

Effect of a Diode Laser on Wound Healing by Using Diabetic and Nondiabetic Mice

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The purpose of this study was to evaluate a 980-nm gallium-aluminum-arsenide diode laser for wound healing. Using genetically diabetic and nondiabetic mice, two 6-mm wounds were created on the back of each mouse by using a punch biopsy. The mice were assigned to 1 of 4 subgroups for laser treatment at different fluence and frequency of treatment: 5 W (18 J/cm²) every 2 days, 5 W (18 J/cm²) every 4 days, 10 W (36 J/cm²) every 2 days, and 10 W (36 J/cm²) every 4 days. In addition, control mice were used and the wounds were allowed to heal naturally. Wound healing was evaluated on days 5, 12, and 19 by percentage of wounds healed and percent wound closure. A maximum of 5 mice per subgroup were killed at days 7, 14, and 21, and histology was conducted on the wound sites. For diabetic mice receiving 5 W every 2 days, the percentage of wounds healed after 19 days was 100% versus 40% in the control group. Only 20% of wounds in the 10-W diabetic subgroups achieved healing during the same period. For the subgroups whose wounds did not completely heal, all but the 10 W every 2 days subgroup had average closure of >90%. The 100% closure for the 5 W every 2 days subgroup was significantly greater than the other subgroups. For nondiabetic mice, 100% of the wounds in the 5 W every 4 days and control subgroups were completely healed, whereas 90% of the wounds from the 5 W every 2 days and the 10 W every 4 days subgroups were completely healed. In the latter 2 subgroups, wound closure was 99.4% and 98.8%, respectively. These differences were not significant. The histologic results confirmed these findings. In conclusion, treatment at 18 J/cm² shows a beneficial effect on wound healing in diabetic mice and does not have a detrimental effect in nondiabetic mice. (The Journal of Foot & Ankle Surgery 43(4):214-220, 2004)

Key words: diabetic mice, diabetic ulcers, diode laser, wound healing

Since their inception in 1960, lasers have found many applications in medicine, including surgery, dental disease, pain management, and rheumatoid arthritis (1). Interest in the efficacy of lasers as a noninvasive tool for wound healing has also developed. These lasers include helium-neon, gallium-arsenide, gallium-aluminum-arsenide (GaAlAs), Nd:YAG, carbon dioxide, ruby, and argon dye lasers (1, 2).

Approximately 11 million Americans are currently diagnosed with diabetes, and there are more than half a million new cases diagnosed each year (4). The leading cause of hospitalization for diabetes mellitus is foot ulceration with

infection (3). Approximately 15% of all individuals diagnosed with diabetes will be afflicted with at least 1 ulcer of the lower extremity during their lifetime (3–5). Although patients with diagnosed diabetes represent only 3% of the United States population, they account for greater than 50% of all lower-limb amputations that occur (3, 5). In 1994, diabetes-related lower-extremity amputations accounted for almost 100,000 days of hospital stays, and the average length of hospitalization approached almost 2 weeks. The vast majority of these amputations are attributed to skin ulcerations and the failure of a wound to heal (3). Amputation of 1 limb predisposes a patient to subsequent amputation on the same or opposite limb within 5 years at a rate as high as 50%. The 5-year mortality rate after lower-extremity amputations may reach as high as 68% (5). A noninvasive, nondestructive method to promote wound healing by stimulation of the patient's own tissues, irrespective of the underlying cause of the ulcer, could greatly reduce chronic ulcerations and subsequent amputations.

Studies using diabetic animals have been conducted to evaluate the effect of various lasers on wound healing. Yu et al (6) found that treatment with a 630-nm argon dye laser at a fluence (amount of energy passing through a unit area) of 5 J/cm² enhanced the percentage of wound closure over

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time in genetically diabetic mice. Stadler et al (7) examined the effect of daily irradiation with an 830-nm diode laser on the tensile strength of wounds. They discovered that, with a fluence of 5 J/cm², the overall wound strength increased in both genetically diabetic and nondiabetic mice. Reddy et al (8) evaluated the effect of laser treatment with a 632.8-nm helium-neon laser at a fluence of 1 J/cm² on wound healing by using rats in which diabetes was induced by streptozotocin injection. The investigators concluded that, based on biomechanical and biochemical findings, laser treatment enhanced the tissue repair process by accelerating collagen production and promoting connective-tissue stability (8).

