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Effect of Low-Level Laser Therapy (λ660 nm) on Angiogenesis in Wound Healing: A Immunohistochemical Study in a Rodent Model

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The aim of the present investigation was to evaluate the angiogenesis on dorsal cutaneous wounds in a rodent model treated with λ660 nm laser light. New vessel formation is a multistep process involving vessel sprouting, endothelial cell migration, proliferation and tube formation. Although several *in vivo* studies have shown that laser phototherapy influences tissue repair, a fully understanding of angiogenesis mechanisms are not yet known. Twenty-four young adult male Wistar rats weighing between 200 and 250 g were used. Under general anesthesia, one excisional wound was created on the dorsum of each animal and they were randomly distributed into two groups: one control and one treated with laser (λ660 nm, 16 mW, 10 J/cm^2). Each group was subdivided into three subgroups according to the animal death timing (2, 4 and 6 days). Laser irradiation started immediately after surgery and was repeated every other day during the experiment and marked with Sirius Red, specific for collagen, and immunomarked with anti-TGF-β and anti-von Willebrand factor. Marked sections underwent histological analysis by light microscopy and the mean area of the wound of each animal was calculated and analyzed by ANOVA and Tukey's test (α=0.05). Although at some death periods, collagen expression and number of blood vessels on irradiated animals were higher than in the control ones, no significant differences were found at any time in relation to TGF-β expression (p>0.05). It was concluded that laser treatment (λ660 nm) contributed to increase angiogenesis.

Key words: angiogenesis, TGF-β, laser, repair.

Introduction

Tissue repair is an interactive process, involving chemical mediators, cells, and the inflammatory response, characterized by the classic steps of repair: inflammation, granulation, and remodeling (1). The whole process is triggered by a tissue insult that is followed immediately by the migration of neutrophils into the injured site. Later, monocytes also migrate into the wound site in a similar manner to neutrophils. The proliferative phase occurs between the 5th and 14th days following injury; it is characterized by fibrogenesis. Fibroblasts, macrophages, and angiogenesis favor the formation of granulation tissue. Later, the wound enters a maturation period during which remodeling occurs, mainly by the deposition of a more mature and better-organized collagen fiber network (2).

In systemic and local healing, neoangiogenesis and collagen matrix deposition are very important for the outcome of tissue repair. Angiogenesis restores the level of both oxygen and nutrients for the newly forming tissue, supplying the high metabolic demand, favoring cell proliferation and migration as well as protein synthesis (3). Several growth factors show angiogenic potential. The transforming growth factor beta (TGF-β) is produced by most cells found in granulation tissue and increases the production of other cytokines, stimulates angiogenesis and the production of type 1 collagen, and inhibits the production of interstitial collagenase (1,2).

It is known that low-level laser therapy (LLLT), using appropriate protocols, improves wound healing (4). This study evaluated the effects of laser light (λ660 nm) on the maturation of granulation tissue, focusing on neoangiogenesis, TGF-β, and collagen matrix expression in cutaneous wounds in rodents.

Material and Methods

The Animal Experimentation Ethics Committee of the School of Dentistry of the Federal University of Bahia approved this work (Process #029/06).

Twenty-four young adult male Wistar rats, weighing 200-250 g, were obtained from the Central Animal House of the School of Veterinary Medicine of the Federal University of Bahia and kept in individual plastic cages, with wood-chip bedding, maintained at 22 °C on a day/night light cycle and fed with standard pellet laboratory diet (Labina, Agribrands-Purina Ltda., Paulínia, SP, Brazil) and had water available *ad libitum* at the Animal Experimentation

Laboratory of the School of Dentistry of the Federal University of Bahia, Brazil.

After regular quarantining, the animals received intraperitoneal general anesthesia [60 mg/kg of ketamine chlorhydrate (Vetaset; Fort Dodge Animal Health, Campinas, SP, Brazil) and 10 mg/kg of xylazine (Coopazine; Intervet Schering-Plough, São Paulo, SP, Brazil)] and had their dorsum shaven and cleaned with chlorhexidine gluconate 10 mg/mL solution (Merthiolate; Hypermarcas S.A., Barueri, SP, Brazil). One excisional cutaneous wound (1 × 1 cm) was created with a scalpel on the dorsum of each animal and left without suturing or dressing. The animals were then randomly distributed into two groups with 12 animals in each (Control and Laser). Each group was subdivided into three subgroups according to the time of sacrifice (2, 4, or 6 days). Laser therapy was carried out using a Twin Flex Laser (MMOptics Ltda., São Carlos, SP, Brazil) and started immediately after surgery and was repeated at every other day during the experimental period. Laser light was applied at four points around the wound (4 x 2.5 J/cm^2). The laser time was 62 s per point, with 248 s per session. The total dose time at 2, 4 and 6 days after surgery were respectively 248, 496 and 744 s (Table 1). The device automatically controlled the time of the application. The spatial average energy fluency (SAEF) per session used for all groups is summarized in Table 1. If the animal presented any evidence of pain, a non-steroid analgesic was ready for use, but this did not occur in any group.

