DOSIMETRY-RELATED WOUND HEALING RESPONSE IN THE RAT MODEL FOLLOWING HELIUM NEON LASER LLLT

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We report on a controlled investigation into the accelerated healing of artificial wounds in the Sprague-Dawley rat treated with helium neon (HeNe) laser irradiation over a range of doses up to 60 J/cm². With a treatment schedule of 3 or 5 treatments per week, an acceleration of approximately 27% in healing time and 49% in wound size reduction were seen in wounds of 1.26 cm² in area inflicted on 27 week-old rats at a dose of 25 J/cm² compared with unirradiated control animals. Smaller wounds (0.39 cm²) in younger animals (< 12 weeks old) demonstrated time and wound reduction improvement of approximately 33% and 54% respectively, compared with controls. A statistically significant difference (student's t-test) was seen in this group of animals treated with 7 J/cm² compared with unirradiated control animals. Wound acceleration was seen to be dose-dependent, increasing up to the optimum value, whereafter the stimulatory effect was observed to decrease (maximum dose of 60 J/cm²). Investigations into the treatment schedule revealed saturation after 6 treatments per week. Skin reflection was investigated, and losses of around 74% were observed, requiring higher doses to achieve an effective result. Varying the power density (3.7 - 15.9 mW/cm²) while keeping the dose constant had no statistically significant effect on the result.

Key words: dosimetry; stimulatory effect; wound healing; HeNe laser

Introduction

Biostimulation can be defined as the photochemical or photophysical reaction caused by laser tissue interaction which generates a group of biological phenomena without involving a temperature rise in tissue.⁽¹⁾ One such phenomenon is the photoacceleration of wound healing.

This renewed interest of mankind in the knowledge of the ancients regarding the health-giving effect of visible light action has been supported recently by a variety of clinical information.⁽²⁻⁵⁾ Other research groups, however, have been unsuccessful in achieving the same effects.⁽⁶⁻⁹⁾ Most of these studies were conducted using helium-neon (HeNe) and argon lasers with total dosages of from 1 J/cm² to 4 J/cm².⁽⁶⁻⁸⁾ Whether these beneficial effects have been confirmed by all investigators⁽¹¹⁻¹⁴⁾ or not,⁽⁶⁻⁹⁾ it remains evident that further controlled systematic studies to add to the understanding of this phenomenon are certainly required.⁽¹⁵⁻¹⁶⁾

The aim of this study is to investigate the laser dosimetric parameters of power, spot size, and expo-

sure time in the form of the power density (mW/cm²) and dose(J/cm²) with respect to other possible contributory variables such as animal age, wound size and treatment schedule, for a series of experiments evaluating the possible acceleration of wound healing both with respect to time and size reduction, using HeNe laser irradiation. An attempt has been made to define the dosimetric parameters affecting this phenomenon.

Materials and Methods

A number of male Sprague-Dawley rats were used this study, chosen according to their age and weight. The animals were originally supplied by Charles River Co., Ltd, UK, in 1984, but are now bred and provided by the Animal Research Facility of the King Faisal Specialist Hospital and Research Centre (KFSH&RC).

The rats were anaesthetized with ketamine (Ketalar) (up to 50 mg/kg) and lidocaine (Xylocaine) (up to 20 mg/kg) administered intraperitoneally. The surgical site was shaved and followed by a hair removing lotion spread for few minutes to clean the skin of hair roots (minimizing the reflection loss) and disinfected with an isopropyl alcohol swab. A full thickness elliptical

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skin wound was made with a scalpel on the shaved areas of the animal in the gluteus maximus. Wound sizes varied according to experimental requirements.

Laser tissue penetration

Knowledge concerning the transmission properties of the HeNe laser in rat skin was addressed, since the laser tissue absorption around the wound could have an added influence. This was achieved by taking a full thickness skin dissection from a freshly killed rat. The skin specimen was placed on the outside rim of an integrating sphere power meter to measure the transmission, and on the base of the integrating sphere to measure the scattering.⁽¹⁷⁾

Temperature measurement

Experiments on possible temperature variations during treatment were accomplished using thermocouple needles (Doric model 450) embedded below the full skin thickness and thermocouple probes (Tegam Model 820) laid on the skin surface.

