Braz Dent J (2002) 13(2): 109-112 ISSN 0103-6440

Does LLLT Stimulate Laryngeal Carcinoma Cells? An *In Vitro* Study

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Low level laser therapy (LLLT) has been used successfully in biomedicine and some of the results are thought to be related to cell proliferation. The effects of LLLT on cell proliferation is debatable because studies have found both an increase and a decrease in proliferation of cell cultures. Cell culture is an excellent method to assess both effects and dose of treatment. The aim of this study was to assess the effect of 635nm and 670nm laser irradiation of H.Ep.2 cells *in vitro* using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). The cells were obtained from squamous cell carcinoma (SCC) of the larynx and were routinely processed from defrost to the experimental condition. Twenty-four hours after transplantation the cells were irradiated with doses ranging from 0.04 to 0.48J/cm^2 for seven consecutive days (5 mW diode lasers: 635nm or 670nm, beam cross-section ~1mm) at local light doses between 0.04 and 0.48J/cm^2. The results showed that 635nm laser light did not significantly stimulate the proliferation of H.Ep.2 cells at doses of 0.04J/cm^2 to 0.48J/cm^2, However, 670nm laser irradiation led to an increased cell proliferation when compared to both control and 635nm irradiated cells. The best cell proliferation was found with 670nm laser irradiated cultures exposed to doses of doses of 0.04 to 0.48J/cm^2. We conclude that both dose and wavelength are factors that may affect cell proliferation of H.Ep.2 cells.

Key Words: cell proliferation, hazards, stimulatory effect.

INTRODUCTION

Low level laser therapy (LLLT) has been used successfully in many medical and dental specialities. The effects of LLLT on wound healing is often attributed to increased cell proliferation (1-3). There is still controversy concerning the effect of LLLT on cell proliferation because there are reports of both stimulatory and inhibitory action of visible laser light on cell cultures (4-6).

In the last 25 years, *in vitro* research has become one of the most used methods for laboratory experiments worldwide, mainly for studies involving drug interaction, anti-tumoral procedures and recently the laser. This method will continue to be the method of choice for research due to difficulties of both human and animal experimentation, because this method allows the study of the effect of several agents on cells of different origins and also LLLT action on different cell lines (1,4,5,7).

H.Ep.2 cells (SCC, type 2), originated from laryngeal carcinomas, have a higher growth rate after two days of culturing when the cell number increases approximately five times (8). The best proliferation rate occurs in a medium with a pH ranging from 7.6 and 8.7. It is also known that H.Ep.1, H.Ep.2, H.Ep.3 and H.Bem.Rh.1 cells are affected by heat and UV light (8). It is very important to be aware of the effect of LLLT on

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malignant cells because of the possibility of exposition to laser light during clinical treatment.

The use of malignant cells in cell cultures has been reported previously (8,9). Increased cell proliferation of cultures from melanoma, cervical and breast cancer, after irradiation with the argon laser (4.2 and 150KJ/m^2, 35 to 500W/m^2) has been reported (10). A study using 805nm laser light (4J/cm^2 and 20J/cm^2) on gingival SCC cultured cells (ZMK), urothelial carcinoma (J82) and normal urothelial cells (HCV 29) observed increased and decreased number of mitosis of HCV29 and J82 with the above mentioned doses, respectively. On the other hand, ZNK cells presented decreased mitotic activity with both doses (11). Stimulation of synthesis of both RNA and DNA was also analyzed using He-Ne laser radiation (10^2J/m^2) on HeLa cells (12). This study concluded that there is expressive cell proliferation on the exponential phase and also increased cell adhesivity to the substrate of human fibroblasts irradiated with He-Ne laser (15 min, 1W/m^2) (4).

There is controversy about the results observed previously because visible laser light may present inhibitory or stimulatory effects on the dependence of photoreceptors which are part of the respiratory chain and depend upon external factors such as the amount of energy, wavelength, potency and irradiation time, meaning that the effect is dose-dependent (4). The reduction of the amount of energy has a positive influence on cell metabolism, because increased amounts of energy and products resulting from photosensitization lethally damage cells (4).

The aim of this study was to assess the effect of 635nm and 670nm laser light on H.Ep.2 cultured cells.

