INCREASED PROLIFERATION OF UNDIFFERENCIATED CELLS INDUCED BY OPTICAL RADIATION: *IN VIVO* STUDY IN PLANARIA "DUGESIA TIGRINA"

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Abstract -Today's scientific interest in tissue engineering for organs transplantation and regeneration from stem cells, allied with recent observations on biostimulation of tissues and cells by laser radiation, stands as a strong motivation for the present work, in which we examine the effects of the low power laser radiation onto planarians under regenerative process. To investigate those effects, a number of 60 amputated worms were divided in three study groups: a control group and two other groups submitted to daily 1 and 3 minutes long laser treatment sections at 0,91 mW/mm² power density. A 685 nm diode laser with 35 mW optical power was used. Samples were sent to analysis histological at the 4th, the 7th and the 15th days after amputation. A remarkable increase in stem cells counts for the fourth day of regeneration was observed when the regenerating worms was stimulated by the laser radiation. Our findings encourage further research works on the influence of optical radiation onto stem cells and tissue regeneration.

Introduction

The effects of coherent and non-coherent light in biological tissues and cells have been subject of many research works [1-8]. A variety of beneficial effects is attributed to the photostimulation of tissues, such as improved healing of diabetic and other types of ulcers [0], analgesic [0] and anti-inflammatory effects [0], stimulation of the proliferation of cells of different origins [0, 0, 0] and stimulation of bone repair [0, 0]. Some research works, like qualitative evaluation of wound healing, can be conducted in human subjects, as the low power visible and near infrared radiation has no side effects when safety limits are respected. However, deeper investigations on the mechanisms of action of the light stimulus and other quantitative works that requires biopsies or destructive analysis has to be carried out in animal models or in cell cultures. In vitro studies have been performed with fibroblasts [0, 0], myofibroblasts [0], cancer cells [0] and with bacteria as well [0].

Today's scientific interest in tissue engineering for organs transplantation and regeneration from stem cells stimulated us to search for a model to examine the effects of the low power laser radiation onto embryonic or undifferentiated cells. It seemed to us, since the very beginning of this research, that an organism with strong regenerative capability of lost body parts would be a natural choice for the intended in vivo study. Planarians are well known organisms with such an amazing capability and was the choice for the present study, a choice that was also supported by the easy of handling of those organisms in the laboratory environment.

The aim of this work, based upon the exposed ideas, was to look at the effects of the low power laser light at 685 nm onto stem cells, by using planarians as an in vivo animal model. Our findings suggest that the biomodulatory effects of optical radiation may be of great interest to people in the field of stem cell research, and to people studying the biology of the planarian regenerative processes.

Methods

For this work, adult organisms (*Dugesia tigrina*) were collected from their natural habitat, a lake situated in the city of Paulínia, state of São Paulo – Brazil. The collected animals were selected for their perfect morphology and maintained individually inside small plastic containers, with non-chlorinated water, in the laboratory environment (19 - 21°C) over a 6 months period prior to the experiments. The worms were fed fresh bovine liver once a week. Ambient light was turned on during day time and off at night.

After starvation for one week, 60 worms measuring from 10 to 15 mm in length were cut transversally at a position posterior to the auricules. For cutting, a block of ice was used for anesthesy.

The posterior fragments were then allowed to regenerate and were subject of this study.Three study groups were established, according to the exposition time to the low power laser light. A first set of samples received no laser treatment and was nominated as the control group (group C).The other two groups were daily exposed to laser radiation with exposition times of 1 minute and 3 minutes, adopting as a rule, a 24 hour interval between treatment sections.These groups will be hereafter referred to as G1 and G3, respectively.The first laser treatment section for G1 and G3 happened right after the cutting.

For the irradiation process, the worm fragments were placed once a time in a petri dish with a thin water layer and the diodo laser was mounted into a mechanical support for best reproducibility of the beam spot size at the plane of the animal body.The laser beam illuminated an area of 38.5 mm^2 at the petri dish, resulting in an optical power density at the animal plane of 0,91 mW/mm².

Therefore, the applied cumulative dosages per treatment section were 54,5 mJ and 164 mJ per square centimeter of the worm fragment surface for the groups G1 and G3, respectively.

