

## Original Research

In vitro response of Human Pre-osteoclasts to low intensity  
Laser irradiation**Authors:**

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**ABSTRACT:**

In vitro and in vivo studies have demonstrated that low intensity laser irradiation stimulates growth and cell differentiation of precursor cells, promoting dental movement and alveolar bone remodeling. But the information about the effect of laser irradiation on human pre-osteoclasts is limited.

**Objective:** To evaluate the effects on the viability of the pre-osteoclasts and cell proliferation in cultures of human pre-osteoclasts, after irradiation with low intensity laser.

**Method:** Poietics™ Human Osteoclast Precursors Cat No. 2T-110 Cambrex-Lonza Inc. were irradiated with low intensity laser (As-Ga-Al) of 832.79 nm wavelength. A cytotoxicity test was performed using the Lactate dehydrogenase (LDH) technique, measuring absorbance, 6 and 24 h after the treatment, in the Stat Fax-2100 at a wavelength of 492 nm. The pre-osteoclast cell density was measured by the absorbance every 24 h for 6 days, using a microplate reader. (Cell proliferation with *Tetrazolium salts*: kit XTT, Roche).

**Results:** The average cytotoxicity at 24 h was twice the observed at 6 h (59% difference) in the experimental group treated with laser; Triton cytotoxicity in the positive control group was seven times higher at 24 h (86.3% difference). After 6 h the laser was 30 times less cytotoxic than Triton and after 24 h was 89 times less cytotoxic than Triton (96,6% difference for 6 h and 98.8% difference for 24 h). These time differences are statistically significant ( $p < 0.001$ ). In the cell proliferation test the differences between groups were not statistically significant during the six days follow-up. Both cultures presented the same biological response, according to the cell cycle under study.

**Conclusions:** Low level laser irradiation does not have a cytotoxic effect that affect the cell viability in normal human Pre-osteoclasts cells cultured *In vitro*.

**Keywords:**

Pre-osteoclasts, Low energy laser, Cytotoxicity, Cell cycle, Orthodontic movement.

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## INTRODUCTION

During normal or stimulated dental movement some biochemical processes within cells must be triggered. (Roberts *et al.*, 1981, Norton, 2000, Meikle, 2006, Krishnan and Davidovitch, 2006, Masella and Eister, 2006, Cardaropoli and Gaveglio, 2007) cells participating in dental movement stimulated by an applied force are essentially periodontal fibroblasts, osteoblasts and osteoclasts (McCulloch, 1995, Roodman, 1996, Dolce *et al.*, 2002, Pompermaier *et al.*, 2008, Krishnan and Davidovitch, 2009).

Trying to potentiate those effects in order to accelerate the rate of dental movement it was previously demonstrated that the application of low energy laser beams indeed accelerates some cell changes related to orthodontic movement, both in animal models (Saito and Shimizu, 1997, Seifi *et al.*, 2007, Yamaguchi *et al.*, 2010) and in Human subjects with different protocols: (Abello and Valbuena, 1996): 904 nm, 110 W, 20 sec per tooth, (Cruz *et al.*, 2004): 780 nm, 20 mW and 5 J/cm<sup>2</sup>, (Dominguez and Velasquez, 2010): 830 nm, 100 mW, 80 J/cm<sup>2</sup> (2,2J), 44 sec (Sousa *et al.*, 2011): 780 nm, 20 mW, 5 J/cm<sup>2</sup>, and 10 sec per tooth.

The biological basis of the clinical effects of laser, (Dominguez *et al.*, 2009) had been partially explained by the results of in vitro studies in fibroblasts (Dominguez *et al.*, 2008, Lopes *et al.*, 2001, Pereira *et al.*, 2002, Marques *et al.*, 2004, Kreisler *et al.*, 2003, Vinck *et al.*, 2003) and osteoblasts (Coombe *et al.*, 2001, Fujihara *et al.*, 2006, Ozawa *et al.*, 1998, Dominguez *et al.*, 2009, Pires *et al.*, 2008). Laser effects on Osteoclast studies in animals reported that a low intensity laser facilitates bone resorption. In rats, (Kawasaki and Shimizu, 2000) found that in the tension side the numbers of osteoclasts are significantly increased with respect to unirradiated control. These reports suggest that the application of laser can accelerate the orthodontic movement through bone remodeling. This is consistent with previous research that