MATERIAL AND METHODS

H.Ep.2 cultured cells (Departamento de Antibióticos/UFPE) were kept frozen at -80°C (10) in a medium containing Dubecco's modified Eagle's medium, 10% fetal bovine serum (FBS), 1% L-glutamine and a 1% antibiotic solution (250 µg/ml streptomycin and 80 mg/ml gentamycin sulfate. Twenty percent FBS was used after defrosting and the pH was kept at 7.8 using a 10% sodium bicarbonate solution. The cells were cultured on plastic culture flasks as described previously (13,14). Cell cultures were re-planted twice a week. Before any experimentation, all cultures were observed under light microscopy and the viability of the culture was confirmed using the Tryptan exclusion test, because this substance easily penetrates on non-viable cells and normal cells remain colorless (15). In order to calculate cell viability the following equation was used: viability = number of living cells x 100/number of dead cells. Twenty-four hours before irradiation a cell suspension (5 x 10^4 cells/ml) was prepared. Prior to irradiation, 100 µl of the solution was placed in 24 wells of a 96-well culture plate in order to avoid light dispersion to others. The suspension was then irradiated with doses ranging from 0.03 to 0.48J/cm^2 for seven consecutive days (5 mW diode lasers: 635nm or 670nm, beam cross-section ~1mm) at local light doses between 0.04 and 0.48J/cm^2. The assessment of cell proliferation was carried out using the MTT method. Spectroscopy (540nm) was used to determine the optical density (OD) of both irradiated and control samples as described by Nascimento (14). Analysis of variance was used for statistical evaluation.

RESULTS

The proliferation of non-irradiated H.Ep.2 cultures remained as reported in the literature (14) during the experimental period. However, all irradiated cell cultures behaved differently. Figure 1 shows a similar tendency for cell proliferation for both 635nm irradiated and control groups. The average OD of irradiated cultures was 0.72 and controls 0.71. Analysis of variance did not show significant differences between the two groups ($p = 0.455$). On the other hand, there was a tendency to increased cell proliferation of samples irradiated with 670nm laser light when compared to controls (Figure 2). The average OD of these cultures was 0.80 compared to 0.74 for the controls. Analysis of variance showed a significant difference between groups ($p = 0.014$). When the two irradiated groups were compared, there was a difference for the tendency of cell proliferation (Figure 3). The average OD of 635nm irradiated samples was 0.72, and 0.80 for 670nm irradiated samples. Analysis of variance also showed a significant difference between the two irradiated groups ($p = 0.004$).

DISCUSSION

No previous literature was found concerning the

use of LLLT on H.Ep.2 cells. Although there is consensus amongst authors regarding the contra-indication of the use of LLLT on proliferative lesions and glandular tissue, we must also consider the inclusion of premalignant lesions and lesions which may not be diagnosed properly at an early stage. This aspect increases the possibility of such lesions being irradiated during LLLT.

It is understood that the behavior of cancerous cells significantly differs from that of normal ones. It is also well accepted that the observation of LLLT beneficial effects requires some type of deficiency because normal cells do not show any changes in their function after irradiation. In this study we used optimal conditions for experimentation using normal cell cultures in order to avoid additional variables that would prevent a more precise analysis of the data as abnormal cells have an atypical behavior.

Although our data show that the proliferation of non-irradiated H.Ep.2 cells followed the same pattern observed in previous studies (14), others have reported a different pattern (8,16). Our results also show that H.Ep.2 irradiated cell cultures behave differently from non-irradiated ones. We agree with Karu (4) that LLLT effects on cells is still a controversial topic. Our data also demonstrate that cell proliferation is influenced by the amount of energy given to the medium as suggested by Karu (12). We were also able to detect differences regarding the wavelength used for irradiation of the cells (4).

Wavelength influence indicates that 635nm laser light does not significantly stimulate the proliferation of H.Ep.2 when using doses ranging from 0.04J/cm^2 to 0.48J/cm (16). However, other doses may present different results. On the other hand, the data on the proliferation of 670nm irradiated samples (0.05 to 0.6J/cm^2) did show differences when compared to 635nm irradiated and non-irradiated cells. When comparing the irradiated groups, there was a tendency to increased proliferation of cells irradiated with 670nm from doses of 0.08J/cm^2. This was different from the results observed previously in a study carried out by our team in which the irradiation with 670nm laser light of *Candida albicans* had no significant effect on proliferation in comparison to samples irradiated with 635nm laser light (17).