Following macroscopic examination, each animal was sacrificed with an anesthetic overdose (300 mg/kg of ketamine hydrochloride and 50 mg/kg of xylazine hydrochloride) at 2, 4, or 6 days after surgery. Specimens were taken and kept in 10% phosphate-buffered formalin for 24 h and were then routinely cut and processed in wax. Five-micrometer-thick sections were stained with hematoxylin and eosin, and Sirius red for collagen identification. Antigen retrieval for the slides for anti-TGF-β staining was accomplished through incubation with pepsin 1%, pH 1.8, at approximately 37 °C for 30 min. For anti-von Willebrand factor, slides were steamed in Tris/EDTA solution, pH 9.0. They were then incubated overnight at 4 °C in a humidified chamber with polyclonal anti-von Willebrand factor antibody (A0082, DAKO-Denmark, 1:200) and a polyclonal anti-TGFβ antibody (1:4), with a background reducer (DAKO antibody diluent with background reducing components, S3022) using the EnVision system (DAKO, K4061), the DAB substrate chromogen system (DAKO, K3466), and Meyer's hematoxylin. Tissue sections were counterstained with hematoxylin, dehydrated, and mounted with Canada balsam. Granulation tissue acted as positive controls and negative control used PBS buffer instead of the primary antibody.

An experienced pathologist evaluated the immunostained sections histologically in a blind manner. A light microscope (Motic B5 Professional Series with a camera Moticam 2000 and the Motic Image Advance 3.0 software; Motic Instruments Inc., Richmond, BC, Canada) was used to capture images of three consecutive fields in each slide. The areas of collagen and TGF-β expression were measured using the Motic Image software. The number and area of vessels marked with anti-von Willebrand factor antibody were quantified. There was no restriction concerning the size of vessels; small endothelial cell islands were included, considering the bifurcations and longitudinal sections of microvessels. The mean area of each specimen was calculated using Excel for Mac. The mean area of each sub-group was calculated similarly. Data were analyzed using the Minitab software, version 15 (Globaltech, Belo Horizonte, MG, Brazil). For statistical analysis, ANOVA and Tukey's test were used to compare mean areas among groups with a significance level of 5%.

Table 1. Summary of the laser parameters used in the study

Parameter	Laser 1 (2 dias)	Laser 2 (4 dias)	Laser 3 (6 dias)
Wavelength (nm)	660	660	660
Intensity (mW)	40	40	40
Spot (cm^2)	0.1256	0.1256	0.1256
Irradiance (mW/cm^2)	318.4	318.4	318.4
Illuminated area (cm^2)	1	1	1
Time (per point) (s)	62	62	62
Time (per session) (s)	248	248	248
Total treatment time (s)	248	496	744
Fluence (per point) (J/cm^2)	19.74	19.74	19.74
Fluence (per session) (J/cm^2)	78.98	78.98	78.98
Fluence (treatment) (J/cm^2)	78.98	157.96	236.94
Energy (per point) (J)	~2.5	~2.5	~2.5
Energy (per session) (J)	~10	~10	~10
Energy (treatment) (J)	~10	~20	~30
SAEF (per session) (J/cm^2)	~10	~10	~10
SAEF (treatment) (J/cm^2)	~10	~20	~30

Results

Immunohistochemical staining for von Willebrand Factor showed elevated numbers of vessels in surgical wounds subjected to laser therapy, compared with the controls, although the differences were only statistically significant on the second day (Table 2, Figs. 1 and 2). A statistically significant difference was found at days 2 and 4 between the control groups ($p<0.001$, ANOVA) and between the laser groups ($p = 0.01$, ANOVA). Table 2 shows the distributions of areas of vessels in the laser treatment *versus* control groups at days 2, 4, and 6 post-treatment.

Table 2. Mean area of TGF- β, collagen and blood vessels on control and laser groups

Day	Group	Mean TGF- β area (mm^2)	Mean collagen area (mm^2)	Mean blood vessel area (mm^2)
2	Control[a]	0.01898	0.007415	0.000044
	Laser[b]	0.01334	0.01768[**(bf)]	0.001463
4	Control[c]	0.01520	0.01750	0.00666
	Laser[d]	0.01125	0.02410	0.01217[****(bd)]
6	Control[e]	0.01219	0.02314[*(ae)]	0.005935
	Laser[f]	0.016314	0.03791[***(ef)]	0.00808

* (ae): statistically difference significant between Control groups at days 2 and 6 (p=0.049). ** (bf): statistically significant difference between Laser groups at days 2 and 6 (p=0.049). ***(ef): difference statistically significant between Laser and control groups at day 6 (p=0.012). ****(bd): statistically significant difference between Laser groups at days 2 and 4 (p=0.00).