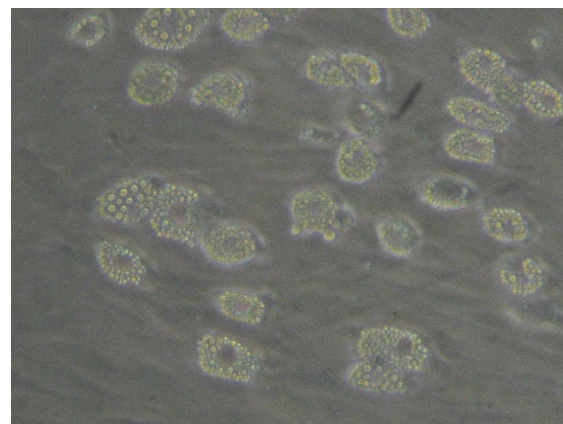
reported several differences in the amount of irradiated osteoclasts vs the control side 3, 5 and 7 days after the initiation of the treatment, promoting dental movement and alveolar remodeling (Sun *et al.*, 2001).

Bone resorption clearly depends on the osteoclastic activity during orthodontic dental movement (Tsay *et al.*, 1999, Noxon *et al.*, 2001, Rody *et al.*, 2001, Xie *et al.*, 2008); therefore it is important to know the effects of laser irradiation upon the precursor cells (Boyle *et al.*, 2003, Mikan and Oliveros, 2007). The only previous report on this subject was obtained in cultures of pre-osteoclasts isolated from rat fetal calvaria (Aihara *et al.*, 2006) finding that laser irradiation induced differentiation and activation of osteoclasts via effects on the RANK system of signalling, but there are no previous studies on human pre-osteoclasts treated with any kind of laser.

Therefore, the purpose of this study was to evaluate the effects on the viability of the pre-osteoclasts and cell proliferation in cultures of human pre-osteoclasts, after the irradiation with low intensity laser, during a follow-up period of six days.

## MATERIALS AND METHODS

The cell culture used was the Poietics™ Human Osteoclast Precursors Cat No. 2T-110 Cambrex-Lonza Inc. (Walkersville, USA)



**Figure 1** Poietics™ Human Osteoclast Precursors from Cambrex-Lonza Inc. (3X)

**Preparation of media**

The culture medium for Osteoclast precursors is prepared by adding only culture medium components (10% bovine fetal serum, glutamine 2 mMol, Penicillin 100 units/mL and Streptomycin 100 µg/mL) and pre-heated at 37°C

**Cell culture**

The in vitro human pre-osteoclast cells have an average increase in cell density in 4 days, in T-25 bottles with an initial seed concentration of 2x10<sup>4</sup> cells per cm<sup>2</sup>. The cells growth in suspension, hence the growth is not described as monolayer but in terms of cell density. The cryovial provided by Cambrex-Lonza Inc. (Walkersville, USA) containing 1x10<sup>6</sup> cells, was defrosted and heated to 37°C in a water-bath.

The cells were recovered by centrifugation at 200x g for 15 min at room temperature.

The number of cells was measured by the Trypan Blue exclusion method and adjusted to a concentration of 2x10<sup>4</sup> cells per cm<sup>2</sup> in culture flasks T-25 Falcon™.

For the cytotoxicity assay the final cell concentration was adjusted to 2x10<sup>4</sup> cells per 200 mL, and for the cell proliferation assay it was adjusted to 4.000 cells per 200 mL. The vials were placed in the 32 central wells of the 96 well plate. The microplates containing the cells were incubated at 37°C under an atmosphere of 5% CO<sub>2</sub>, 90% humidity during 24 h. (Figure 1)

**Effects on the viability of the osteoclasts with LDH assay**

The LDH activity in the supernatant is measured with the LDH kit (Roche) by a microplate reader spectrophotometer (ELISA reader) at 492 nm.