A preliminary clinical study was conducted by Kawalec et al (9) to evaluate the effect of a 980-nm GaAlAs diode laser applied once every 2 weeks to healing ulcers on the foot or lower legs. Both diabetic and nondiabetic patients were enrolled in the study. At each visit for laser treatment, the ulcers were cleaned, debrided with a scalpel to remove hyperkeratotic tissue, and then treated with the laser set at a power of 5 W. The patients were then treated with their previously prescribed wound-care treatment, including medication and dressing. Nineteen ulcers from 16 patients were included in the study. Twelve of the ulcers were from patients with diabetes, whereas 7 were from patients without diabetes. Seven of the 19 ulcers, or 36.8%, were completely healed during the course of laser treatment. This included 6 of the 12 diabetic ulcers, or 50%. The average time until complete wound closure was 8.3 weeks. These findings suggested that the diode laser did enhance wound healing, particularly in patients with diabetes.

The results from the clinical study indicate that further study of the effect of the 980-nm GaAlAs diode laser on wound healing is warranted. The purpose of this study was to evaluate the effect of a 980-nm diode laser on diabetic and nondiabetic ulcers, using a controlled animal model. Specifically, the study examined the effect of fluence (based on the power setting on the laser) and frequency of treatment.

Materials and Methods

The animal models used for this study were genetically diabetic and nondiabetic C57BLKS/J mice (Jackson Laboratory, Bar Harbor, ME). One hundred fifty mice were initially evaluated, but 4 were lost to anesthesia complications (3 diabetic mice and 1 nondiabetic mouse) and 3 were lost to infection (1 diabetic mouse and 2 nondiabetic mice). This left 143 mice to be included in the analysis: 71 diabetics and 72 nondiabetics. The diabetic and nondiabetic mice were each divided into 5 subgroups based on the power and frequency of laser treatment (Table 1): mice in the 5W2d subgroup received laser treatment at 5 W every 2 days, mice in the 5W4d subgroup received treatment at 5 W every 4 days, mice in the 10W2d subgroup received laser treatment at 10 W every 2

TABLE 1 Experimental subgroups

Subgroup	Treatment/ Frequency	Diabetic Mice in Subgroup (N)	Nondiabetic Mice in Subgroup (N)
5W2d	5 W every 2 d	14	14
5W4d	5 W every 4 d	15	15
10W2d	10 W every 2 d	13	14
10W4d	10 W every 4 d	15	14
Control	No laser treatment	14	15

days, and mice in the 10W4d subgroup received treatment at 10 W every 4 days. The mice in the control subgroup did not receive laser treatment. The protocol was reviewed and approved by the Ohio College of Podiatric Medicine Institutional Animal Care and Use Committee.

Each mouse was anesthetized with a cocktail containing ketamine HCl, xylazine HCl, acepromazine, and sterile saline. Its dorsum was shaved and cleansed with Betadine solution (Purdue Pharma, Stamford, CT). Using aseptic technique, 2 full-thickness circular skin-punch biopsies, approximately 6 mm in diameter, were created, 1 on each side of the spine. All wounds were created by the same surgeon in an attempt to limit variability in the depth of each wound. Both wounds received treatment according to the subgroup to which the mouse was assigned. Laser treatments began the same day that wounding occurred. The Ceralas D15 diode laser (Biolitec, Inc, East Longmeadow, MA) was used to induce photostimulation, or stimulation by light energy. This laser is a GaAlAs diode laser that delivers up to 15 W of optical power at a wavelength of 980 nm. Laser energy was applied via a 600- μ m optical fiber.