In animals treated with the laser, vasodilation was notable at every post-treatment time-point compared with control animals, although at days 2 and 4 the differences were statistically significant only between the laser groups. Table 2 shows the distribution of TGF-β area in laser-treated *versus* control groups at days 2, 4, and 6 post-treatment. At days 2 and 4, laser-treated wounds showed lower TGF-β levels, while at day 6, TGF-β synthesis was elevated in the laser treatment group compared with the control, although the differences were not significant (Figs. 3 and 4). Table 2 shows the distribution of collagen area in the laser and control groups at days 2, 4, and 6 post-treatment. Statistically significant differences were found at days 2 and 6 between the control (p=0.049) and the laser (p=0.049) groups. At the 6th day, laser-treated wounds showed elevated levels of collagen compared with the control group (Figs. 5 and 6, $p = 0.012$, ANOVA).

Discussion

This study assessed whether granulation tissue in cutaneous wounds would be influenced by the use of laser light because the formation of new blood vessels involves phenomena such as the migration of endothelial cells, proliferation, tube formation and survival (5). The degradation involving mast cells represents an important step in the reduction of vessel formation in the first 24 h after laser application. Immunohistochemical staining of von Willebrand factor showed lower numbers of vessels until the first 24 h in surgical wounds submitted to laser therapy compared with the control group. In animals treated with laser, vasodilation was notable, especially at the first 12 h post-treatment, compared with control animals (6).

In a study of the number of vessels formed with and without laser application (670 nm), there was an increase

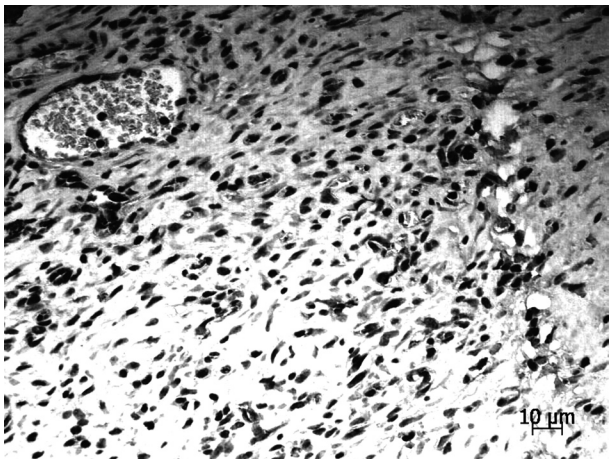

Figure 1. Expression pattern of endothelial von Willebrand positive cells in immunohistochemical staining. Control group showing a small number of vessels, day 2.

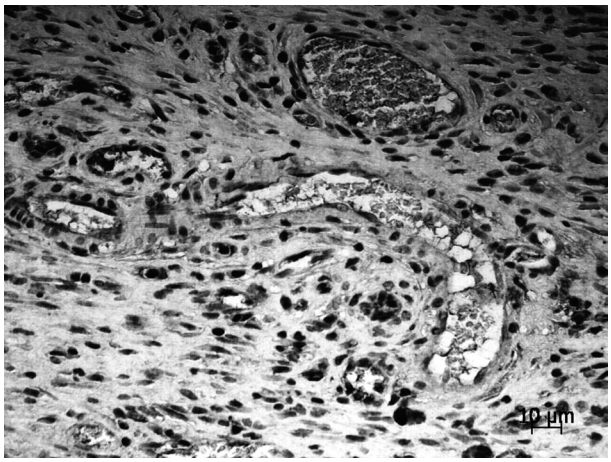

Figure 2. Von Willebrand positive cells in immunohistochemical staining. Laser group exhibiting elevated number of vessels and larger vessels lumen in comparison with the control group, day 2.

in vessels in the laser group compared to the control group on only the 3rd day (6). In the present investigation, the number of vessels and relative area of the laser group was superior at all periods. The pro-angiogenic effect of the use of LLLT we report here was characterized by a higher number of newly formed blood vessels at the wound site, mainly on the 2nd day following injury. Our findings are consistent with the report of Corazza et al. (7), who used 5 J/cm^2 and 20 J/cm^2 (λ = 660 nm, 40 mW, 31 s and 126 s), also on cutaneous wounds. A recent study in Wistar rats found similar results (λ = 670 nm, 9 mW, 4 J/cm^2 and 124 s) (6).