The analysis histological were realized in three experimental periods the 4^{th}, the 7^{th} and the 15^{th} days of regeneration. For improved results in the maintenance of histologial tissues structures from this *Platyhelminthes* , Bouin's fluid was used for fixation. Bouin's fluid has been described as a fixative solution for planarians in the literature[0]. The serial sections were performed whit 5µm thickness and stained with Haematoxilin and Eosin (HE).

Results

The histological analysis has been centered to those sagittal cuts from the median portion of the worm fragments.

Figures 1-3 show the neoblast counts for the three experimental groups, obtained from histological analysis performed at the 4^{th}, the 7^{th} and the 15^{th} days after amputation. The results for the 4^{th} day of the experiment (Figure1) show that the average count for the group submitted to 1 minute long daily laser treatment sections triplicates, as compared to the average count for the non-irradiated control group. A less remarkable result can be observed for the group that received 3 minutes long laser treatment sections, for which the average neoblast count was a little above twice the average count for the control group, suggering that an optimum radiation dosage does exist. Indeed, published works on low power laser therapy conducted on cell cultures pointed out that excess radiation might have an inhibitory effect on cell proliferation [0]. Further research is required to determine the optimum dosage for planarian stem cell photobiostimulation.

At the 7^{th} and 15^{th} days of the experiment neoblast counts for the untreated and laser treated groups showed no differences. When are compared, we can see that the average neoblast count in the sampled area at day 4 was about 18 for the control group and about 60 for the group G1. At the day 7 of the experiment, the counts for the control group raised to about 40 and the counts for G1 decreased to about 40. Group G3 that presented an average neoblast count of about 48 at day 4 also decreased slightly to 40, a number that, at a first analysis, remained approximately constant from day 7 to day 15 for all treated and untreated groups.

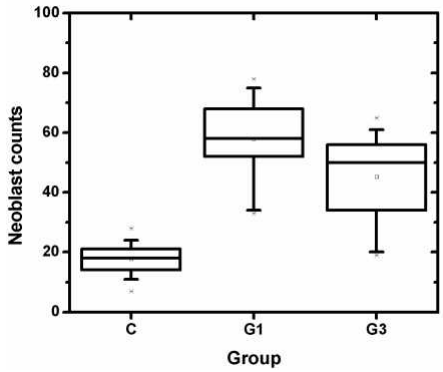

Figura 1: .Neoblast count in a standard 6600 μm^2 area delimited in the regeneration site for the three experimental groups at the 4^{th} day of the experiment.

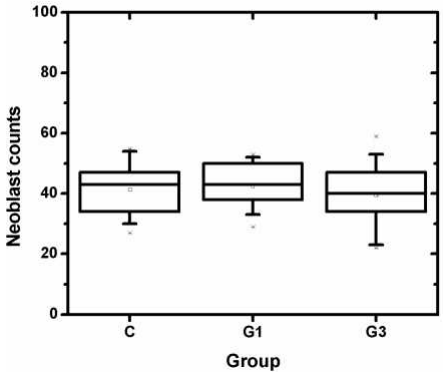

Figura 2: .Neoblast count in a standard 6600 μm^2 area delimited in the regeneration site for the three experimental groups at the 7^{th} day of the experiment.

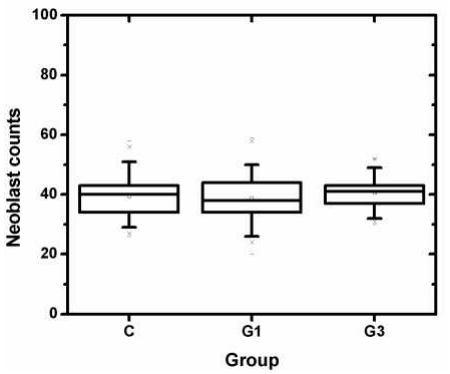

Figura 3: Neoblast count in a standard 6600 μm^2 area delimited in the regeneration site for the three experimental groups at the 15th day of the experiment.

Discussion

Several research works have reported on biomodulating effects of the low power laser (LPL) light. Nicolau and co-workers observed an increased mineral apposition rate in rat bone repair upon low power laser treatment [0]. Those authors also concluded in their report that the laser treatment applied during the inflammatory period of the bone repair process increases normal cell activity (resorption and formation). Most of the contemporary research on LPL therapy is particularly focused on the photobiostimulation of wound healing.