Laser system

Three HeNe laser systems were utilized in the present study, with maximum continuous wave outputs of 5 mW, 18 mW and 35 mW at a wavelength of 632.8 nm. The laser beams had linear polarization (manufacturer's information). The laser beam was directed by a system of reflectors, expanders and/or fibreoptics. The output power was measured continuously using Laser Guide 633 and Scientech 364 or 365 power meters.

For the purposes of the present study, the dose (J/cm²) was defined as the energy density of the incident laser beam, calculated as the measured power in mW, multiplied by the treatment time in seconds and divided by the area in cm² of the laser spot aimed at the wound. The dose rate was defined as the power density of the beam expressed in mW/cm², calculated by dividing the incident power (in mW) by the area of the laser spot on the target tissue (in cm²).

Experimental procedures

The treated sub-groups of animals were restrained in a Plexiglas cage specifically designed for this purpose. Treatment commenced without anaesthesia on the day

of injury and was continued at the rate of 3 or 5 times per week until the wound had completely closed. The control sub-groups were carefully subjected to the same manipulated environment received by the treated animals, except for the laser exposure. The wounds of all the rats were measured several times every alternate day (unless otherwise specified) with a caliper, until complete healing (wound closure) and the wound area calculated as for an ellipse as follows:

$$\text{Area} = \frac{L}{2} \cdot \frac{W}{2} \pi \text{ (cm}^2\text{)}$$

where L and W are the length and the width of the wound respectively in centimetres.

The percentage acceleration of wound healing days (AccD) relative to control was calculated as follows:

$$\text{AccT} = [1 - \frac{\text{TD}}{\text{TC}}] \times 100 \text{ (\%)}$$

where TD and TC are the days required for complete healing of the treated and control animals, respectively.

The percentage acceleration of the size (AccS) of the healing wound relative to controls was calculated as follows:

$$\text{AccS} = [1 - \frac{\text{AT}}{\text{AC}}] \times 100 \text{ (\%)}$$

where AT and AC are the areas of the wounds on the treated and control animals, respectively, calculated as above and measured every alternate day.

The relative percentage of overall wound healing (WH_R) was calculated as follows:

$$\text{WH}_R = [1 - \frac{\text{A}_n}{\text{A}_z}] \times 100 \text{ (\%)}$$

where A_n is the wound area on day (n) postoperatively and A_z is the wound area on day zero.

The percentage acceleration of wound healing will be referred to as acceleration in size reduction or days, wherever applicable. We adopted complete separation between the assessment process and the treatment process. The animals in the treatment process were divided into seven sub-groups each with their own controls, and every sub-group was further divided according to dosage (See Table 1).

Table 1: The treatment process as related to dosimetric parameters.

Exp No.	Age (wks)	Initial wound size (cm ²)	Dose rate (mW/cm ²)	Treatment schedule	Dose (J/cm ²)
1	27	1.26	3.7	5/week	4, 7, 11
11	27	1.26	10.5	3/week	20, 30, 50
10	27	1.26	10.5	3/week	40, 60
9	27	0.39	10.5	3/week	5, 20, 40, 60
4	12	0.39	15.9	5/week	1, 4, 7
12	27	1.26	10.5	1 - 5/week	10
13	27	1.26	10.5	1 - 10/week	20

Fig 1: The relative percentage of wound healing as a function of healing time (in days) for control animals: group 1, wound size 0.39 cm², age 12 w.o.; Group 2, wound size 0.39 cm², age 27 w.o.; and group 3, wound size 1.26 cm², age 27 w.o.

Graphs were then prepared for the data of relative wound healing (WH_R) against postoperative days for experimental and control animals, WH_R against incident dosage, and wound healing size reduction against dose, wound size, animal age, and treatment schedules. As other reports have found no real long-term differences in the quality of healing, calculations were also prepared for the data at 80% of total wound healing as a more meaningful reference point for the comparison of control and experimental data.

Results

Preliminary control experiments

Figure 1 presents the relative percentage of wound healing (at the 80% of total healing mark) as a function of the healing days for control animals. The natural healing dependence of wound size closure from the animal parameters (age and initial wound size) reveals the difference caused by initial wound size and age used in this study: elliptical wounds with an area of 1.26 cm² demonstrated a slower wound closure (approx. 8%) and longer healing time (approx. 2.4 days) than wounds of 0.39 cm² in the 27 week-old animals (average weight = 438 ± 39 g). There was no significant difference between the 12 w.o. and 27 w.o. animals with 0.39 cm² wounds.

