Original

The effects of low-level diode laser treatment and dental pulp-capping materials on the proliferation of L-929 fibroblasts

Vanessa Ferriello¹⁾, Miriam R. Faria¹⁾ and Bruno N. Cavalcanti^{1,2)}

¹⁾Biodentistry Postgraduate Program, Ibirapuera University, São Paulo, SP, Brazil
²⁾Department of Cariology, Restorative Sciences and Endodontics, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

(Received 7 May and accepted 14 November 2009)

Abstract: Low-level laser therapy (LLLT) has been reported to improve tissue healing and might therefore be useful in dental pulp capping after trauma. We evaluated the effects of a low-level diode laser (λ = 680 nm) and dental pulp-capping substances on cell proliferation. Calcium hydroxide and adhesive resin were applied as conditioned media to cultures. Half of the samples received irradiation with the diode laser at a fluence of 4 J/cm² for 60 s. Using a hemocytometer, cells were counted at 1, 3, 5, and 7 days, and the data were analyzed by ANOVA. All cultures exhibited continuous growth, except those treated with adhesive resin. As compared to the other two groups, cell proliferation was significantly lower in cultures treated with adhesive resin; it was also significantly lower in cultures treated with calcium hydroxide, as compared to the control group. When combined with dental pulpcapping materials, LLLT had no effect on L-929 cell proliferation. (J Oral Sci 52, 33-38, 2010)

Keywords: calcium hydroxide; cell culture; dental pulp capping; laser.

Introduction

There is an ongoing search for dental materials that maintain pulp vitality and integrity after trauma. Calcium

Correspondence to Dr. Bruno Neves Cavalcanti, Av. Paul Harris, 661, Sao Jose dos Campos, 12242-170 SP, Brazil Tel: +55-12-91350725 Fax: +55-12-39236370 E-mail: brunocav@umich.edu hydroxide, in all its forms, is used extensively as an option in pulp capping, because it induces mineralization and is antibacterial (1,2).

Although calcium hydroxide has proven to be effective and less cytotoxic than other materials, some researchers have recommended the used of dental adhesives because they obviate microleakage, which is a possible cause of pulp-capping failure (3,4). However, the use of adhesives that are directly applied to the pulp can be harmful to the tissue, because such adhesives are cytotoxic, inhibit cellular mechanisms, and induce necrosis (5-9).

Recently, low-level laser therapy (LLLT) has been used to stimulate healing and edema regression via its antiinflammatory action, which may be beneficial after a trauma-related pulp exposure. The effects of LLLT have been seen in all the health sciences – not only in dentistry (10-13). Moreover, the effect of LLLT is localized, resulting in no harm to adjacent structures (14). Because of these advantages, LLLT biostimulation is already improving treatment results in many research areas; however, there is no reference in the literature to its use in dental pulp capping. Indeed, there are no published data regarding the use of LLLT in any type of dental pulp therapy. Due to this lack of information, it is important to begin studies of both therapies, since the use of a low-level laser could be useful in improving pulp capping. In addition, it should be stressed that because LLLT is quite effective in a number of health applications, it could also support conservative dental pulp therapy, which is the subject of the present report.

The use of cell cultures provides an excellent method for a preliminary study, as it allows for the control of pH, temperature, O_2 and CO_2 tension, and sample characterization and homogeneity (15). Thus, the purpose of this study was to evaluate the combined effects of a lowlevel diode laser ($\lambda = 680$ nm) and dental pulp-capping materials on cell proliferation. In addition, the cytotoxicity of calcium hydroxide and a dental adhesive were analyzed and the results were compared to a control group.

Materials and Methods

The cytotoxicity of materials used in direct pulp-capping procedures was measured *in vitro*. This analysis was based on long-term survival, which was measured by calculating the remaining self-renewal capacity of the cells.

Experimental groups and preparation of the materials

Three experimental groups were established:

- Control (fresh culture medium);
- Calcium hydroxide powder (Hidroxil; Inodon, Porto Alegre, Brazil);
- Adhesive resin (Single bond, 3M ESPE, St. Paul, USA).

