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Effects of Laser at 810 Nm on Wound Healing in Albino Mice

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Abstract

Many researches focused on laser therapy of wound healing in different animal models due to the lack of a standard protocol in the application of such phototherapy. Objective: To study the effects of 810nm laser at a constant irradiance of 41.63 mw/cm^2 and exposure (illumination) time of 5,15 minutes on wounds created on Albino mice (BALB/c).

Skin wound with elliptic shape and full thickness was created on the dorsal side of '45 mature male albino mice. Irradiated animals were divided into two main groups based on irradiation time, the first was irradiated for 5 min and the second for 15 min, each was subdivided into three subgroups (n=5) according to number of treatment days (3, 5 and 10 days). Both treated and respective control (n=15) subgroups were sacrificed on days 3, 5 and 10 posttreatment. Laser therapy was applied using a 810 nm diode laser with a continuous wave, an output power of 400 mw, and irradiance of 41.63. The 5 min dose was 12 .5 J/cm^2, whereas the 15 min dose was 37.4 J/cm^2. The shape of the laser beam was fitted with a convex lens as 'beam expander' to irradiate a circular area of 3.4 cm diameter. Laser therapy was started after surgery and repeated for 3, 5 and 10 days, while its effects were examined by histological evaluation. Results: At day 3 of treatment with near infrared 810nm laser at doses of 12.5J/cm² and 37.4J/cm², there was no evidence of wounds healing in irradiated groups which showed no differences with the respective control groups. At day 5 of treatment, the results showed an important increase in the scores of the parameters of wound healing (formation of granulation tissue and collagen deposition) in the irradiated groups. Near infrared 810nm laser had photobiostimulation effects on wound healing at irradiance of 41.63mW/cm² and doses of 12.5J/cm² for 5 minutes and 37.4J/cm² for 15 minutes exposure time. A complete picture of wound healing response appeared in all irradiated groups within 10 days of treatment, as expressed by complete 're-epithelialization', moderate granulation tissue formation, and presence of collagen fibers, while incomplete wound healing response was observed in un-irradiated control groups within the same period. The study showed that 810 nm laser therapies had significant effects on wound healing, especially at a dose of 37.4J/cm².

Keywords: laser, wound healing

تاثيرات ليزر 810 نانـــومتر على التـــئام الـــجروح بالفـــئران

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الخلاصة

ركزت العديد من البحوث على معالجه الجروح باليزر في نماذج حيوانيه مختلفه. وذلك لعدم وجود بروتوكولات ثابته في تطبيق هذه المعالجه الضوئية . هدفت الدراسه الى تقيم تاثيرات الليزر ذو الطول

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الموجي 810 نانومتر بكثافه اشعاع $41.63\ mw/cm^2$ وبزمن تعريض (5 و 15) دقيقه على جروح مستحدثه في الفئران البيض BALB/C. تم عمل جروح جلديه بسمك كامل وذات شكل بيضوي على منطقه الظهر في ذكور بالغين للفئران البيض. المجموعه المعرضه للاشعاع قسمت الى مجموعتين رئيسيتين الاولى بزمن تعريض 5 دقائق والمجموعه الثانيه بزمن تعريض 15 دقيقه. كل مجموعه رئيسيه قسمت الى مجاميع ثانويه اعتمادا على ايام المعالجه. مجموعه معالجة لمده 3 ايام ومجموعه معالجه لمده 5 ايام ومجموعه معالجه لمده 10 ايام. عدد مجاميع السيطره (15 فأر) قتلت في الايام 3و5و10 وكذلك المجاميع المعرضه لليزر قتلت في 3و5و10 بعد المعالجه. المعالجه بالليزر تمت باستخدام ليزر الدايود وبموجه مستمره. حيث ان شده الاشعاع $41.63\ mw/cm^2$ وبجرعه $12.5\ J/cm^2$ لمده تعريض 5 دقائق وجرعه $37.4 J/cm^2$ لمده 15 دقيقه. حيث تم استخدام عدسه محدبه(موسع للحزمه) لجعل حزمه الليزر دائريه بقطر(3.4 cm). بدئت المعالجه بعد اجراء الجراحه وتم تكرار المعالجه لمده 3و5و 10 ايام وتم استخلاص النتائج من خلال الفحص النسيجي. حيث أظهرت النتائج عند المعالجه لمده ثلاث ايام وعند الجرعتين $12.5 J/cm^2$ و $37.4 J/cm^2$ لايوجد التئام ولايوجد فرق بين المجموعه المعرضه لليزر ومجموعه السيطره. اما المجموعه التي تم علاجها لمده خمسه ايام اظهرت زياده في العوامل المسؤوله عن التئام مثل تكون النسيج الحبيبي واعاده الظهاره وتراكم الكولاجين. الاستجابه الكامله للالتئام الجروح ظهرت لكلا الجرعتين عند المعالجه لمده عشره ايام. حيث اظهرت عوده الظهاره الكامله والنسيج الحبيبي المعتدل ووجود خيوط الكولاجين بينما الجروح في مجموعه السيطره لم تظهر التئام كامل ضمن نفس مده التعريض. ان العلاج باليزر ذو الطول الموجي 810 نانومتر له تاثير ملموس على علاج الجروح وخاصه عند الجرعه $37.4\ J/cm^2$.