Table 2: Percentage of 632.8 nm transmission and reflection for different skin thicknesses in Sprague-Dawley rats.

Percentage of Transmission				% Reflection
1.0 mm*	1.5 mm*	2.0 mm*	2.5 mm*	1.0mm ~ 2.5 mm*
10.3 ± 40.7	9.26 ± 0.4	7.61 ± 0.8	5.08 ± 0.5	74.19 ± 2.1

* Thickness in mm of the skin specimens

Fig 2: Relative percentage of wound healing as a function of healing time (in days), comparing control and experimental animals with wounds 0.39 cm² (●) and 1.26 cm² (▲). The dotted lines indicate the values in healing time at 80% of complete healing

Control and experimental results

Figure 2 shows the relative wound healing (WH_R) of the control and the treated animals (20 J/cm² - 10.5 mW/cm², 31.7 min) for both the 0.39 cm² and 1.26 cm² initial wound in 27 w.o. rats (448 ± 40 g) as a function of the healing time. Figure 2 presents a difference in healing of ≈ 19% and ≈ 3.15 days faster than the control for the 0.39 cm² area animals, ≈ 17% and ≈ 3.3 days faster than the control for 1.26 cm². Figure 2 also indicates the method of calculating the percentage acceleration of wound healing size reduction with respect to the control as already demonstrated in the equations in the *Materials and Methods* section, giving (at the 80% wound healing time) ≈ 49% and ≈ 46% greater size reduction and a reduction of ≈ 29% and ≈ 26% in healing days for 0.39 cm² and 1.26 cm² groups, respectively.

Laser tissue penetration

The transmission and reflection losses (Table 2) were measured for different skin thicknesses. They indicated that the HeNe laser penetration is ≈ 5% for 2.5 mm thick skin and varies with skin thickness. The reflection losses were very high (≈ 74%). The Plexiglas cage correction percentages were 7.09 ± 0.7%. Accordingly, the percentage of dermal absorption and scattering was calculated from this Table as 13.64% ± 1.1.

Fig 3: Percentage of acceleration of wound closure as a function of delivered dose compared over 2, 4, 7, 9 and 11 days postoperatively.

Fig 4: Percentage of acceleration of wound closure as a function of dose calculated at the 80% stage of total healing.

Temperature measurements

No appreciable thermal effects were noticed for any of the dosages. Temperature differences of up to $1.15 \pm 0.07^\circ\text{C}$ were recorded subcutaneously and for the surface the readings were $0.25 \pm 0.07^\circ\text{C}$.

Percentage acceleration of wound healing

Figure 3 shows the combined results of three experiments, evaluating the acceleration in size reduction as a function of dosage, measured at different healing days (2, 4, 7, 9 and 11 days postoperatively); (experiments 1, 10 and 11 in Table 1) in 27 w.o. (467 ± 42 g) rats. This figure indicates that acceleration in wound size reduction is dose dependent, with maximum ac-

Fig 5: Percentage acceleration of wound closure and time (in days) as a function of dose for different ages, wound sizes and treatment schedule.

celeration seen at an optimum value, whereafter reduction in the stimulatory effect occurs for larger dosages, tested up to 60 J/cm^2 (10.5 mW/cm^2 for 95 min). This figure also shows the reduced effectiveness of dose rates and treatment schedules within the values used in the present study.

The acceleration in size reduction and days presented in Figure 4 for experiments 1, 10 and 11, calculated at the 80% healing time, shows maximum values of $\approx 49\%$ and $\approx 27\%$ respectively, at the optimum dose of 25 J/cm^2 . The assumption of continuity refers to the belief that there is a smooth curve which could be appropriately matched to the obtained experimental points. The equation was applied to the data using Graph Master version 1.31 supplied by Visual Business Systems Inc., run on a Macintosh Quadra 700. The resultant polynomial equation was as follows:

$$\% \text{ wound acceleration} = a + bx + cx^2 + dx^3$$

where x is the dose limited to from 4 - 60 J/cm^2 ; $a = 10.011$; $b = 3.434$; $c = -0.08$ and $d = 0.001$ for the size reduction; the values for the healing time for the same dose range are $a = -1.292$; $b = 2.613$; $c = -0.071$; and $d = 1.0$.