Calcium hydroxide was prepared by mixing 1 g of powder in 350 μ l of sterile distilled water on a sterile glass plate and adding the solution to the bottom of a 50ml centrifuge tube. The adhesive resin was applied to the bottom of the centrifuge tube and light cured with a 600 mW/cm² halogen light (Dabi Atlante, Ribeirão Preto, SP, Brazil) for 20 s.

Culture medium conditioning

In order to obtain conditioned media (ie, media containing dissolved calcium hydroxide or adhesive resin), the test tubes containing the capping materials were filled with Dulbecco's modified Eagle culture medium (DMEM; Sigma, St Louis, MO, USA). Conditioning was carried out for 1 h, at 37°C, using 0.2 g of each substance per ml of fresh medium (5). In the adhesive group, light polymerization was performed after filling the tube with fresh medium, and dissolution of materials was allowed before and during this procedure. The stock conditioned media obtained during this step were diluted (10%) and then applied to the cell cultures. The control group underwent no media conditioning, which allowed us to evaluate the effects of the laser in the absence of a pulp-capping material.

Cell culture

The mouse fibroblast cell line L-929 was cultured in DMEM, supplemented by 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% antimycotic-

antibiotic solution (10,000 units of penicillin, 10 mg of streptomycin, and 25 μ g of amphotericin B per ml in 0.9% sodium chloride; Sigma). The cells were kept in an incubator at 37°C and a humidified 5% CO₂ atmosphere. Cultures were supplied with fresh medium every other day. Only cells between the 5th and 10th passages were used in experimental procedures. After cell culture, the cells were counted and plated on 6-well plates (1,000 cells per well), 6 wells per group. Half the wells (3 wells per group) were treated with the laser; the other half were treated only with the culture media (conditioned or not). To confirm the results obtained, all experiments were conducted independently 3 times.

Laser treatment

After cell adhesion to the plates, the diode laser (Twin Laser, MM Optics, São Carlos, SP, Brazil) was applied to half the cultures for 60 s at a total fluence of 4 J/cm² (16-18). The irradiation was performed in a standard position -1 cm above the cells and centered to the plate – with the laser in unfocused mode to increase the spread of energy on the cells. The wavelength used was 680 nm.

Cell survival assays

The growth curves for each material were obtained as previously described (15). Briefly, cell counts were determined by counting the viable cells in a hemocytometer, using the trypan blue dye exclusion assay. The number of viable cells harvested from each dish was estimated by the following equation: UC \times D \times 104/nSQ, where UC, unstained cell count (viable cells); D, the dilution of cell suspension; and nSQ, number of counted squares of the hemocytometer.

Statistical analysis

Each data point corresponded to the mean \pm standard deviation of cell counts from 3 wells. The data were compared by 2-factor (laser treatment and capping material) ANOVA (P < 0.05) and Tukey's post hoc test (P < 0.05).

Results

Cell proliferation in all tested groups is illustrated in Fig. 1. All groups had continuous growth, except those treated with the bonding system. There were no significant differences (Table 1) with respect to laser treatment (P = 0.107); however, there were significant differences with respect to material (P < 0.001). Tukey's post hoc test revealed significant differences in all comparisons of the tested materials (Table 2). The overall cell viabilities of the test groups and control groups are illustrated in Fig. 2.

Table 1 Analysis of variance

Source of Variation	SS	df	MS	F	P-value
Material*	49145.49	3	16381.83	14.33	< 0.001
Laser treatment	1653.13	1	1653.13	1.45	0.2335
Interaction	270.49	3	90,16	0.08	0.9712
Within	731:	55.56	64		1143.06
Total		12	24224.65		71

*Statistically significant difference

Table 2Statistically significant differences among the tested materials,
Tukey's test

Comparison	Difference between means	Significance
Control \times Ca(OH) ₂	21.88	Yes
Control × Bonding system	79.79	Yes
$Ca(OH)_2 \times Bonding system$	57.92	Yes

Tukey's critical value = 17.28



Fig. 1 Growth curves, by test group. Cell count (×1,000).

Discussion

It has been suggested that the healing properties of lowlevel laser therapy can minimize the cytotoxic effects of substances leaching from pulp-capping materials (10-13). However, in the present study, we found that the growth curves of cells treated with a low-level laser were not significantly better than control curves. In addition, we found that calcium hydroxide was less cytotoxic than adhesive resin.

