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Effectiveness of Laser Photobiomodulation at 660 or 780 Nanometers on the Repair of Third-Degree Burns in Diabetic Rats

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ABSTRACT

Objective: **The aim of this investigation was to compare by light microscopy the effects of laser photobiomodulation (LPBM) at $\lambda = 660$ nm and $\lambda = 780$ nm on third-degree burns in diabetic Wistar rats.** ***Background Data:*** **Burns are severe injuries that result in fluid loss, tissue destruction, infection, and shock, that may result in death. Diabetes is a disease that reduces the body's ability to heal properly. LPBM has been suggested as an effective method of improving wound healing.** ***Materials and Methods:*** **A third-degree burn measuring 1.5 × 1.5 cm was created in the dorsum of each of 55 animals, and they were divided into three groups that were or were not treated with LPBM ($\lambda = 660$ nm or $\lambda = 780$ nm, 35 mW, $\phi = \sim 2$ mm, 20 J/cm^2). The treatments were started immediately post-burn at four points within the burned area (5 J/cm^2) and were repeated at 24-hour intervals over 21 d. The animals were humanely killed after 3, 5, 7, 14, and 21 d by an overdose of intraperitoneal general anesthetic. The specimens were routinely cut and stained and analyzed by light microscopy.** ***Results:*** **We found that healing in the animals receiving 660-nm laser energy was more apparent at early stages, with positive effects on inflammation, the amount and quality of granulation tissue, fibroblast proliferation, and on collagen deposition and organization. Epithelialization and local microcirculation were also positively affected by the treatment.** ***Conclusion:*** **The use of 780-nm laser energy was not as effective as 660-nm energy, but it had positive effects at early stages on the onset and development of inflammation. At the end of the experimental period the primary effect seen was on the amount and quality of the granulation tissue. The 660-nm laser at 20 J/cm^2, when used on a daily basis, was more effective than the 780-nm laser for improving the healing of third-degree burns in the diabetic rats beginning at the early stages post-burn.**

INTRODUCTION

HEALING IS A COMPLEX PROCESS that involves a dynamic series of events including clotting, inflammation, granulation tissue formation, epithelialization, collagen synthesis, and tissue remodeling, and it still attracts the attention of many scientists, especially with regard to factors that delay or hinder it.

Diabetes mellitus is a syndrome with multiple etiologies, and is characterized by the absence of insulin. Impaired wound healing is a debilitating complication of diabetes and poses a serious challenge in clinical practice.

It is well known that the most important repair failures are those that occur in the initial stages, and may lead to edema, reduced vascular proliferation, and decreased numbers of leukocytes, macrophages, and fibroblasts.

Diabetes mellitus is a metabolic disease characterized by increased blood sugar levels resulting from impaired secretion and/or action of insulin. Diabetes is associated both clinically and experimentally with impaired wound healing.[1] The condition is prevalent worldwide, and many sufferers are not aware that they have the disease.[2] Thus diabetics often remain untreated or have poor medical control of their condition.[3]

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Poor wound repair in diabetic patients is well documented in the literature. The exact pathogenesis of poor wound healing in diabetes is not completely understood, but evidence from studies involving both human and animal models of diabetes reveals several abnormalities in the various phases of the wound healing process.

Diabetes-induced impairment of wound healing is characterized by inhibition of the inflammatory response, poor angiogenesis and fibroplasia, and defects in collagen deposition and differentiation of the extracellular matrix. These alterations result in impaired collagen synthesis, and also increase the risk of infection.

Several methods of improving wound healing in diabetics have been extensively reported elsewhere. On the other hand, the study of burns in diabetic patients has been poorly reported, and the use of phototherapy in these cases is rare. Laser photobiomodulation (LPBM) has been studied and shown to be effective in improving healing of both soft and mineralized tissues. Studies of excisional wounds in diabetic rats have also shown beneficial effects of LPBM.[4]

The present study aimed to determine the effects of LPBM on the healing process of third-degree burns in diabetic and nondiabetic Wistar rats.