Introduction

Wound healing is one of the issues that gained most of the interest of researchers among today's medical problems, especially in the field of surgery. For decades, various types of treatment for wound repair and prevention of infection has been proposed [1]. A large number of animal models were used to study the influence of cold laser therapy (CLT) on many chronic and acute diseases. Many research attempts applied CLT to enhance wound healing in different animal models because no standard procedure is available for the application of such phototherapy. CLT is used in three principle fields that focus on the enhancement of wound healing, reduction of inflammation and edema resulting from injury, and its usage as analgesic for pain relief [2].

There is no heat effect while using CLT, i.e., the energy from the absorbed photons is not transformed into heat, but into photo- biological effect. CLT is believed to affect all three phases of wound healing (the inflammatory phase, the proliferative phase and the remodelling phase). It also activates local discharge of chemokines, cytokines, and other modifiers of biological response, leading to the decrease of the time needed for wound closure [3].

The controversy in the use of CLT is fundamentally due to two reasons; first, the uncertainty about the principle cellular and molecular mechanisms responsible for transforming of energy from incident photons on the cells to the biological response that happen in the irradiated tissue. Also, dosimeter parameters (wavelength, power density, coherence and pulse structure) and the delivered dose (irradiation time and repetition regimen affect the therapeutic outcome. Many of the previous investigations revealed that the negative outcomes of CLT were mostly due to inappropriate wavelength and dose selection [4].

The objective of the present study is to investigate the effects of 810nm laser at constant irradiance of 41.63 mw/cm^2 and exposure (illumination) times of 5 and 15 min on wounds created on Albino mice (BALB/c).

Subjects and methods

Study subjects

Forty five BALB/c mice with weight range of 18-32 grams were included in this research. Animals were kept in hygiene conditions in individual plastic cages, maintained at $22c^o$ in a day/night light cycle with wood chip bedding, fed with standard pelted laboratory diet, and provided with water *ad libitum*. The study was conducted at the animal house of the National Centre for Drugs Control Researches / Iraq, and approved by the ethical committee in the centre.

Diode laser

During the study protocol, all equipment was calibrated in the beginning of the study to make sure of proper dose dwlivery. Before starting the experiments, the method of irradiation was standardized. Continuous wave, low energy semiconductor laser from 'UK scientific Ltd' was used in all experiments of irradiation. The parameters of lasers used in this study are listed in Table-1. Power meter (SOLOPE Genetc- EoInc, Canada) was used to measure the output laser power used.

Laser was fitted with a beam expander at the distal end to irradiate a circular area, which integrated the wound and some surrounding intact skin. A convex lens was used to produce the homogenous laser spot which was approximately 3.4 cm in diameter for 810nm, and the distance between the lens and the surface of the mouse was kept fixed. This apparatus was kept constant for all irradiation regimens (Table-1, Figures-(1 and 2).

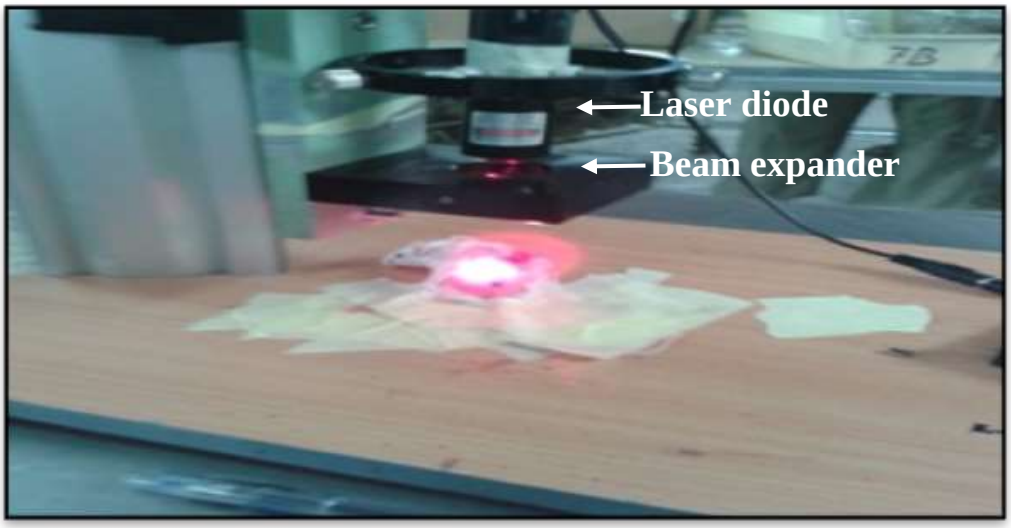


Figure 1-Diode laser and beam expander.