We used cell culture in this study because it allows for the control of pH, temperature, O_2 and CO_2 tension, and sample characterization and homogeneity (15). A commercially available mouse fibroblast cell line (L-929) was chosen for its reliability and because its use allowed comparison with other studies of cytotoxicity and the



Fig. 2 Cell counts per group, normalized to untreated control group value. Cell count, normalized to untreated control group value.

effects of laser treatment. It should be noted, however, that results from primary culture may differ according to the laboratory responsible for cell culture.

The main purpose of vital pulp therapy is to assure pulp integrity and functionality. Minimizing damage to this tissue is clinically advantageous because it reduces the risk of postoperative complications and avoids radical endodontic treatment (7,19). In this context, the choice of a pulp-capping material is difficult, because no material fulfills all requirements regarding tissue compatibility and long-term protection. Calcium hydroxide has been recommended due to its biological effects, which aid in tissue healing and dentin bridge formation (1,2); its superior biocompatibility makes this material the first choice for direct pulp capping (2,8,9,20). The results of the present study accord with previous findings (5,20), ie, cell counts in the calcium hydroxide group were always approximately 80% of those observed in control cultures (Fig. 2).

In contrast, the use of a dental adhesive is not recommended for pulp capping, because of its high cytotoxicity, which has been noted in previous studies (5,6,19). In the present study, the performance of dental adhesive was poor, even with the slight increase in cell numbers that occurred after LLLT. Cell counts were less than 20% of those observed in control cultures, which indicates that the adhesive killed most of the cells, and blocked their proliferation (Fig. 2).

In addition to our investigation of pulp-capping materials, we attempted to determine whether low-level laser treatment could improve the performance of the abovementioned materials in cell cultures. A diode laser ($\lambda = 680$ nm) was chosen because of its stimulatory effects, as previously described in the literature (11,16,18), and because diode laser devices are inexpensive and readily available for use in private practice. In general, a low-level diode laser is used as an alternative to biomodulation, which is associated with wound healing (10,16,17) and improved cellular effects (11,14,17,21). Stabholz et al. (13) maintained that these effects are due to an increase in ATP in mitochondria, which leads to an increase in DNA and RNA synthesis after diode laser irradiation. This increase in DNA and RNA synthesis can result in a superior and faster cellular response to injuries, via the enhanced production of proteins related to healing and repair.

In the present study, we analyzed cell proliferation because it is essential to biological activity (22). Fluence is an important parameter in low-level laser therapy, as very low and very high doses cannot produce the desired biological effect (17,22). Although fluence does not precisely quantify the amount of energy absorbed by cells, it is a highly standardized parameter for describing the amount of energy delivered to cells. Based on the methods and results of previous studies (16-18), we used a fluence of 4 J/cm², which is considered to be very effective for lowlevel laser therapy. However, cell proliferation was only slightly higher in laser-treated groups than in control groups; the difference was not statistically significant. Many types of laser devices are available for clinical use,

37

and altering wavelengths and other parameters can lead to different cellular effects (11,14,22). In addition, because this was the first study to explore the use of LLLT for dental pulp therapy, all the test parameters and methods were developed for the present study. Our results indicate that, under the present parameters, low-level laser treatment does not improve the performance of dental pulp-capping materials. Further studies using different laser wavelengths and parameters are recommended in order to explore the possibility of therapeutic support for vital pulp therapy, which may result in a protocol for clinical use. In addition, our findings do not support the use of dental adhesive for direct pulp capping, as it is considerably more cytotoxic than calcium hydroxide.

In conclusion, under the present experimental conditions LLLT did not increase L-929 cell proliferation when combined with dental pulp-capping materials. In addition, calcium hydroxide is less cytotoxic than dental adhesive.