During laser treatment, the energy was applied for 1 second with the tip of the optical fiber held approximately 1 cm from the surface of the wound, resulting in a spot size of 9 mm. As a result, the wounds in the 5-W groups received a fluence of 18 J/cm² per treatment, whereas those in the 10-W groups received a fluence of 36 J/cm² per treatment. After initial wounding, the wounds were covered with Tegaderm semi-transparent dressing (3M, St. Paul, MN) for a maximum of 4 days to prevent infection.

Two perpendicular diameters of each wound were measured with digital calipers on the day of wounding and at days 5, 12, and 19. The measurements were determined primarily by 1 individual (a biomedical engineer) throughout the course of the experiment to reduce variability in data collection. The areas were then calculated, using the equation for an ellipse ($A = \pi r_1 r_2$). An ellipse was chosen because, although the biopsy punch was circular, the resulting wound was often more elliptical in shape as a result of the thin mouse skin. Percent wound closure (%WC) was calculated for each day that a measurement was determined, using the following formula:

$$\%WC = \frac{(\text{area on day } x - \text{area on day } 0)}{\text{area on day } 0} \times 100$$

The percentage of wounds that had completely healed was also determined at each measurement day. The wounds were labeled completely healed if they were examined and observed to be clinically closed.

Mice from each subgroup were then killed at days 7, 14, and 21. Histologic slides of the wound area were prepared to assess healing. The specimen containing the wound was sectioned, mounted in paraffin, and stained with hematoxylin & eosin. All sections were scored on a 0 to 4 scale for degree of epithelialization, cellular content, granulation tissue, collagen deposition, and vascularity (Table 2). The scoring was completed by a blinded evaluator who did not know the fluence or the frequency of treatment the specimen had received. The blinded evaluator was a highly qualified individual with a specialty in laboratory animal pathology. The scores for each category were added together to determine a total score and then the average total score was calculated for each subgroup. The total score ranged from 0 to 20, with degree of healing defined as follows: 0.0 to 5.0, poor healing; 5.1 to 10.0, fair healing; 10.1 to 15.0, moderate healing; and 15.1 to 20.0, excellent healing. Statistical analysis was conducted for the average percent wound closure, using the Student 2-tailed *t* test (2 sample unequal variance). Significance was defined as *P* < .05.

Results

Percentage of Wounds That Completely Healed

Diabetic mice. Results for the percentage of wounds in diabetic mice that had completely healed based on visual

inspection at the time of wound measurement are shown in Fig 1. No wounds from any of the subgroups had completely healed by day 5. By day 12, 11.1% of all wounds in the 5W2d subgroup, 10.0% of all wounds in the 5W4d subgroup, and 10.0% of all wounds in the control subgroup were completely healed. By day 19, 100% of the wounds in the 5W2d subgroup were completely healed, as compared with 40% in the control subgroup. Thirty percent of the wounds in the 5W4d and 20% of the wounds in the 2 subgroups that received laser treatment at 10 W were completely healed at this day.

Nondiabetic mice. Results for the percentage of completely healed wounds for nondiabetic mice are shown in Fig 2. By day 5, 3.6% of all wounds in the 10W2d subgroup and 3.3% in the control subgroup were completely healed. All other subgroups showed no evidence of complete heal-

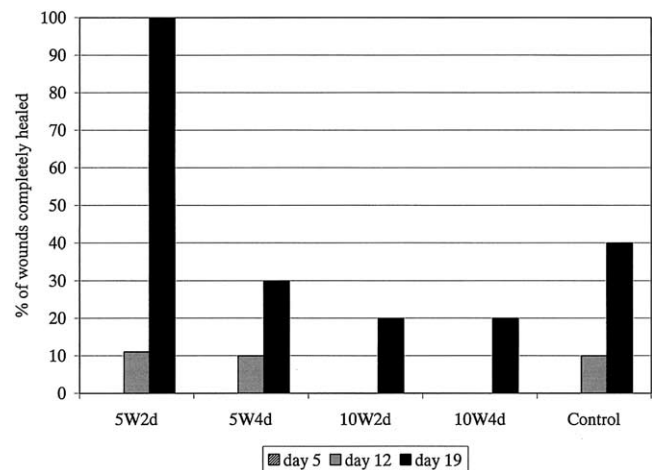


FIGURE 1 Percentage of wounds completely healed in diabetic mice.