Table 1-Characteristics of diode laser used in the study

Central wavelength(nm)	Operating mode	Output power	Output power with beam expander
810nm	cw	500mW	400 mW
nm= nanometer **cw =** continuous wave **mw =** milli watt , Focal length of beam expander used was 50mm.			

The laser used in the present study was a continuous wave, portable, semiconductor laser with Gallium Aluminium, Arsenide NIR (Ga-AI-As) and 810nm . Near infrared laser (810nm) was powered using a battery which was fully charged before the beginning of irradiation of wounds in each exposure.

Table 2-Treatment parameters for diode laser used in the study

wavelengths	810nm	
Output power (mW)	400mW	
*Beam spot size(irradiated area) (cm^2)	9.0cm^2	
Irradiance measured at the target area	41.632 mW/cm^2	
Exposure time(min)/ day	5 min	15 min
Dose J/cm^2	12.5 J/cm^2	37.4 J/cm^2

Laser was organized in metal holders which fix the laser perpendicular to and at a fixed distance from the wound surface (Figure-2). Laser therapy was initiated directly after wounding and continued

over 3, 5, and 10 days. The protocol was selected according to the conventional clinical approach to laser therapy for wounds in 3 and 5 exposures per week for 24 hours [5, 6].

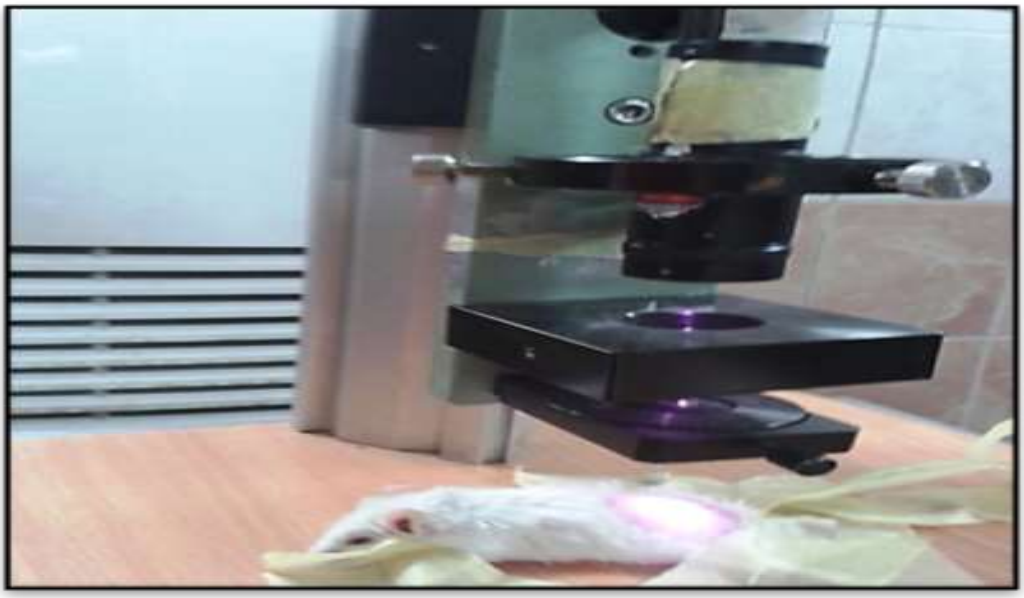


Figure 2-Diode laser set up.

Wound model

Following sterilization with 70% alcohol, hair was shaved at the cervical to mid-lumbar dorsum of mice back and incisions were made, resulting in 1.5-3 cm long wounds with full-thickness and elliptic shape over the adjacent musculature and the thoracic spinal column. During the whole period of experiments, the wounds were left uncovered [5, 6].

Study design

Animals were divided into two principal groups; the control (15 animals) and the irradiated (30 animals) groups.

Irradiation treatment of wounds started at the beginning of day 1 and continued for 3, 5, and 10 days, after which the treated mice were saccrificed. This protocol was selected because it is commonly considered by previous studies of wound healing [5, 7, 8, 9]. The data of experimental groups are shown in Table-3.