The amount of supernatant taken for the assay is 100 µL per well. To each well 100 µL of reagent are added and the mixture is incubated for 30 min. After that time of incubation the cells of the experimental group are irradiated.

Any increase in death cells or cell membrane breakdown will be reflected by a proportional increase in LDH activity measured by the amount of formazan produced, whose absorption is measured at 500 nm

To calculate the percentage of cytotoxicity it is necessary to read three controls. (Table 1)

To determine the percentage of cytotoxic activity, the average values of absorbance from three readings is first calculated and then the average absorbance is subtracted from the absolute control, which in this case was <0.200, and therefore was neglectable.

The measured values were substituted in the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Low Control}}{\text{High Control} - \text{Low Control}} \times 100$$

**Cell proliferation evaluation**

Cell proliferation was determined using the XTT kit from Roche (Roche; St Louis, MO, USA).

The pre-osteoclast cells are placed in the microplate wells to a final volume of 200 mL with fresh culture medium and incubated at 37°C, in a humidified atmosphere with 5% CO<sub>2</sub>, for 24 h.

After the incubation time laser is applied and then 50 ml of the XTT reactive are added to have a final concentration of 0.3 mg/mL XTT. The plates are incubated for 24 to 144 h in humidified atmosphere

**Table 1 Reagent Distribution in wells for the LDH assay**

	Control Absolute	Control Low	Control high	Toxicity	Assay
Medium	200 uL	100 mL	-	-	-
Tritón X-100 (2%)	-	-	100 mL	100 mL Tritón 100%	-
Cells	-	+	+	+	+
Irradiation	-	-	-	-	+

**Table 2 Laser Parameters**

Power	Wavelength	Dose	Time
36.73mW	832.79 nm	3.75 J/cm <sup>2</sup>	32.40 sec/per well

(37°C, 5% CO<sub>2</sub>) and the absorbance was measured every 24 h for six days. The absorbance is proportional to the cell density.

**Irradiation Protocol**

The protocol used is the same described in previous studies for gingival and periodontal fibroblasts (Domínguez *et al.*, 2008) and for human osteoblasts (Domínguez *et al.*, 2009). The laser parameters are summarized in Table-2.

The source was an infrared laser Photon LASE II (As-Ga-Al. DMC Equipamentos; Sao Carlos, Brazil) at a wavelength of 832.79 nm. It was adapted with a convergent lens at a focal distance of 5 cm from the dispositive output and at 7.0 cm away of the cell culture surface.

The power output was maintained at 36.73 mW in continued mode. The total time of irradiation per well was 32.40 sec. The flow of energy was 3.75 J/cm<sup>2</sup>.

The irradiation of culture media was performed during the proliferation phase, 24 h after seeding. After the application of laser the cultures were incubated again to evaluate cell proliferation after 24, 48, 72, 96, 120 and 148 hours and the cell cytotoxicity was evaluated after

**Table 3 Cytotoxicity Results at 6 h**

Group	High Control	Low Control	Triton	Laser
Absorbance	0,423	1,218	0,754	1,064
	0,381	0,754	0,927	1,023
	0,532	1,102	0,979	0,951
	0,526	0,934	0,639	0,98
	0,439	0,976	0,829	0,964
	0,71	0,754	0,96	0,823
			0,963	0,991
			0,597	0,982
			1,179	0,964
			1,69	0,823
		0,924	0,951	
		0,675	0,942	
Mean	0,502	0,956	0,926	0,955
Std deviation	0,118	0,186	0,293	0,07

six and 24 h.

**Ethical issues**

According to the Colombian Norm 008430 of 1993, this study was classified as FREE OF RISK for the participants as there was not any intervention on subjects because the study was made in human pre-osteoclasts from a cell line already established.

**RESULTS**

**Cell Cytotoxicity**

The LDH assay results are given as average data for absorbance at six hours in Table 3.