MATERIALS AND METHODS

Following approval by the Animal Experimentation Ethics Committee of the School of Dentistry of the Federal University of Bahia, 55 young adult male Wistar rats weighing 200–230 g were obtained from the Centro de Criação de animais da Faculdade de Medicina Veterinária da Universidade Federal da Bahia, and were kept at the Animal Experimentation Laboratory of the School of Dentistry of the Federal University of Bahia. The animals were kept in individual plastic cages lined with wood chips and maintained at 22°C in a day/night light cycle. The animals were fed a standard laboratory diet and had water available *ad libitum*. After a regular quarantine period, the animals were randomly distributed into three groups as follows: G1: control (no treatment, n = 15); G2: LPBM (λ = 660 nm, n = 20); and G3: LPBM (λ = 780 nm, n = 20). Each group was then divided into five subgroups according to the time of sacrifice of the animals. The animals were kept unfed for 15 h and then injected with streptozotocin diluted in citrate buffer (0.1 M, pH 4.5, 60 mg/kg).[1] Forty-eight hours after injection, the blood sugar levels was taken, and only animals with blood sugar levels of 350 mg/100 mL or higher entered the study.[1] Under intraperitoneal general anesthesia (0.10 mL/100 g of ketamine and 0.25 mL/100 mg of xylazine) the animals had their dorsum shaven and cleaned. While under general anesthesia, a specially designed instrument measuring 1.5×1.5 cm[9] was heated until red and incandescent and then applied to the skin for 20 s to induce formation of a third-degree burn. In case an animal showed evidence of pain, a nonsteroidal analgesic was kept at hand, but it was not needed in any of the animals. LPBM was performed on group G2 (λ = 660 nm, 20 J/cm^2, 35 mW, ϕ = 2 mm) (Bio Wave LLLT, Kondortech, São Carlos, SP, Brazil) and group G3 (λ = 780 nm,

Table 1. Criteria Used for Light Microscopy Analysis

Criterion	*Score*		
Re-epithelialization	Absent	Present: Covering <50% of the wound Present: Covering >50% of the wound Present: Covering 100% with irregular thickness Present: Covering 100% with regular thickness	
Acute inflammation	Mild: <25% neutrophils seen in the cells in the field	Moderate: <25%–50% neutrophils seen in the cells in the field	Intense: >50% neutrophils seen in the cells in the field
Chronic inflammation	Mild: Presence of <25% chronic inflammatory cells in the field	Moderate: <25%–50% chronic inflammatory cells in the field	Intense: >50% chronic inflammatory cells in the field
Granulation tissue	Mild: Few fibroblasts, collagen fibers, and inflammatory cells present (400× magnification)	Moderate: Moderate numbers of fibroblasts, collagen fibers, and inflammatory cells present (400× magnification)	Intense: Many fibroblasts, collagen fibers, and inflammatory cells present (400× magnification)
Number of fibroblasts	Mild: <25% young and less differentiated fibroblasts among other cell types	Moderate: <25%–50% young and less differentiated fibroblasts among other cell types	Intense: >50% young and less differentiated fibroblasts among other cell types
Numbers of collagen fibers	Mild: Sirius red staining is less intense than that observed in the healthy adjacent tissue	Moderate: Sirius red staining is similar that observed in the healthy adjacent tissue	Intense: Sirius red staining is more intense than that observed in the healthy adjacent tissue
Neoangiogenesis	Mild: Less than that seen in the healthy adjacent tissue	Moderate: An amount similar to that seen in the healthy adjacent tissue	Intense: More than that seen in the healthy adjacent tissue

20 J/cm^2, 35 mW, ϕ ~2 mm) (Bio Wave LLLT; Kondortech, São Carlos, SP, Brazil). The choice of these treatment parameters was due to conflicting results in the literature regarding the effects of different treatment parameters on the efficacy of LPBM. The use of two different wavelengths was due to differences in the absorption and penetration of the laser energy, as both superficial and deep tissues were affected by the burns. LPBM was begun immediately post-burn, and was repeated daily until the day before the animal's sacrifice. The laser energy was applied transcutaneously in four equidistant points around the wound's margin. The dose per point was 5 J/cm^2 and the total dose per session was 20 J/cm^2. At each time point chosen (3, 5, 7, 14, and 21 d post-burn), the animals were humanely killed by an overdose of general anesthetic. Specimens were taken and kept in 10% formalin for 24 h. The specimens were routinely cut and embedded in wax. The slides were stained with hematoxylin and eosin and sirius red. The specimens were analyzed with light microscopy by an experienced pathologist. The criteria used for the analysis are shown in Table 1.