Table 3-Experimental groups

Irradiated groups				
Groups	**Exposure time 5 minutes**	**Exposure time 15 minutes**	**Control groups**	
	Number of animals	**Number of animals**	**Groups**	**Number of animals**
Group irradiated for 3days	5	5	Group sacrificed after 3days	5
Group irradiated for 5days	5	5	Group sacrificed after 5days	5
Group irradiated for 15 days	5	5	Group sacrificed after 10 days	5
Total number of irradiated groups = 30 animals.			Total number of control group =15 animals	

Histopathological estimation:

The tissue (whole skin) specimens were stained with hematoxlin and eosin and examined with a semi-quantitive method to evaluate histopathological parameters, including fibroblasts, granulation tissue formation, re-epithelialization, and polymorphic nuclear leucocytes [5, 7, 10].

Mice were randomly selected for each group at 3, 5 and 10 days after wounding and sacrificed by ether inhalation.

Glass slides were prepared and evaluated by two pathologists who were not aware of the sample codes. The sections were evaluated by two observers and estimated on a scale of 0-3 by using a light microscope (Olympus, Japan). Sections were graded for wound healing according to the following seven elements associated to acute inflammatory response and repair: formation of granulation tissue (angiogenesis and fibroblast), collagen deposition, inflammatory reaction (macrophages and leucocytes,), and evidence of 're-epithelialization'. Each parameter was semi-quantitatively evaluated (from 3 = prominent or marked, to 0 = absent or no evidence) according to McMinn [10].
Wound healing process is described as complete healing, incomplete healing and no healing responses [7, 5, 10, 11].

- **Complete healing**

Complete healing is characterized by moderate to marked granulation during tissue formation, complete re-epithelialization, presence of collagen fibres, and scattered to mild inflammatory cell infiltration.

- **Incomplete healing**

It is characterized by incomplete re-epithelialization, mild to moderate granulation, presence of collagen fibres, and mild to moderate inflammatory cell infiltration.

- **No evidence of healing response**

It is characterized by no evidence of re-epithelialization, no evidence of mild granulation during tissue formation, absence of collagen deposition and of moderate to marked inflammatory cell infiltration.

Statistical analysis

Data were analysed using the statistical package for social science (Spss V.20) computer software. Histopathological parameters were correlated via chi-square test. The association between two categorical variables was assessed by chi- square test of independence.

Results

- **Control groups**

Nearly all histological sections of the control mice showed incomplete healing response. In addition, responses in mice sacrificed after 5 days of wounding were characterized by incomplete re-epithelialization, mild granulation tissue formation and absence of collagen fibres (Table-4, Figure-3), while those in mice sacrificed after 10 days were associated only with incomplete re-epithelialization Tables-(4, 5), Figures-(3, 4, and 5).

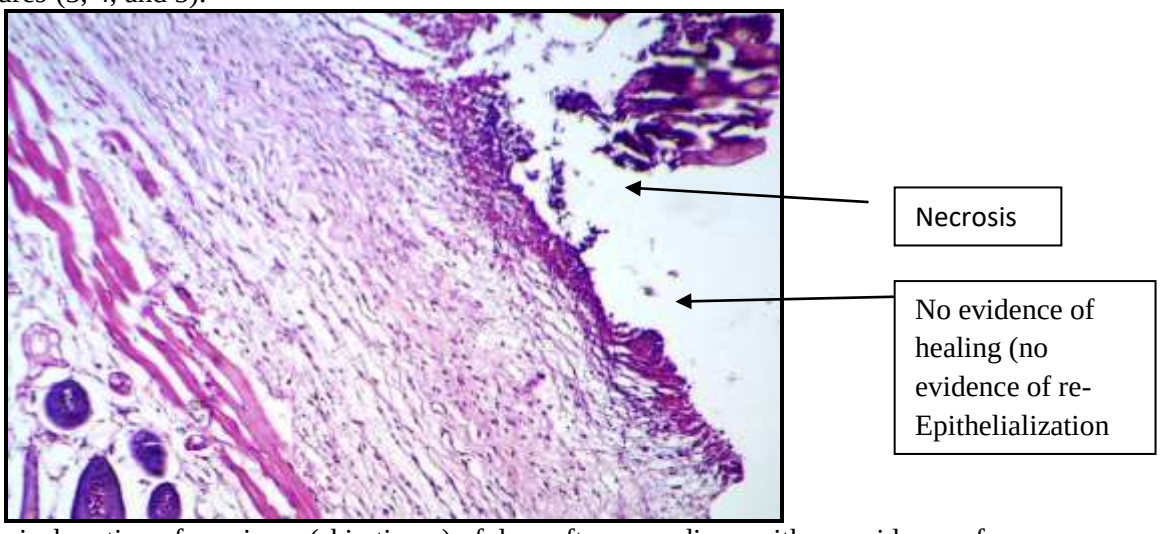


Figure 3-Histological section of specimen (skin tissue) of days after wounding, with no evidence of healing (control group). (H&E staining, X10 magnifiication).