TABLE 2 Scoring sheet for histologic sections

	0	1	2	3	4	Score
Epithelialization	None to very minimal	Minimal to moderate	Completely epithelialized; thin layer	Thicker epithelial layer	Thick epithelium	
Cellular content	None to very minimal	Predominantly inflammatory cells, few fibroblasts	More fibroblasts	Predominately fibroblasts	Fewer mature fibroblasts in dermis	
Granulation tissue	None to sparse amount at edges	Thin layer at edges	Thin layer across wound	Uniformly thick	Uniformly thicker	
Collagen deposition	None	Few collagen fibers	Moderate collagen fibers	Extensive collagen fibers	Dense, organized mature fibers	
Vascularity	None	Few capillaries	Moderate neovascularization	Extensive neovascularization	Well-defined capillary system	
						Total score →

Note. Based on a scoring system reported in Yu et al (6).

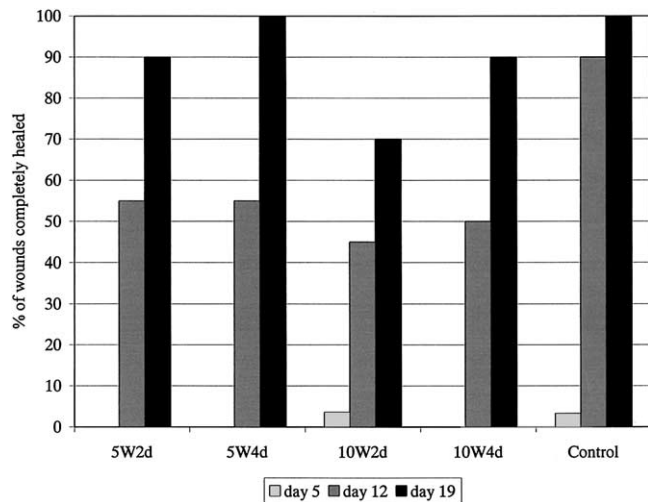


FIGURE 2 Percentage of wounds completely healed in nondiabetic mice.

ing. By day 12, 90% of the wounds from the control subgroup were completely healed, compared with 55% of all wounds in the two 5 W subgroups. Fifty percent of the wounds in the 10W4d subgroup and 45% of those in the 10W2d subgroup were healed. By day 19, all of the wounds from the 5W4d and control subgroups were completely healed, and 90% of the ulcers in the 5W2d and the 10W4d subgroups were completely healed. In contrast, only 70% of the wounds in the 10W2d subgroup were completely healed by day 19.

Percent Wound Closure

Diabetic mice. The results for percent wound closure for diabetic mice are shown in Fig 3. On average, the wounds from all subgroups were smaller than the initial size of the ulcer by day 5. However, only the difference between the 5W4d group (27.0%) and the 10W2d group (40.8%) was significant ($P = .038$). By day 12, the average percent wound closure was 75% to 83% for all but 1 subgroup. The wounds from the 10W2d subgroup experienced only a mean 64.5% closure, which was significantly less than the 5W2d subgroup ($P = .001$), the 5W4d subgroup ($P = .042$), the 10W4d subgroup ($P = .020$), and the control subgroup ($P = .001$). By day 19, only the wounds in the 5W2d subgroup attained 100% closure. All other subgroups, with the exception of the 10W2d subgroup, experienced an average of >90% closure. The wounds from the 10W2d subgroup obtained an average of <80% closure. However, the values for all subgroups were significantly less than the 100% obtained in the 5W2d subgroup ($P = .012$ for the 5W4d subgroup, $P = .010$ for the 10W2d subgroup, $P = .021$ for the 10W4d subgroup, and $P = .023$ for the control subgroup).