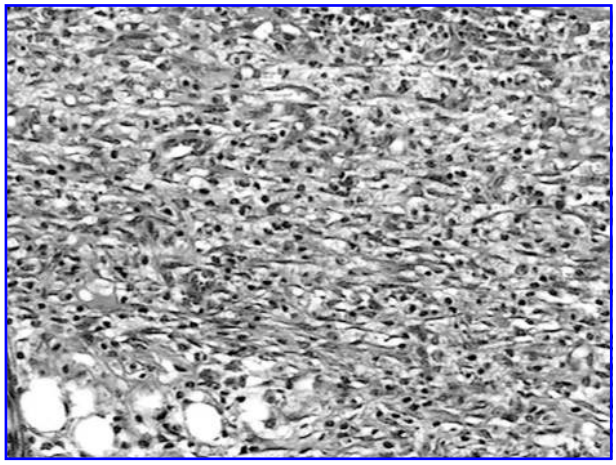

FIG. 2. Photomicrograph made 14 d post-burn of a specimen from a diabetic animal that had 780-nm LPBM. It shows granulation tissue with an intense mixed inflammatory infiltrate, and tortuous blood vessels can also be seen (hematoxylin and eosin, approximately 100×)

RESULTS

Three days after surgery, all specimens showed an extensive area of coagulative necrosis. While control specimens showed moderate to severe acute inflammatory reactions, in most subjects treated with both wavelengths of laser energy, inflammation was moderate in most cases48. Control subjects did not show signs of fibroblastic activity at this time. However, the use of both wavelengths of laser energy resulted in the presence of small numbers of fibroblasts that were migrating toward the area under repair. The amount of granulation tissue was also small in the laser-treated groups, and the amounts of collagen deposition and neoangiogenesis were small.

At day 5, all groups still showed coagulative necrosis extending down to the dermis, as well as inflammation (Fig. 1). Most subjects treated with laser energy had crust on the wound surface and intense inflammation. Chronic inflammation and small amounts of granulation tissue were present in all groups. While most laser-treated subjects showed a moderate number of fibroblasts at this stage, in control animals the number was smaller. Amounts of granulation tissue, collagen deposition, and neoangiogenesis were slight in all groups.

FIG. 1. Photomicrograph made 5 d post-burn of a diabetic control specimen, showing that necrosis of the dermis remained and inflammation could be seen (hematoxylin and eosin, approximately 100×).

At day 7, the necrosis was still present and extended down to the subcutaneous tissue in control animals. In the 660-nm laser-treated subjects it was seen extending down to the epidermis. In the 780-nm laser-treated subjects it was also seen extending down to the dermis, and viable skin cells could also be seen at this stage. Crust formation was seen in most of the laser-treated animals. While the acute inflammatory reaction was mostly intense, the chronic inflammatory reaction was less in control animals. In the subjects treated with 660-nm laser energy, the acute inflammatory reaction was moderate in all subjects, and the chronic inflammatory reaction was either slight or moderate. In the 780-nm laser-treated animals the acute inflammatory reaction was slight. Most specimens showed only a slight chronic inflammatory reaction at this stage. The number of fibroblasts was moderate in all groups, and the amount of granulation tissue was slight in the controls and moderate in the laser-treated subjects. Amounts of collagen deposition and neoangiogenesis were slight in all groups.