References

- 1. Cox CF, Suzuki S (1994) Re-evaluating pulp protection: calcium hydroxide liners vs. cohesive hybridization. J Am Dent Assoc 125, 823-831.
- 2. Schuurs AH, Gruythuysen RJ, Wesselink PR (2000) Pulp capping with adhesive resin-based composite vs. calcium hydroxide: a review. Endod Dent Traumatol 16, 240-250.
- 3. Cox CF, Suzuki S, Suzuki SH (1995) Biocompatibility of dental adhesives. J Calif Dent Assoc 23, 35-41.
- Liebenberg WH (1999) Intentional pulp capping: a clinical perspective of the adhesive experience. J Adhes Dent 1, 345-363.
- 5. Cavalcanti BN, Rode SM, Marques MM (2005) Cytotoxicity of substances leached or dissolved from pulp capping materials. Int Endod J 38, 505-509.
- 6. Hebling J, Giro EM, Costa CA (1999) Biocompatibility of an adhesive system applied to exposed human dental pulp. J Endod 25, 676-682.
- 7. Heyeraas KJ, Sveen OB, Mjör IA (2001) Pulpdentin biology in restorative dentistry. Part 3: Pulpal inflammation and its sequelae. Quintessence Int 32, 611-625.
- 8. Costa CA, Mesas AN, Hebling J (2000) Pulp response to direct capping with an adhesive system. Am J Dent 13, 81-87.
- Trope M, McDougal R, Levin L, May KN Jr, Swift EJ Jr (2002) Capping the inflamed pulp under different clinical conditions. J Esthet Restor Dent

14, 349-357.

- Amir A, Solomon AS, Giler S, Cordoba M, Hauben DJ (2000) The influence of helium-neon laser irradiation on the viability of skin flaps in the rat. Br J Plast Surg 53, 58-62.
- Almeida-Lopes L, Rigau J, Zangaro RA, Guidugli-Neto J, Jaeger MM (2001) Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and same fluence. Lasers Surg Med 29, 179-184.
- 12. Guzzardella GA, Fini M, Torricelli P, Giavaresi G, Giardino R (2002) Laser stimulation on bone defect healing: an in vitro study. Lasers Med Sci 17, 216-220.
- Stabholz A, Sahar-Helft S, Moshonov J (2004) Lasers in endodontics. Dent Clin North Am 48, 809-832.
- 14. Kreisler M, Christoffers AB, Willershausen B, d'Hoedt B (2003) Effect of low-level GaAlAs laser irradiation on the proliferation rat of human periodontal ligament fibroblasts: an in vitro study. J Clin Periodontol 30, 353-358.
- 15. Schmalz G (1994) Use of cell cultures for toxicity testing of dental materials advantages and limitations. J Dent 22, S6-S11.
- Amorim JC, de Sousa GR, de Barros Silveira L, Prates RA, Pinotti M, Ribeiro MS (2006) Clinical study of the gingiva healing after gingivectomy and low-level laser therapy. Photomed Laser Surg 24, 588-594.
- Pugliese LS, Medrado AP, Reis SR, Andrade Zde A (2003) The influence of low-level laser therapy on biomodulation of collagen and elastic fibers. Pesqui Odontol Bras 17, 307-313.
- Ferreira AN, Silveira L, Genovese WJ, de Araujo VC, Frigo L, de Mesquita RA, Guedes E (2006) Effect of GaAlAs laser on reactional dentinogenesis induction in human teeth. Photomed Laser Surg 24, 358-365.
- Demarco FF, Tarquinio SB, Jaeger MM, de Araújo VC, Matson E (2001) Pulp response and cytotoxicity evaluation of 2 dentin bonding agents. Quintessence Int 32, 211-220.
- 20. Alliot-Licht B, Jean A, Gregoire M (1994) Comparative effect of calcium hydroxide and hydroxyapatite on the cellular activity of human pulp fibroblasts in vitro. Arch Oral Biol 39, 481-489.
- Ohbayashi E, Matsushima K, Hosoya S, Abiko Y, Yamazaki M (1999) Stimulatory effect of laser irradiation on calcified nodule formation in human

38

dental pulp fibroblasts. J Endod 25, 30-33.

22. Sroka R, Schaffer M, Fuchs C, Pongratz T, Schrader-Reichard U, Busch M, Schaffer PM, Dühmke E, Baumgartner R (1999) Effects on the mitosis of normal and tumor cells induced by light treatment of different wavelengths. Lasers Surg Med 25, 263-271.