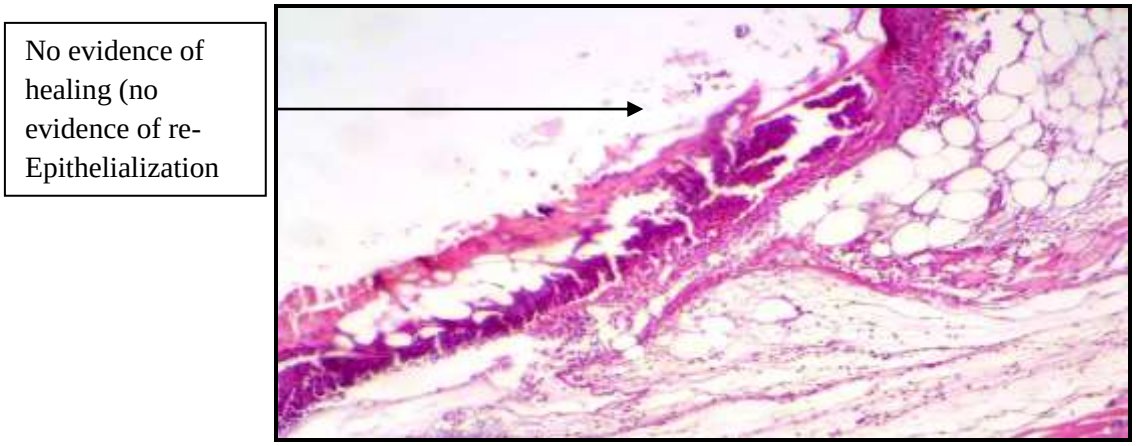


Figure 4-Histological section of specimen (skin tissue) of 5 days after wounding. Incomplete healing with incomplete re-epithelialization, (H and E, X10)

Table 4-Main histological features of control groups

	Control groups
Day 3	Epithelialization: No evidence Granulation tissue: Mild Inflammation: Moderate Collagen: Absent Necrosis: Present
Day 5	Epithelialization: Incomplete Granulation tissue: Mild Inflammation: Mild Collagen: Absent Necrosis: Absent
Day 10	epithelialization: Incomplete granulation tissue : Moderate Inflammation: No evidence Collagen: Present Necrosis: Absent

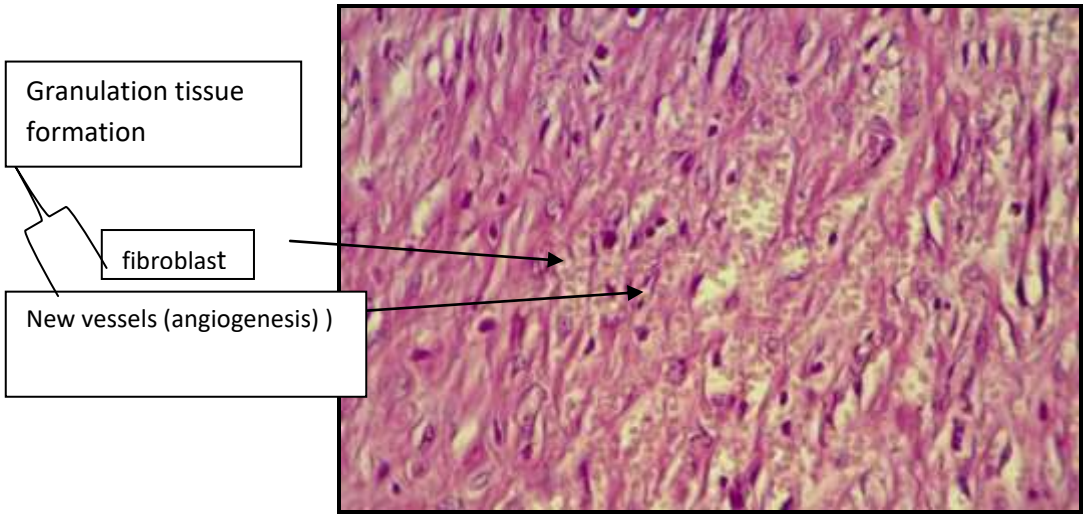


Figure 5-Histological section of a specimen of control mice 10 days after wounding. Incomplete healing response, granulation tissue formation) (fibroblast and angiogenesis). (H and E X40)