We conclude that these preliminary results indicate that cell proliferation increases in H.Ep.2 cells irradiated with 670nm laser light using doses from 0.08 J/cm^2, and that there is a dose/wavelength dependency.

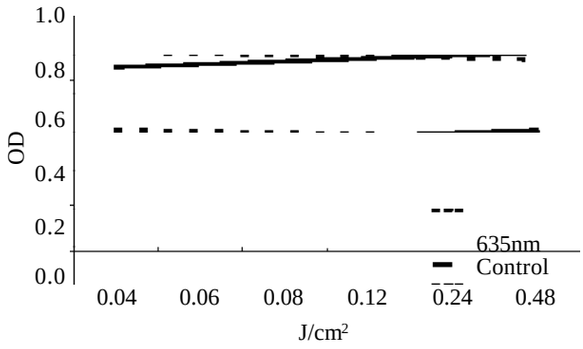

Figure 1. Tendency curve of proliferation of H.Ep.2 cells irradiated with 635nm laser light compared to control.

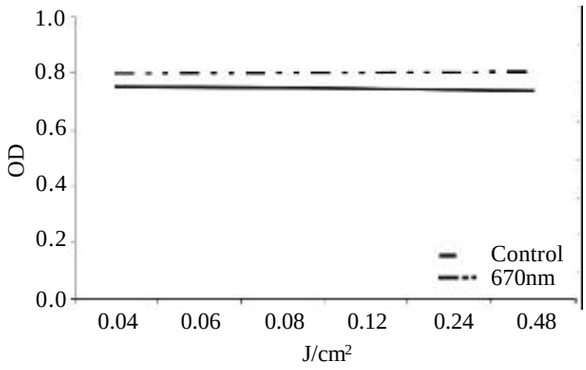

Figure 2. Tendency curve of proliferation of H.Ep.2 cells irradiated with 670nm laser light compared to control.

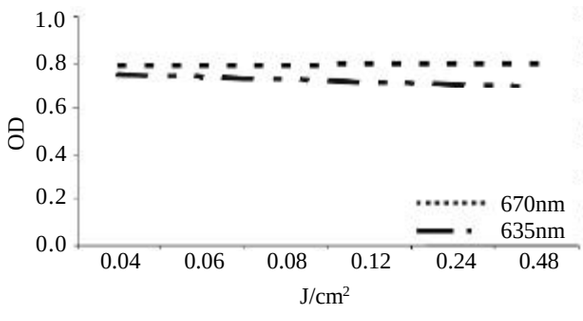

Figure 3. Tendency curve of proliferation of H.Ep.2 cells irradiated with 635nm laser light compared to H.Ep.2 cells irradiated with 670nm laser light.

RESUMO

Pinheiro ALB, Nascimento SC, Vieira ALB, Rolim AB, Silva PS, Brugnera Jr A. A laserterapia não cirúrgica estimula células de carcinoma de laringe? Un estudo *"in vitro"*. Braz Dent J 2002;13(2):109-112.

As irradiações com lasers não-cirúrgicos têm sido usadas com sucesso em diversas especialidades biomédicas. A ação do laser na atividade proliferativa celular é um assunto controverso já que a luz laser tem ora estimulado e ora inibido a proliferação de células em culturas. As culturas de células *"in vitro"* constituem um excelente meio para o estudo dos efeitos de diversos agentes permitindo verificar as reações entre dose e efeito. O objetivo deste trabalho foi avaliar através do M.T.T. (dimetil-tetrazólio) o efeito da irradiação de células H.Ep.2 por lasers de 635nm e 670nm de comprimento de onda, *"in vitro"*. As células foram dissociadas a partir de tumores de câncer de laringe, e foram mantidas acondicionadas congeladas em frasco de cultura a -80ºC. Após o preparo das placas e 24 horas após o transplante, foram submetidas a doses de 0.04 a 0.48J/cm^2 durante sete dias (635 ou 670nm, CW, 5 mW, (f ~1mm beam), 0.04, 0.06, 0.12, 0.24, 0.48J/cm^2). Os resultados demonstraram que a luz laser de 635nm não exerce um efeito estimulativo significante na proliferação das células H.Ep.2, dentro da faixa energética de 0,04J/cm^2 e 0,48J/cm^2 e que as culturas irradiadas com o laser de 670nm tiveram sua atividade proliferativa aumentada em comparação aos grupos controle e aos irradiados com o laser de 635nm. Conclui-se que a irradiação de células H.Ep.2 com lasers de 670nm resulta em um aumento da proliferação celular; que a proliferação celular em culturas irradiadas com o laser de 670nm foi melhor observado com doses a partir de 0.08J/cm^2; que a dose e o comprimento de onda são fatores que podem influenciar no processo proliferativo das células H.Ep.2.