Fourteen days post-surgery, the necrosis remained down to epidermis. Below the necrotic area, the muscular tissue was infiltrated by neutrophils. The connective tissue was comprised of a moderate number of congested blood vessels in all control groups. In the 660-nm laser-treated animals, the ulceration was covered by crust in all animals. Below it, the connective tissue was comprised of a moderate number of fibroblasts. In the 780-nm laser-treated group the ulceration was covered by an irregular fibrinous crust. Below the crust, edema, neutrophils, and macrophages were present. There was also granulation tissue comprised of well-organized fibroblasts located parallel to the wound surface, some tortuous newly-formed blood vessels, a

mixed inflammatory infiltrate, and a collagen matrix (Fig. 2). The collagen fibers were immature and were delicate and sometimes fragmented. Under light microscopy, a small to moderate number of collagen fibers was seen, and the fibers were parallel to the wound surface. In some areas, fatty tissue was also seen. Control animals showed large numbers of fibroblasts that were mostly fusiform or oval in shape, and were dispersed in a collagen matrix. The inflammatory infiltrate was mononuclear (Fig. 3). In laser-treated subjects, the amount of neoangiogenesis was moderate, and the newly-formed blood vessels were congested and tortuous in all cases. Acute inflammation was slight in all subjects, and the chronic inflammatory infiltrate was either moderate or intense.

At day 21, control specimens still had ulceration covered by a crust at the dermis, and re-epithelialization was only partial in all cases (Fig. 4). Most 660-nm laser-treated subjects showed advanced re-epithelialization (Fig. 5). No 780-nm laser-treated specimens showed re-epithelialization at this stage. Both acute and chronic inflammation was slight in the control animals. In most of the laser-treated subjects, acute inflammation was mostly slight, and the amount of chronic inflammatory infiltrate was moderate. While control specimens had many fibroblasts, the number was only moderate in the laser-treated animals. In all groups, collagen deposition was mostly moderate and the amount of neoangiogenesis was intense. A summary of all the results can be seen in Table 2.

DISCUSSION

Despite the literature documenting many studies of the use of LPBM on wound healing in diabetic patients, its use is not as well documented with regard to the healing of third-degree burns. A previous study by Al-Watban and Andres[6] reported the use of LEDs to promote burn healing in diabetic rats. The laser parameters we used in this study are the same as those we previously used in a model of systemic impairment.[7] Al-Watban and Delgado[8] found that different laser parameters have different effects on the outcome of treatment using LPBM on burns.[8] Enwemeka et al.[9] also pointed out that other treatment parameters may also influence the results of such treatment.

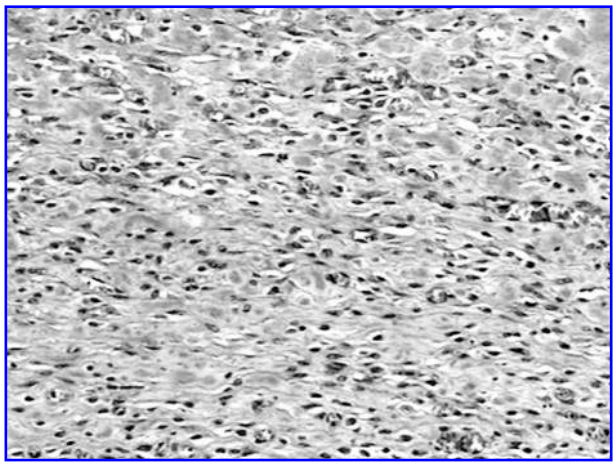

FIG. 3. Photomicrograph made 14 d post-burn of a specimen from a diabetic control animal, showing oval, fusiform fibroblasts, and a mixed inflammatory infiltrate scattered in a collagen matrix (hematoxylin and eosin, approximately 100×).

FIG. 4. Photomicrograph made 21 d post-burn of a specimen from a diabetic control animal. The granulation tissue was covered by a fibrinous crust and early re-epithelialization can be seen (hematoxylin and eosin, approximately 40×).

In the present investigation, we found that the effects of 660-nm LPBM of third-degree burns in diabetic rats are more evident at the early stages. During this part of the healing process, the laser energy promoted an accelerated inflammatory response, with increased numbers of mononuclear cells, as well as increases in granulation tissue formation and neoangiogenesis.