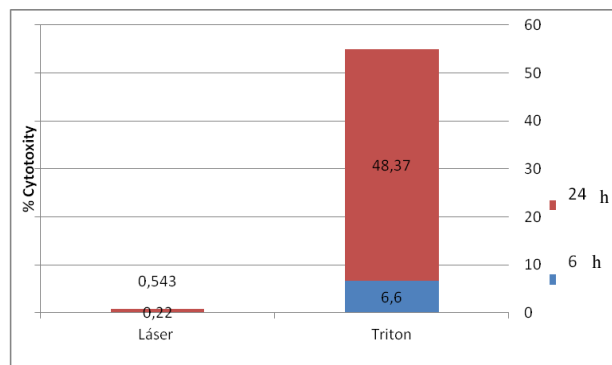
The irradiated cells presented 0.22% of cytotoxicity at 6 h, which corresponds to 44 cells out of 20000 damaged during that period of time. The triton treated group (Triton X-100, 100%) gave 6.6% cytotoxicity, which are 1320 lysed cells after 6 h.

The average absorbance results after 24 h are presented in Table 4.

After 24 h the laser group presented 0.543% of cytotoxicity against 48.37% for Triton X-100, which means that in 24 h from the initial amount of 20,000 cells, only 108 were lysed in the experimental group while in the Triton X-100 group 9674 cells were damaged. (Table 5)

The difference between groups in the percentage of cytotoxicity is significant for 6 and 24 h (P<0.001).

No significant difference was found between laser and high control in terms of cytotoxicity:



**Figure 2 Comparative percentage of Cytotoxicity laser Vs Triton**

**Table 4 Cytotoxicity Results after 24 h**

Group	High Control	Low Control	Triton	Laser
Absorbance Values	0,34	1,003	0,988	0,87
	0,727	0,956	0,811	0,861
	0,775	0,987	0,903	1,049
	0,773	0,819	0,878	0,924
	0,934	1,197	0,851	0,904
	1,129	0,819	0,958	0,809
			0,997	0,902
			0,865	0,93
			0,901	1,139
			0,767	1,104
Mean Standard deviation			0,817	0,869
			0,765	1,193
	0,78	0,964	0,875	0,963
	0,262	0,14	0,078	0,125

After 24 h the difference between laser group and low control was 0.1%. In contrast, the difference between high control and low control was 19%. After 6 h, the difference between laser and low control was 0.1%. The difference between high and low control was 49%. That means the cytotoxicity of the laser group is similar to the low control after 24 and 6 h (the respective averages are 0,964 and 0,963. Also at 6 h the averages are: 0.956 and 0.955.).

The average cytotoxicity measured after 24 h is twice the observed after 6 h (59% difference) in the laser treated group; with Triton the cytotoxicity was seven times higher after 24 h (86.3% difference). For six hours, the laser was 30 times less cytotoxic than Triton and for 24 h it was 89 times less cytotoxic than Triton (96,6% difference after 6 h and 98.8% difference after 24 h). These are statistically significant differences

**Table 5 Average cytotoxicity (%)**

Hours	Laser	Triton
6	0,22	6,6
24	0,543	48,37

( $p < 0.001$ ).

The only significant difference at a significance level  $p = 0,05$  is between laser and Control high at 6 h ( $p < 0,00001$ ).

For the 24 h interval there are no significant differences ( $p > 0,05$ ).

Comparison between groups are in Table 6.

**Cell viability**

No significant differences between the irradiated and the control group were detected at any time after the treatment.

Data about the proliferation results are in Tables 7-8.

Due to the non-parametric distribution of the data the Kruskal-Wallis test was applied, to detect a significant difference between the time periods evaluated ( $p < 0.0001$ ).

The Mann-Whitney test also detected significant differences between days 1, 2 and 3 and not significant between the 4, 5 and 6 days.

After three days the effect does not continue to increase in a significant way.