Another finding in the irradiated animals was vasodilatation, which was observed at all time points in comparison with the control animals and was significantly higher in irradiated subjects on both days 2 and 4. Although the difference between the groups was not statistically significant, it is possible that the laser contributed to the increase in vessel area, possibly due to the effects of light on cellular receptors (8,9). This phenomenon was also reported in subcutaneous tissue in which the inflammatory reaction was increased by laser irradiation (λ = 670 nm, 9 mW, 4 J/cm^2, 124 s) (8).

Growth factors, proteins, components of the coagulation/fibrinolytic pathways, extracellular matrix proteins, and platelets interact with endothelial cells and pericytes adjacent to blood vessels, regulating the formation of new blood vessels (10). Of the growth factors involved in angiogenesis, VEGF, PDGF, bFGF, TNFα, EGF, PDECGF and TGF-β were detected. VEGF is a determining factor for the differentiation of endothelial cells and for the development of vascularity in the wounded area. The role of the other growth factors, such as TGF-β, seems to be complementary (10). It was found lower TGF-β expression in irradiated animals in comparison with the control groups during the early phases of the repair process. Increased expression of this factor was observed on only the sixth day. Despite the absence of a significant difference between the groups, TGF-β expression may be considered a signal of the inhibition of both immunological and inflammatory responses. A previous report of healing of gingival lesions in rodents showed that the use of LLLT (λ = 632.8 nm, 17 mW, 7.5 J/cm^2, 300 s) increased PDGF and TGF-β gene expression. However, the expression of some cytokines, such as IL-1β and IFNγ, was reduced (11).

TGF-β acts as a regulator of growth and differentiation of cells, as well as playing an important role in the formation of the extracellular matrix (12). In the present study, we found increased collagen deposition in irradiated subjects, which was significantly higher on the sixth day. This is consistent with previous reports in which LLLT accelerated both angiogenesis and wound healing (13). *In vivo* and *in vitro* studies using different protocols have shown that LLLT modulates many of the cells involved in the healing process (6,14-21). A previous study using λ = 830 nm and/or λ = 685 nm laser light and doses of 20 J/cm^2 and 50 J/cm^2 showed increased collagen production in irradiated animals in comparison with the controls (λ = 830 nm, 50 J/cm^2) (22). The findings of a recent study evaluating the influence of LLLT on bone volume and bone implant contact interface around implants inserted in bovine or autologous bone grafts in the femurs of rabbits revealed that the use of LLLT stimulated new bone formation with consequent increase of bone-implant interface in both xenografts and autografts (23).

The results of the present study indicate that LLLT positively influences angiogenesis, TGF-β expression and collagen deposition. However, the TGF-β area increased significantly only from the 6th day on, while the collagen area increased progressively throughout the study period. This finding suggests that other growth factors are involved in collagen synthesis.

Resumo

O objetivo do trabalho foi avaliar a angiogênese em feridas cutâneas no dorso de ratos tratados com o laser de λ660 nm. A neovascularização é um processo que envolve o aparecimento vascular, a migração das células endoteliais, a proliferação e a formação tubular. Embora diversos estudos *in vivo* demonstrem que a fototerapia laser influencia no reparo tecidual, uma compreensão completa dos mecanismos da angiogênese ainda não é conhecida. Foram utilizados 24 ratos Wistar novos, machos e adultos pesando entre 200 e 250 g. Uma ferida excisional foi criada no dorso de cada animal sob anestesia geral e os animais foram distribuídos aleatoriamente em dois grupos: G0 (controle) e G1 (laser λ660 nm, 16 mW, 10 J/cm^2). Cada grupo foi subdividido em três subgrupos de acordo com o sincronismo da morte dos animais (2, 4 e 6 dias). A irradiação laser foi iniciada imediatamente após a cirurgia, sendo repetida diariamente durante a experiência, avaliada por meio de vermelho de Sirius, específico para o colágeno e avaliação imunológica com anti-TGF-β e o Fator anti-von Willebrand. As seções marcadas foram submetidas à análise histológica no foto-microscópio, onde a área média de cada subgrupo foi calculada e analisada usando o teste ANOVA e de Tukey (α=0,05). Os valores dos animais irradiados foram maiores em certos períodos da morte, na expressão do colágeno e no número de vasos em comparação com os grupos controles. Nenhuma diferença significativa foi encontrada na expressão do TGF-β entre os grupos nos períodos. Conclui-se que o tratamento com laser λ660 nm contribuiu para o aumento da angiogênese.

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