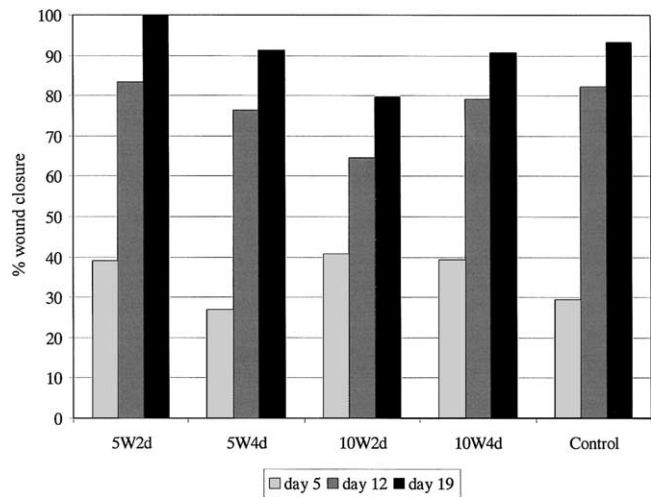


FIGURE 3 Percent of wound closure in diabetic mice.

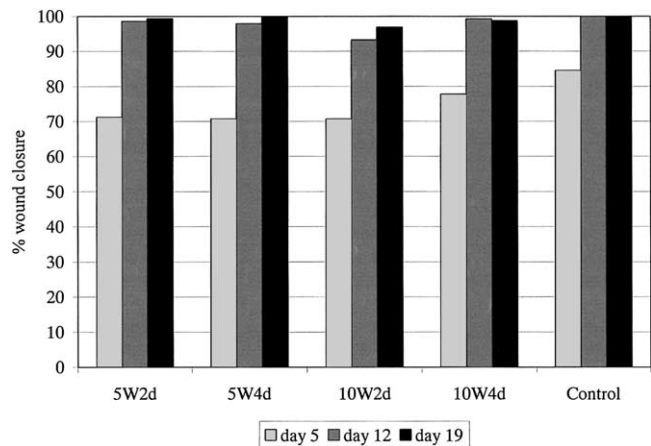


FIGURE 4 Percent of wound closure in nondiabetic mice.

Nondiabetic mice. The results of percent wound closure for nondiabetic mice are shown in Fig 4. The findings indicate that the ulcers obtained an average of 70% to 80% closure by day 5 in all treated subgroups. The control subgroup experienced 84.5% closure. This was significantly greater than the 5W2d subgroup ($P = .0001$), the 5W4d subgroup ($P = .00002$), and the 10W2d subgroup ($P = .0001$). It was not significantly greater than the 10W4d subgroup, which obtained an average of 77.8% closure.

By day 12, the average percent closure for all subgroups was >93.3%. However, there were some significant differences. The control subgroup obtained an average 100% closure, which was significantly greater than the 5W2d subgroup ($P = .040$), the 5W4d subgroup ($P = .002$), the 10W2d subgroup ($P = .003$), and the 10W4d subgroup ($P = .018$). In addition, the average 93.3% closure in the 10W2d subgroup was significantly less than the 5W2d subgroup ($P = .018$), the 5W4d subgroup ($P = .036$), the

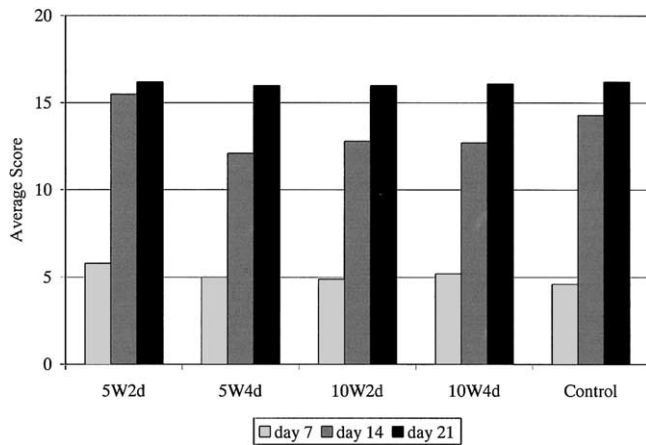


FIGURE 5 Average healing scores from histologic analysis in diabetic mice.