Figure 5 gives the results of the combination of five experiments (1, 11, 10, 9 and 4 in Table 1) where experiment 9 was with 27 w.o. (455 ± 23 g) animals and experiment 4 with 12 w.o. (337 ± 20 g) animals. This comparison indicates the effects of the physical parameters of animal age and wound size used in this study and shows also the repeating effect of dose dependence, with decreasing acceleration for higher dosages above the optimum. In this figure the wound size difference gives no more than from 2 to 5% higher

Fig 6: Percentage acceleration of wound closure rate (size reduction) and time (in days) as a function of doses of 10 J/cm² (▲) and 20 J/cm² (*).

acceleration for the smaller wound group, whereas the age difference gives a difference of from 5 to 9% for low doses. By extrapolation, the predicted acceleration for 12 weeks with wounds of 0.39 cm² in area gives a 33% acceleration in time and a 54% acceleration in size reduction at a dose of 20 J/cm².

Treatment schedule

The results of experiments 12 and 13 presented in Figure 6 using 27 w.o. animals (466 ± 23 g) with acceleration in wound healing as a function of the treatment schedule, at doses of 10 J/cm² and 20 J/cm², indicates an optimum treatment schedule of 6 times per week. The difference between 3 and 6 times a week is from 3% to 6% improvement, where the difference between 1 and 6 times could reach between 10% and 15% acceleration for time and size reduction, respectively. It is possible that, above 5 treatments per week, saturation point was reached at both the above doses. Treatments up to 10 times week (i.e. twice a day, morning and afternoon, Monday - Friday) were carried out. The approximate experimental equation was applied to these data using Graph Master version 1.31, as mentioned earlier, and the resultant power formula equation is as follows:

$$\% \text{ wound acceleration} = a \cdot x^b$$

where x is the treatment schedule in times per week (from 1 to 10), $a = 35.799$ and $b = 0.169$ for size reduction; and for time, $a = 16.217$ and $b = 0.279$.

Statistical analysis

The students t-test was used to compare the treated and control groups for significance of difference. P-values were considered to be significant at values of < 0.05. The treated groups when compared to the

controls were significant at $p < 0.05$ with doses > 7 J/cm², and insignificant with doses of < 4 J/cm². Comparison between the treated groups show significant results for dissimilar wound sizes (0.39 cm² and 1.26 cm²) but identical ages (27 w.o.), and insignificant results for different age groups (12 and 27 w.o.) with the same wound size (0.39 cm²).

Discussion

HeNe laser wound healing acceleration is dose dependent and increases with a higher incident dose until it reaches an optimum value of 25 J/cm², then a reduction in the stimulatory effect appears, tested up to 60 J/cm². Optimum results achieved a maximum of 27% acceleration in wound healing time and 49% acceleration in wound healing size reduction relative to the control at the optimum dose (Figure 4), for 1.26 cm² wounds in animals 27 w.o. With the smaller wound size and animals younger than 12 w.o., we could predict not more than 33% acceleration in time and 54% size reduction (Figure 3). This is expected to decrease for larger wound sizes and older animals.

The higher percentage of size reduction compared to the healing days could possibly be attributed to the effective biostimulation in the granulation tissue production phase of the wound healing repair mechanism. The optimum treatment schedule is 6 times per week, higher appears to show a saturation effect, and lower reduces the acceleration in healing time percentage, up to approximately 10% (for once per week) (Figure 6) and 15% for size reduction. This may indicate that the accumulation of the dosage has no effect on the healing phenomenon.

The large incident dosages required in this study related to the high natural reflection losses in these animals of 74% (Table 2) even when the utmost care was taken in hair removal. This might also influence the treatment schedule effect and the uncertainty in the measurements, particularly at low incident dosages below 7 J/cm². Accordingly, the dermal absorption and scattering of the optimum incident dose accounts for approximately 3.46 J/cm².