Histological evaluation for groups treated with 810nm near-infrared laser

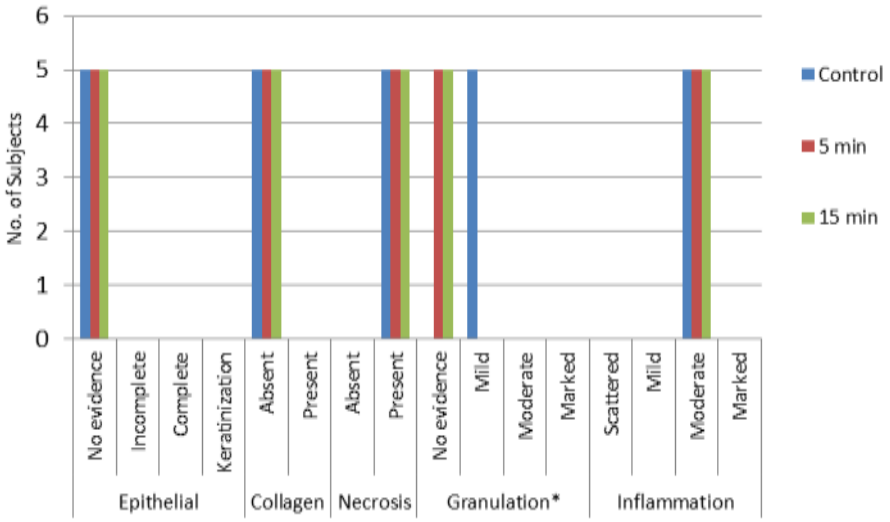
Irradiated groups were classified into two groups according to laser dose.

Group 1: irradiated for 5 minutes exposure time at a dose of 12.5J/cm².

Group 2: irradiated for 15 minutes exposure time at a dose of 37.4J/cm².

- **Histological assessment 3 days after treatment by NIR laser**

Three days after treatment, no evidence of healing response was seen in all irradiated groups and the respective control group (Table-5, Figure-6).



*** Significant (Chi-square=15, df= 2, P=0.001)**

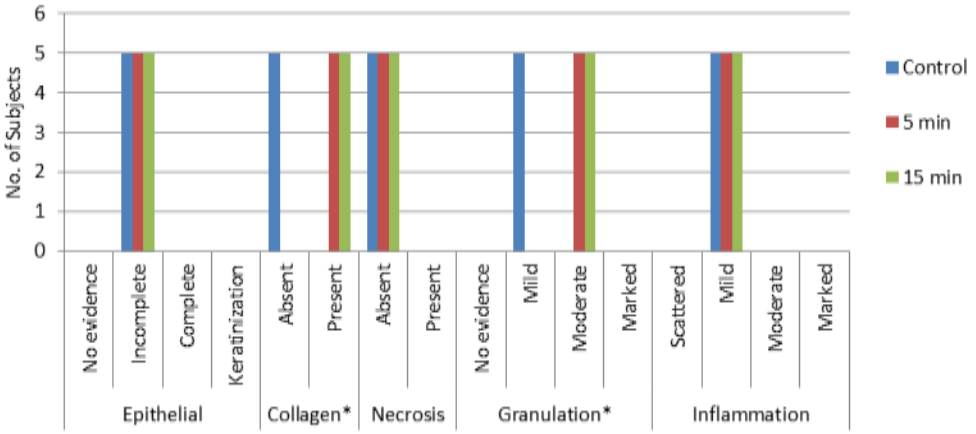
Irradiated groups for 3 days after treatment (NIR 810nm laser)

Figure 6-The semi quantitative histopathological evaluation 3 days after wounding. Groups irradiated by 810nm NIR laser.

- **Histological assessment 5 days after treatment by NIR**

Five days after treatment, incomplete healing response was seen in irradiated groups and the respective control group, as expressed by incomplete re-epithelialization, enhancement in granulation tissue formation and collagen deposition. (Table-5, Figures-(7 and 8).

Significant differences in granulation tissue and collagen formation was seen between irradiated and control groups (p=0.001) (Figure-7).



*** Significant (Chi-square=15, df=2, P=0.001)**

irradiated groups 5 days after treatment(NIR 810nm laser)

Figure 7-Semi-quantitative histopathological evaluation 5 days after wounding. Groups irradiated by 810nm NIR laser.

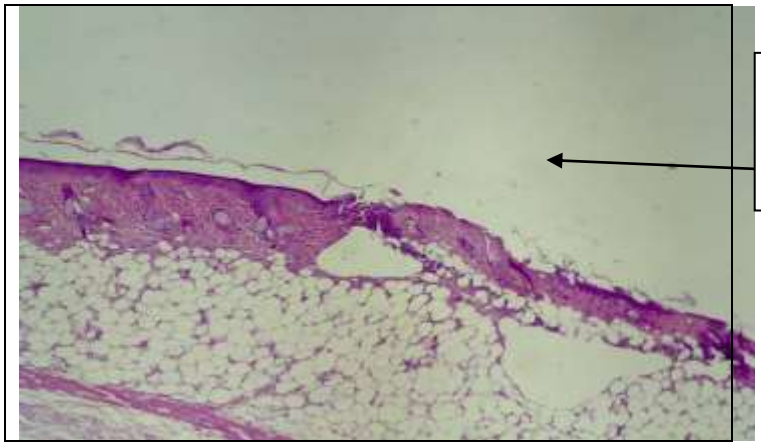


Figure 8-Histological section of the 5 days after wounding mice irradiated by NIR 810 nm (15 minutes exposure time, 37.4 J/cm^2 dose) showing incomplete healing with incomplete Re-epithelialization, (H and E, X10**).**