10W4d subgroup ($P = .008$), and the control subgroup ($P = .003$). Finally, the average 99.3% closure in the 10W4d was significantly greater than the average 97.9% closure in the 5W4d subgroup ($P = .048$).

By day 19, wounds in the control and 5W4d subgroups obtained an average of 100% closure, whereas those in the 5W2d and 10W4d subgroups achieved an average of 99.4% and 98.8% closure, respectively. The ulcers in the 10W2d subgroup achieved the lowest percent closure, with an average of 96.9%. There were no significant differences among any of the subgroups.

Histology

Diabetic mice. The histology results for the diabetic mice are shown in Fig 5. The microscopic observations from histology sections indicated that there appeared to be enhanced healing in the 5W2d subgroup, with an average score of 5.8 by day 7. The possibility of enhanced healing was also observed in the 10W4d subgroup, with an average score of 5.2. This was characterized by increased vascularization and collagen content. By day 14, there appeared to be enhanced healing for the 5W2d subgroup, as compared with all other subgroups, because the average score increased to 15.5. This was again characterized by an increase in vascularization and collagen content of the wound. By day 21, there was no discernable difference in the healing stages between all subgroups because the mean scores ranged from 16.0 to 16.2.

Nondiabetic mice. The histology results for the nondiabetic mice are shown in Fig 6. The histologic results indicated that all subgroups with the exception of the 10W2d subgroup achieved a score of 11.5 by day 7. Those in the subgroup 10W2d received an average score of 9.8. By day 14, all subgroups received an average score of 15.9 to 16.7. By day 21, little additional difference was observed from

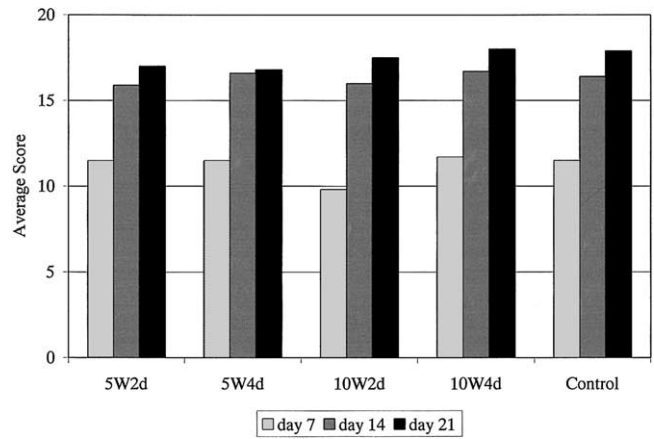


FIGURE 6 Average healing scores from histologic analysis in nondiabetic mice.

the previous time period, with average scores ranging from 16.8 to 18.0.

Discussion

For the diabetic mice, all wounds show a reduction in size over time, regardless of the subgroup to which they belonged. Treatment at 5 W (18 J/cm^2) every 2 days results in all of the wounds achieving complete closure after 19 days, whereas less than half of those in the control subgroup were healed. All other subgroups showed a smaller percentage of wounds completely healed than the control subgroup. This suggests that treatment at 5 W (18 J/cm^2) every 2 days enhances wound healing in diabetic mice as compared with those wounds not receiving treatment. However, with the exception of the 10W2d subgroup, the average percent closure for wounds at day 19 was 90% to 93%. A reduction in healing for the wounds receiving treatment at 10 W (36 J/cm^2) every 2 days could be the result of eschar formation caused by the high fluence level and frequency of treatment.