In changing the dose rate (3.7 mW/cm², 10.5 mW/cm² or 15.9 mW/cm²), we noticed no effect, particularly when compared with the incident dose. This may be attributed to the non-thermal nature of the phenomenon, as also temperature measurements up to the maximum incident dose of 60 J/cm² confirm this finding (within the thermal limit).

A reduction in the stimulatory effect appears to occur above the optimum dose and increases as the dosages rise. Due to the low power limitation of lasers used, it is difficult to work with higher doses, which would require larger and impractical experimental

times to reveal if there is any further inhibitory effect. However the precepts of the Arndt-Schultz law would appear to hold true in the present study.

The approximate quadratic relationship for HeNe laser effects shows that wound healing acceleration in both time and size reduction are dose dependent. The constants a , b , c , and d relate to the dosimetric laser and animal parameters. These require further investigation. Also the power relationship between wound acceleration and time schedule at a fixed dose should be a subject for additional future experimentation.

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References

- 1: Berns HW, and Nelson F (1988): Laser Applications in Biomedicine. *Journal of Laser Applications*, **1**: 34-39.
- 2: Mester AF, and Mester A (1989): Wound healing. *Laser Therapy*, **1**: 7-15.
- 3: Labbe RF, Skogerboe KJ, Davis HA, and Rettner RL (1990): Laser photobioactivation mechanisms. *Lasers in Surgery and Medicine*, **10**: 201-207.
- 4: Karu TI (1989): Photobiology of low-power laser effects. *Health Physics*, **56**: 591-704.
- 5: Mester E, Mester AF, and Mester A (1985): The biomedical effects of laser applications. *Lasers in Surgery and Medicine*, **5**: 31-9.
- 6: Surinchak JS, Alago ML, Bellamy RF, Stuck BE, and Belkin M (1983): Effects of low-level energy lasers on the healing of full thickness skin defects. *Lasers in Surgery and Medicine*, **2**: 267-74.
- 7: Jongsma FHM, Bogoard AE, Gemert MJ, and Henning JP (1983): Is closure of open skin wounds in rats accelerated by argon laser exposure. *Lasers in Surgery and Medicine*, **3**: 75-80.
- 8: Hunter J, Leonard L, Wilson R, Snider G, and Dixon J (1984): Effects of low energy laser on wound healing in a porcine model. *Lasers in Surgery and Medicine*, **3**: 285-290.
- 9: Colver GB, and Priestley GC (1989): Failure of a helium-neon laser to affect components of wound healing *in vitro*. *British Medical Journal*, **121**: 179-189.
- 10: Gross AJ, and Jelkmann W (1990): HeNe Laser irradiation inhibits the growth of kidney epithelial cells in culture. *Lasers in Surgery and Medicine*, **10**: 40-44.
- 11: Weiss N, and Oron N (1992): Enhancement of muscle regeneration in the rat gastrocnemius muscle by low energy laser irradiation. *Anatomy and Embryology (Berlin)* **186**: 497-503.
- 12: Brengel V, and Bor PP (1992): Power density and exposure time of HeNe laser irradiation are more important than total energy dose in photobiostimulation of human fibroblasts *in vitro*. *Lasers in Surgery and Medicine*, **12**: 528-37.
- 13: Smol'yaninova NE, Karu TI, Fedoseeva GE, and Zelenin AV (1991): Effect of HeNe laser irradiation on chromatin properties and synthesis of nucleic acids in human peripheral blood lymphocytes. *Biomedical Science*, **2**: 121-126.
- 14: Karu TI, Smol'yaninova NE, and Zelmeia A (1991): Long term and short term responses of human lymphocytes to HeNe laser radiation. *Lasers in Life Sciences*, **4**: 167-178.
- 15: Zhang XY, and Al-Watban FAH (1992): Biostimulation effect of low power laser therapy on wound healing. *Research Centre Bulletin KFSH*, **4**: 16-20.
- 16: Al-Wathan FAH, and Zhang XY (1993): Towards optimum dosimetric parameters for the effect of laser therapy on wound healing. *Lasers in Surgery and Medicine; Supplement 5*: 10.
- 17: Anderson RR, and Parrish JA (1982): Optical properties of human skin. In: *Science of Photomedicine*. (JD Regon and JA Parrish, eds). Plenum Press, New York. pp 147-94.