These effects on inflammation were maintained throughout the experimental period. The 660-nm wavelength was more effective following removal of necrotic tissue by phagocytes.

The metabolic impairment did not seem to significantly affect the ability of the laser light to interact with the tissue. There

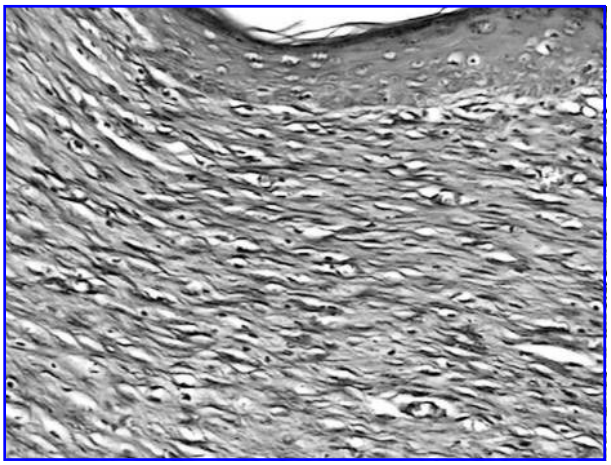

FIG. 5. Photomicrograph made 21 d post-burn of a specimen from a diabetic animal that had 660-nm LPBM. Epithelium can be seen covering the burned area. A large amount of extracellular matrix rich in collagen with a few mononuclear inflammatory cells could be seen on the dermis (hematoxylin and eosin, approximately 100×).

Table 2. Summary of the Results

	3 Days			5 Days			7 Days		
Variable	*G1*	*G2*	*G3*	*G1*	*G2*	*G3*	*G1*	*G2*	*G3*
Acute inflammation	33.3% mild; 66.6% intense	75% moderate; 25% slight	25% absent; 50% mild; 25% intense	33.3% intense; 66.6% moderate	75% intense; 25% mild	75% discrete; 25% intense	33.3% moderate; 66.6% intense	100% moderate	50% moderate; 25% mild; 25% intense
Chronic inflammation	100% absent	75% mild; 25% moderate	25% absent; 50% moderate; 25% mild	33.3% intense; 66.6% moderate	50% mild; 25% intense	25% absent; 50% mild; 25% intense	100% mild	50% mild; 50% moderate	25% absent; 75% mild
Re-epithelialization	100% absent	100% absent	100% absent	100% absent	100% absent	100% absent	100% absent	100% absent	100% absent
Fibroblasts	100% absent	100% mild	50% mild	33.3% intense; 66.6% moderate	100% mild	75% mild; 25% moderate	33.3% mild; 66.6% moderate	100% mild	75% mild; 25% moderate
Granulation tissue	100% absent	75% mild; 25% moderate	100% absent	33.3% moderate 66.6% mild	75% mild; 25% intense	50% absent; 50% mild	33.3% mild; 33.3% moderate	50% mild; 50% moderate	25% absent; 75% mild
Collagen	100% absent	100% mild	50% absent; 50% mild	100% mild	100% mild	100% mild	66.6% mild; 33.3% moderate	100% mild	75% mild; 25% moderate
Neoangiogenesis	100% absent	100% mild	100% absent	33.3% absent; 66.6% mild	100% mild	50% absent; 50% mild	33.35 absent; 66.6% mild	75% mild; 25% moderate	50% mild; 50% moderate

(*continued*)