A number of experiments performed on photobiostimulation have also been conducted on cells. Increased proliferation rate induced by laser radiation have been recently observed on fibroblast cell cultures [0] and human laryngeal carcinoma cells in vitro [0]. The results observed in several cell types prompted us to investigate what would be the effect of the laser radiation onto stem cells.

Planaria was chosen as the animal model due to the well known high density of those undifferentiated cells, referred to as neoblasts, randomly scattered throughout the planarian parenchyma [0,0,0]. Pearson highlights that creatures, like planarians, with remarkable regeneration capacity may hold clues for researchers developing human cellular therapies. It is pointed out that organisms that can regenerate body parts, such as this flatworm, could aid human stem cell research [0]. To the authors' knowledge, this is the first report on the effect of low power laser biostimulation on stem cells.This is also the first report on photobiostimulation of wound healing in planarians, although the evaluation of regeneration speed under laser stimulus was not the objective at this time.

In contrast with several published works exploring cell cultures and unicellular organisms, the animals we have chosen for our work are among the simplest organisms that have tissue layers and tissues with distinct organs. Planarians have key anatomical features (mesoderm, central nervous system, digestive and excretory system) that might have been platforms for the evolution of the complex and highly organized tissues and organs found in higher organisms [0, 0,0].

In this work a remarkable increase in neoblast counts for the fourth day of regeneration was observed when the regenerating worms was stimulated by the laser radiation. Our results encourage further research works on the influence of optical radiation on tissue regeneration and onto stem cells. Although planarians are known as inhabitant of the shadows, our work suggests that light plays an important role on stem cells proliferation.

In addition, because previous studies pointed out that coherence seems not to be the issue in laser stimulation of biological tissue it may be suggested that indirect natural sunlight might also play a role in the neoblast proliferation dynamics for planarians under regenerative process in their natural habitat.

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BIBLIOGRAPHY

[1] KARU, T. Primary and secondary mechanisms of action of visible to near infra-red radiation on cells. **J. Photochem. Photobiol. B: Biol.**,v.49, p.1-17,1999.

[2] KARU T. Photobiological fundamentals of low-power laser therapy**. IEEE Journal Quantum Electron.**,v.10, p.1703-1717, 1987.

[3] SCHAFER, M.; SROKA, R.; FUCHS, C.; SCHRADER-REICHARDT, U.; SCHAFER, P.M.; BUSCH, M.; DÜHMKE, E.; Biomodulative effects induced by 805 nm laser light irradiation of normal and tumor cells**. J. Photochem. Photobiol. B: Biol.,** v.40, p.253-257, 1997.

[4] SCHAFER, M.; BONEL, H.; SROKA, R.;. SCHAFER, P.M.; BUSCH, M.; REISER, M.; DÜHMKE, E. Effects of 780 nm diode laser irradiation on blood microcirculation: preliminary findings on time-dependent TI-weighted contrast-enhanced magnetic resonance imaging (MRI). **J.**

Photochem. Photobiol. B: Biol., v.54, p.55-60, 2000.

[5] FREITAS, I.G.F.; BARANAUSKAS, V.; CRUZ-HOFLING, M.A. Laser effects on osteogenesis. **Applied Surface Science** p.154–155, 2000.

[6] NICOLAU, R.A.; JORGETTI, V., RIGAU, J.; PACHECO, M.T.T.; REIS, L.M.; ZÂNGARO, R.A. Effect of low-power GaAlAs laser (660 nm) on bone structure and cell activity: an experimental animal study, **Lasers Med Sci,** v.18, p.89-94, 2003.

[7] VINCK, E.M.; CAGNIE, B.J.; CORNELISSEN, M.J.; DECLERCQ, H.A.; CAMBIER, D.C. Increased fibroblast proliferation induced by light emitting diode and low power laser irradiation. **Lasers Med Sci**, v.18, p.95- 99, 2003.

[8] MANTEIFEL, V.; BAKEEVA, L.; KARU, T. Ultrastructural changes in chondriome of human lymphocytes after irradiation with He-Ne laser: appearance of giant mitochondria. **Journal of Photochemistry and Photobiology B: Biology**, v.38, p.25-30, 1997.