The histologic findings for the diabetic mice seem to support these findings. The wounds in all groups, except those in the 5W2d and 10W4d subgroups, showed poor healing at day 7. By day 14, all of the wounds, except those in the 5W2d subgroup, showed moderate healing, whereas those that received treatment at 5 W every 2 days showed excellent healing. By day 21, the wounds in all subgroups showed excellent healing. Histologic evaluation was performed 2 days later than wound-area measurements, which may account for differences in area and histology results at day 21. Histologic findings also suggest that treatment at 5 W every 2 days showed a positive healing effect. This was manifested by increased infiltration of mature loose fibrillar collagen in association with thick layers of subdermal granulation tissue when compared with other subgroups, including the controls. All wounds determined to be closed by

examination were deemed healed by histologic evaluation with varying degrees of reepithelialization.

For the nondiabetic mice, the percentage of wounds in the 10W2d and control subgroups achieving complete closure by day 5 was minimal. By day 12, more than half of the wounds in nearly all subgroups were healed. By day 19, nearly all wounds in almost all subgroups were completely closed. The exception was subgroup 10W2d, in which only 70% of all wounds were completely healed. Again, this may stem from eschar formation, resulting from the high frequency of treatment at a high power level. These findings were confirmed by analysis of percent wound closure. For all subgroups, the average percentage closure was at least 70% by day 5. By day 12, all wounds with the exception of those in the 10W2d group were at least 97.9% closed, and this trend of increased healing continued at day 19.

The histologic findings for the nondiabetic mice seem to support these findings. All subgroups, with the exception of 10W2d, indicated moderate healing after 7 days. Those in the 10W2d subgroup indicated fair healing. By day 14, wounds from all subgroups showed excellent healing, with infiltration of mature collagen. These findings were also seen at day 21. As with the diabetic mice, all wounds that were determined to be completely healed when examined visually were also shown to be healed histologically with varying degrees of reepithelialization. Nondiabetic mice do not have impaired healing, and thus already heal at an optimal rate. Therefore, although treatment with the 980-nm diode laser did not seem to impair healing in healthy mice, it seemed to enhance healing in diabetic mice. This was also observed in a preliminary clinical trial in which the effects of the laser are more apparent in patients with diabetes (9). The mechanism of action that results in this observation is not known and further investigation is warranted.

Although no animal studies have been conducted using a diabetic mouse model to specifically examine the effect of the 980-nm GaAlAs diode laser, the findings from the current study are similar to those obtained by using different lasers (6–8). The results also confirm our preliminary study's findings that the 980-nm diode laser does enhance wound healing (9). Possible mechanisms of wound healing by lasers that have been suggested include collagen synthesis and formation of new blood vessels (10–16). Both of these phenomena have been observed in the current study.

There were some limitations to this study. The fact that the diabetic mice were greatly obese in comparison with the nondiabetic mice might be a potential source of bias, because the surgeon who performed the wounding was able to distinguish the diabetic mice from the nondiabetic mice. This could have influenced the depth of the wound created by the surgeon. The obvious difference in appearance of the 2 types of mice may also have caused potential bias when measuring the size of the ulcer. In addition, the definition of completely healed can vary between individuals, thus intro-

ducing bias in determining if the ulcers were completely healed. Although wound size measurements were determined primarily by 1 individual, on occasion another surgeon took the measurements on a specific date when the primary evaluator was unavailable. Another limitation to the study was that wound assessment was performed on days 5, 12, and 19, whereas histologic evaluations were conducted at days 7, 14, and 21. This would make comparisons between wound size measurements and histology difficult. All forms of analysis should have been conducted on the same day.

Conclusion

In conclusion, treatment with the 980-nm GaAlAs diode laser at 5 W (18 J/cm²) showed a beneficial effect on wound healing in diabetic mice. Furthermore, treatment at 18 J/cm² does not appear to have a detrimental effect on wound healing in nondiabetic mice, which already heal at an optimal rate. Treatment at 10 W, or 36 J/cm², every 2 days seems to be too aggressive, as indicated by decreased healing and the formation of eschars. If laser treatments do enhance healing of diabetic ulcers in mice, they may also be significant in the treatment of humans with diabetes.

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