Table 2. Cont'd

	14 Days			*21 Days*		
Group	*G1*	*G2*	*G3*	*G1*	*G2*	*G3*
Acute inflammation	33.3% mild; 66.6% moderate	100% mild	75% mild; 25% moderate	33.3% absent; 66.6% mild	75% mild	50% mild; 50% intense
Chronic inflammation	100% mild	50% intense; 50% moderate	75% moderate; 25% intense	33.3% mild; 66.6% moderate	75% mild	100% moderate
Re-epithelialization	100% absent	100% mild	75% absent; 25% <50%	100% partial	75% total; 25% partial	75% <50%; 25% >50%
Fibroblasts	33.3% intense; 66.6% moderate	50% moderate; 25% mild; 25% intense	50% mild; 50% moderate	33.3% intense 66.6% moderate	75% intense 25% moderate	100% moderate
Granulation tissue	33.3% mild; 66.6% moderate	50% mild; 25% moderate; 25% intense	100% moderate	33.3% mild; 66.6% moderate	100% moderate	75% intense; 25% mild
Collagen fibers	33.3% intense; 66.6% moderate	50% mild; 50% moderate	50% mild; 50% moderate	100% moderate	50% moderate 25% mild; 25% intense	75% moderate; 25% mild
Neoangiogenesis	100% moderate	100% moderate	100% moderate	100% intense	50% mild; 50% intense	75% intense; 25% moderate

was a decrease in the number of leukocytes and an increase in the number of lymphocytes. The latter are cells responsible for the late humoral response.

Positive responses were also seen in the amount and quality of granulation tissue formed, fibroblast proliferation, and on both collagen deposition and organization of the collagen matrix. This is important, as diabetic rats produce less granulation tissue than normal rats. The scars on diabetic animals have weak mechanical resistance, and these alterations have been attributed to biochemical alterations in the wound caused by insulin-deficiency syndrome.[10]

Researchers studying the effect of various lasers on wound healing in diabetic animals have found a higher percentage of wound closure in genetically diabetic mice.[11] Another group found that overall wound strength was increased when LPBM was performed in both genetically diabetic and nondiabetic mice.[12]

Our results corroborate those of a previous study, in which rats had diabetes induced by streptozotocin injection. Those researchers concluded that based on biomechanical and biochemical findings, LPBM enhanced the tissue repair process by accelerating collagen production and promoting connective-tissue stability.[13] Re-epithelialization and local microcirculation were also positively affected by the treatment. A study carried out recently[14] using HeNe laser energy on diabetic animals, found improved re-epithelialization following the use of 4 J/cm^2 on 4 consecutive days, suggesting that treatment of chronic ulcers in diabetic patients could be improved.

We found that 780-nm laser light had positive effects at early stages on the onset and development of inflammation. At the end of the experimental period, the primary effect seen was on the amount and quality of granulation tissue. These results may be attributed to the impaired metabolic status of diabetics, and this probably affected the ability of the tissues to absorb this wavelength and to process the energy effectively.

Our results are in agreement with those of several others, who found that LPBM accelerates the wound healing process, and increases production of collagen in diabetic subjects, mainly during fibroblast differentiation, which precedes collagen formation.[6,10,12,15]

The mechanism by which LPBM facilitates collagen production in wound healing is not clear. This effect may involve a variety of photobiostimulating mechanisms. One possibility is participation of mitochondria as photoacceptors of light energy. The absorption of energy by the mitochondrial respiratory chain may cause oxidation of reduced nicotinamide adenine dinucleotide (NADH), producing changes in the redox status in both the mitochondria and cytoplasm. Activation of the electron transport chain results in an increase in the electrical potential across the mitochondrial membrane, an increase in the ATP pool, and finally synthesis of nucleic acids.

It is known that laser energy can modulate cell proliferation and the release of growth factors from fibroblasts. Therefore the positive effects of LPBM on wound healing may involve the enhancement of growth factor release, which in turn promotes extracellular matrix production and degradation. Further investigation is needed to determine the precise mechanism of action of LPBM in relation to collagen remodeling and the actions of collagen metabolic enzymes during wound healing.

From the findings of our investigation, one could postulate that LPBM may be capable of increasing collagen, both in diabetics and nondiabetics. These findings are corroborated by those of other studies, that demonstrated the efficacy of LPBM in accelerating the healing process in diabetic and nondiabetic animals, by increasing the quantity of fibroblasts and collagen fibers.[12,14,16,17]

CONCLUSION

The results of this study indicate that 660-nm laser energy at 20 J/cm^2 daily is more effective than 780-nm laser energy in accelerating healing of third-degree burns in diabetic animals, beginning in the early stages post-burn. These findings may also have significant implications for the treatment of wounds in diabetic humans.

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