project and for providing the Pharmacology Laboratory facilities at the Faculty of Health, Universidad del Valle. We also thank Dr. William Criollo for his assistance in laboratory processes, Dr. Carlos Mejía for his realiable assistance in immunohistochemistry, and Dr. Luis Hernández, MSc in Statistics.

DEDICATION

To the memory of our colleague Juan Pablo Rosales Mora, who cooperated in the design and execution of this project.

Contact address: Dr. A. Dominguez, Department of Orthodontics, Faculty of Dentistry, Universidad del Valle Office: Calle 6 No 127-85 Cali, Colombia. Fax: +57-2-5551346. e-mail: adominguez71@gmail.com