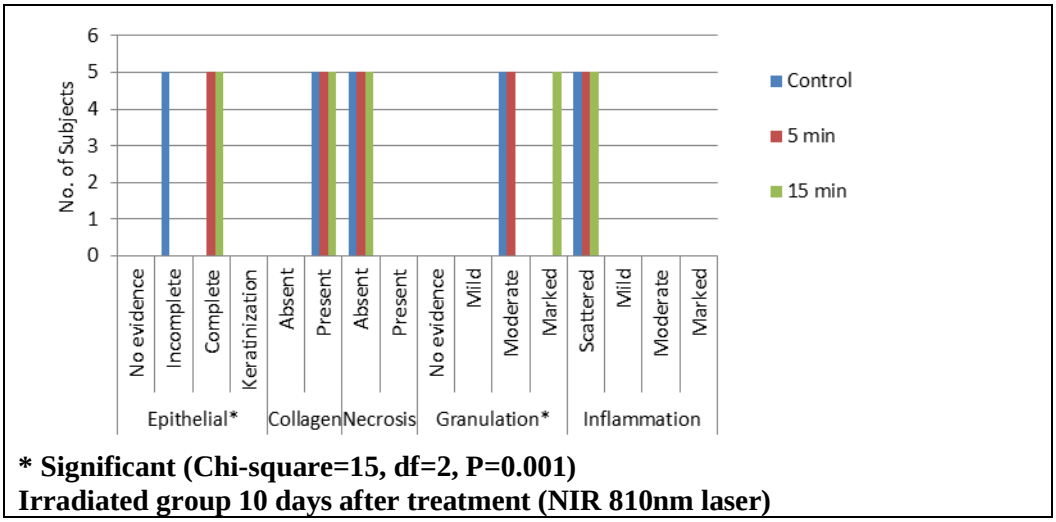
- **Histological assessment 10 days after treatment by NIR laser**

Examining wounds at day 10, irradiated groups showed complete healing responses with complete re-epithelialization. (Table-5, Figures-(9 and 10).

Groups exposed to 37.4J/cm² for 15 min showed marked granulation tissue formation, indicating better healing response, while groups exposed to 12.5J/cm² for 5 min showed moderate granulation tissues (p=0.001). The control group showed incomplete response of healing (Table-5).

Table 5-Main histological features of groups irradiated by 810nm near infrared laser

Flow up period	groups irradiated by 810 nm		
	Irradiated groups		Control
	Exposure time = 5 min Dose =12.5 J/ cm²	Exposure time=15 mint Dose = 37.4 J/ cm²	
Day 3	no evidence of healing response	no evidence of healing response	no evidence of healing response
Day 5	incomplete healing response	Incomplete healing response	incomplete healing response
Day 10	Complete healing response	complete healing response	Incomplete healing response



*** Significant (Chi-square=15, df=2, P=0.001)**
Irradiated group 10 days after treatment (NIR 810nm laser)

Figure 9-The Semi-quantitative histopathological evaluation 10days after wounding. Irradiated by NIR 810nm laser

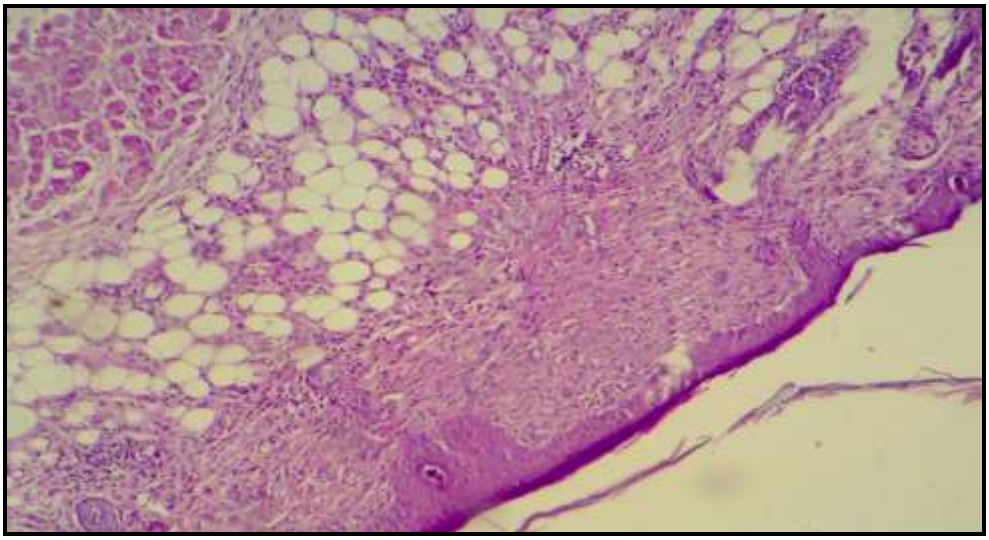


Figure 10- Histological section of mouse wound, 10 days after wounding, irradiated by 810 nm NIR (5 minutes exposure time, 37.4 J/cm^2 dose), showing complete healing with complete re-epithelialization and presence of collagen deposition (H and E, X10).