Unitermos: proliferação celular, riscos, efeito estimulativo.

REFERENCES

1. Lubart R, Wollman Y, Friedmann H, Rochkind S, Laulicht I Effects of visible and near-infrared lasers on cell cultures. J Photochem Photobiol Biol 1992;12:305-310.
2. Pinheiro, ALB: Bases físicas dos lasers. In: Brugnera Júnior A, Pinheiro ALB. Lasers na Odontologia Moderna. São Paulo: Pancast; 1998.
3. Pogrel MA, Chen JW, Zhang K. Effects of low-energy gallium-aluminum-arsenide laser irradiation on cultured fibroblasts and keratinocytes. Lasers Surg Med 1997;20:426-432.
4. Karu TI. Effects of visible radiation on cultured cells. J Photochem Photobiol Biol 1990;52:1089-1098.
5. Lopes LA, Rigau J, Zângaro RA, Guiduli-Neto, Jaeger MM. Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and some fluence. Lasers Surg Med 2001;29:179-184.
6. Originali L. Risultati clinici di stimolazione laser e studi sperimentali circa il mecanismo di azione. Minerva Medica (Budapest) 1981;72:2195-2199.
7. Balboni GC, Brandi ML, Zonefrati R, Repice F. Effects of He-Ne / I.R. Laser irradiation on two lines of normal human fibroblasts "In Vitro". Arch Ital Anat Embriol 1986;91:179-188.
8. Moore AE, Sabachewsky L, Toolan HW. Culture characteristics of four permanent lines of human cancer cells. Cancer Research Philad 1955;15:598-602.
9. Thompson MD, Cupps TL, Wise DS, Wotring LL, Townsend LB. Synthesis and evaluation of 6-(dibromomethyl) 5 nitropyrimidines as potential antitumour agents. J Med Chem 1997;40:766-770.
10. Marchesini R, Dasdia T, Melloni E, Rocca E. Effect of low-energy laser irradiation on colony formation capability in different human tumour cells "in vitro". Lasers Surg Med 1989;9:59-62.
11. Schaffer M, Sroka R, Fuchs C, Schrader-Reichardt U, Schaffer PM, Busch M, Duhmke E. Biomodulative effects induced by 805nm laser light irradiation of normal and tumour cells. J Photochem Photobiol: Biology 1997;40:253-257.
12. Karu TI. Photobiological fundamentals of low-power laser. IEEE J Quantum Electrocs 1987;23:1703-1718.
13. Freshney RI. Culture de célules néoplasiques. In: Adolphe M, Meimon GB. Culture de cellules animales: methodologies applications. Paris: Inserm; 1998.
14. Nascimento SC. Recherche de l'activite antitumorale de produits de synthese ou d'origine naturelle: utilisation du samba pour l'analyse des modifications cellulaires induites. France, Docteur U.F.R. de Pharmacie, Grenoble I, Universite Joseph Fourier; 1993.
15. Weisenthal LM, Marsden JA, Dill PL, Macaluso CK. A novel dye exclusion method for testing "in vitro" chemosensitivity of human tumours. Cancer Res 1983;43:749-757.
16. Boothman DA, Trask DK, Pardee AB. Inhibition of potentially lethal DNA damage repair in human tumor cells by β-lapachone, an activator of topoisomerase I. Cancer Res 1989;49:605-612.
17. Ponzi EAC, Pinheiro ALB, Carvalho DA, Silva MP. The effects of 635 and 670 nm laser irradiation on Candida albicans: Study "in vitro". In: Proceedings of the 6th International Congress on Lasers in Dentistry, Hawaii, 1993.

Accepted November 14, 2001