**DISCUSSION**

Previous studies have shown that low intensity laser is not cytotoxic against gingival and periodontal fibroblasts. Gingival fibroblasts proliferation was greater in the experimental cultures than in controls, although the difference was not statistically significant, while in periodontal fibroblasts the rate of cell proliferation was greater in the control group, but again not significantly different (Domínguez *et al.*, 2009)

Normal human osteoblast cultures (NHOst) are sensitive to low intensity laser irradiation with Photon LASE (As-Ga-Al) during the initial stage of the culture, presenting a significant increase in cell proliferation since the first day (Domínguez *et al.*, 2009). Following the same protocol a reaction of human pre-osteoclasts in vitro against low intensity laser irradiation found no

**Table 6 Comparison between groups-Cytotoxicity**

Groups compared		p
6 h	Laser vs High control	<0,00001
	Laser vs low control	0,99
	Laser vs Triton	0,742
	low control vs Triton	>0,05
24 h	Laser vs High control	0,124
	Laser vs low control	>0,05
	Laser vs Triton	0,742
	low control vs Triton	>0,05

cytotoxic effect. Additionally there was not any significant effect on cell viability.

Pre-osteoclasts are mononuclear cells derived from the lineage of macrophage-monocytes (CFU-GM) that normally are differentiated to osteoclasts, and therefore may increase their cell density as active forms or may be fused and differentiated to mature multinuclear osteoclasts in the presence of specific signals, without undergoing proliferation. It is possible that these work results don't correspond to (Karu, 1988) model about the mechanism of the laser action which conducted to cell proliferation or protein synthesis.

In the present study we found significant differences between the results of cytotoxicity observed at 6 vs 24 h ( $P < 0.001$ ) and between the irradiated group and the group treated with Triton X-100. The cell density was not affected as there are no significant differences between groups, concluding that the biological behavior was the same, according to the expected for the cell cycle observed.

Previous studies in animal models (Saito and Shimizu, 1997, Seifi *et al.*, 2007, Yamaguchi *et al.*, 2010) as well as the study of Kawasaki and Shimizu (Kawasaki and Shimizu, 2010) reported that orthodontic movement is faster in irradiated rats, and that there is an increased amount of osteoclasts in the compression side. The effect of laser irradiation on the speed of orthodontic movement is traced back to differentiation and activation of osteoclasts by the expression of RANK signals and the interaction Rank-Rank-L, a significant increase in TRAP positive

cells (Fujita *et al.*, 2008) and changes in the expression of MMP-9, cathepsin K, and integrins alpha and beta.

Besides an increment is detected in the interaction of M-CSF with the receptor c-Fms in osteoclastic precursors, that stimulate osteoclastic differentiation (Yamaguchi *et al.*, 2010).

The new experimental results reported here evaluate in-vitro effects of low intensity laser on human pre-osteoclasts cells, which must be taken into account to get an insight into the biological processes occurring in vivo.

So far, according to our knowledge there are no reports about the effect of this laser or any other laser type on Human pre-osteoclasts in vitro. This may be due to the difficulty of maintaining the short life cycle of culture pre-osteoclasts culture.

Only one article: (Aihara *et al.*, 2006) suggested that low-energy laser irradiation facilitated differentiation and activation of osteoclasts with up regulation of RANK expressions; Low-energy laser irradiation (Ga-Al-As semiconductor laser) was applied to rat osteoclasts precursor cells but not the, no human cells.

This is the final work about the effect of low level laser on cells related to orthodontic movement with the same protocol of irradiation (Dominguez *et al.*, 2009, Dominguez *et al.*, 2008, Dominguez *et al.*, 2009). The reason to use only one dose is that the clinician can't radiate with different parameters in the same zone. Correlating the present results with previous studies in osteoblasts, in which these cells had a significantly

higher proliferation (Domínguez *et al.*, 2009), it is likely that the osteoclastic stimulation is due to cell interactions between osteoblasts-osteoclasts rather than by a direct effect on the normal activity of pre-osteoclasts (density cell was not affected). The results obtained do not exclude the fact that low intensity laser irradiation benefits the process of bone remodeling during the orthodontic dental movement.

### CONCLUSIONS

- Low intensity laser irradiation does not have a significant cytotoxic effect on human pre-osteoclasts cells in vitro.
- Low level laser therapy is not affecting the cell viability of normal human pre-osteoclasts cells cultures in vitro.

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