Discussion

The fundamental aim of this study is to examine the impact of 810nm laser radiation on wound healing in mice.

The results showed a significant improvement in histological parameters of healing (granulation tissue formation, re-epithelialization, and collagen deposition) in the irradiated wounds, with the effect being dose dependent.

Epithelialization is an essential structure of wound healing that is used as an important parameter of wound healing development, and without epithelialization, wound cannot be considered as healed. For these reasons, this research concentrated on re- epithelialization as an essential parameter for histopathological assessment of wound healing, in addition to the formation of granulation tissue, collagen deposition and inflammatory cells infiltration [12].

The irradiated groups showed a significant enhancement in the percentage of wound closure and histopathological parameters when assessed at day 5 after treatment, indicating that cold laser was efficient in healing wounds. This finding is in conformity with the results reported by Chung *et al*. [13], Al-watban *et al*. [8], Lee *et al*. [14], Ferreira *et al*. [15], and Fekrazad *et al*. [16].

Previous reports showed that various types of cells respond differently to laser irradiation, depending upon wavelength, power density (irradiance) and dose. Different types of cells are involved in the wound repair and tissue regeneration, i.e. fibroblasts and macrophages, (essential cells for granulation tissue formation and angiogenesis) and keratinocytes (fundamental cells for skin re-epithelialization [11].

In the field of wound research, fibroblasts are correlated to the granulation tissue formation which is the most essential component of extracellular matrix responsible for wound tensile strength.

Macrophages are attracted to such circumstances with the general essential growth factors, resulting in energetic angiogenesis and augmentation of fibroblasts at wound margins [17] [18].

As an essential explanation for the stimulation of wound healing in mice by using cold laser in this study, such an effect is possibly due to absorption of laser light with specific wavelength by the target tissue, leading to improvement of fibroblast proliferation (increased fibroblastic activity) and the subsequent progress in the formation ofgranulation tissue and extracellular matrix along with the progress of collagen metabolism. This explanation is in accordance with the results of previous studies which proposed an increased fibroblast activity and proliferation in irradiated groups when compared with non- irradiated subjects. This indicates an increased proliferation of fibroblast in *in vivo* conditions [19] [20].

In this research, the laser beam was delivered through a beam expander to cover the whole area of the wound, and this method was used in many previous studies [8, 7,5,20, 21].

Positive results were also obtained using 810nm near infrared laser at two doses of 12.5J/cm^{2}and 37.4J/cm^2. These doses lie in the range generally used with near infrared wavelengths, and its advantage is in agreement with the study of Castano *et al* who found a positive result using an 810nm laser with a beam expander at a dose of 30J/cm^2 in the treatment of inflammatory arthritis in rats [21]. Several studies proposed that the enzyme cytochrome c oxidase is among the important chromophores located in the mitochondria, representing a unit in the respiratory chain located in the inner membrane of mitochondria. Absorption bands of this protein lie in the visible to near-infrared regions; incident photons absorbed by cytochrome c oxidase increase the activity of this enzyme [22] [23]. The impact of cold laser therapy depends on the stimulation action on cytochrome c oxidase, while the increase in the strength of the response depends on the availability of enough time to have actual influences on the cells. Cold laser therapy depends on improving and recognizing the reactivity of cytochrome c oxidase [22]. Some previous reports showed that irradiance and dose (exposure time) were important in establishing the effect of cold laser therapy.

Conclusions

1. At day 3 of treatment with 810nm near infrared laser at doses of 12.5J/cm² and 37.4J/cm², there was no evidence of wounds healing in irradiated and control groups.
2. At day 5 of treatment, the experiments showed a significant increase in wound healing parameters (granulation tissue formation, re-epithelialization and collagen deposition) in irradiated groups.
3. Near infrared 810nm laser had photobiostimulation effects on wound healing at irradiance of 41.63mW/cm² and doses of 12.5J/cm² for 5 minutes and 37.4J/cm² for 15 minutes exposure time.
4. A complete picture of wound healing response appeared in all irradiated groups within 10 days of treatment, as expressed by complete re-epithelialization, moderate granulation tissue formation and presence of collagen fibers, while incomplete wound healing responses were observed in un-irradiated control groups within the same period.

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