[9] LAGAN, K.M.; MC DONOUGH, S.M.; CLEMENTS, B.A.; BAXTER, G.D. A case report of low intensity laser therapy (LILT) in the management of venous ulceration: potential effects of wound debridement upon efficacy. **J Clin Laser Med Surg**, v.18, p.15-22, 2000.

[10] TAM, G. Low power laser therapy and analgesic action. **J Clin Laser Med Surg**.v.17, p.29-33,1999.

[11] FREITA, A.C.; PINHEIRO, A.L.B.; MIRANDA, P.; THIERS, F.A.; VIEIRA, A.L.B. Assessment of anti-inflammatory effect of 830 nm laser light using c-reactive protein levels, **Braz. Den. J.,** v.12, p.187–190, 2001.

[12] KREISLER, M.; CHRISTOFFERS, A.B.; WILLERSHAUSEN, B.; HOEDT, B.D. Low-level 809 nm GaA1As laser irradiation increases the proliferation rate of human laryngeal carcinoma cells in vitro. **Lasers Med Sci**, v.18, p.100-103, 2003.

[13] OZAWA, Y.; SHIMIZU, N.; KARIYA, G.; ABIKO, Y. Low-Energy Laser Irradiation Stimulates Bone Nodule Formation at Early Stages of Cell Culture in Rat Calvarial Cells. **Bone**, v.22, p.347–354, 1998.

[14] ALMEIDA-LOPES, L.; RIGAU, J.; ZÂNGARO, R.A.; GUIDUGLI-NETO, J.; JAEGER, M.M.M. Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and same fluence. **Lasers in Surgery and Medicine**, v.29, p.179-184, 2001.

[15] MEDRADO, A.R.A.P.; PUGLIESE, L.S.; REIS, S.R.A.;ANDRADE, Z.A. Influence of low level laser therapy on wound healing and its biological action upon myofibroblasts. **Lasers in Surgery and Medicine**, v.32, p.239-244, 2003.

[16] NUSSBAUM, E.L.; LILGE, L.; MAZZULLI, T. Effects of 810 nm laser irradiation on in vitro growth of bacteria: Comparison of continuous wave and frequency modulated light. **Lasers in Surgery and Medicine**, v.31, p.343-351, 2002.

[17] SILVA, N.M.S.; ZANCHET, A.M.L.; HAUSER, J. Analysis of the efficiency of different solutions for the fixation of *Girardia tigrina* (Turbellaria, Tricladida, Paludicola). **Braz. J. Morphol. Sci**.,v.14, p.271-274, 1997.

[18] ALVARADO, A.S.; NEWMARK, P.A.; ROBB, S.M.C.; JUSTE, R. The Schmidtea mediterranea database as a molecular resource for studying platyhelminthes, stem cells and regeneration. **Development**, v.129, p.5659-5665,2002.

[19] CEBRIÀ, F.; BUENO, D.; REIGADA, S.; ROMERO, R. Intercalary muscle cell renewal in planarian pharynx. **Dev Genes Evol**, v.209, p.249–253, 1999.

[20] NEWMARK, P.A.; ALVARADO, A.S. Bromodeoxyuridine Specifically Labels the Regenerative Stem Cells of Planarians. **Developmental Biology**, v.220, p.142–153, 2000.

[21] PEARSON, H. The regeneration gap. **Nature**, v.414, p.388-390, 2001.

[22] NEWMARK, P.A. and ALVARADO, A.S. Not your father's planarian: a classic model enters the era of functional genomics. **Nat. Rev Genet**, v.3, p.210-219, 2002.

[23] MÄNTYLÄ, K.; HALTON, D.W.; REUTER, M.; MAULE, A.G., LINDROOS, P.; SHAW, C.; GUSTAFSSON, M.K.S. The nervous system of Tricladida. IV. Neuroanatomy of *Planaria torva* (Paludicola, Planaridae): an immunocytochemical study. **Hydrobiologia**, v.383, p.167–173, 1998.

[24] CEBRIÀ, F.; NAKAZAWA, M.; MINETA, K.; IKEO, K.; GOJOBORI, T.; AGATA, K. Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. **Develop. Growth Differ,** v.44,p135–146,2002.