Photo-Stimulatory Effect of Low Level Energy Laser Irradiation On The Progress Of Wound Healing In Mice

Hala Moustafa Ahmed [1] and Harbi A. Sayed [2]

[1]Medical Biophysics, Faculty of Applied Medical Science, October Six University,
[2]Biology Department, Vacsera Company,
Bakar_tarek_76@hotmail.com

Abstract: Many studies have demonstrated that low level laser therapy (LLLT) can promote the wound healing on diabetic and non-diabetic animals. This study aimed to evaluate the photo-stimulatory effect of low energy 650 nm Diod laser irradiation on excisional diabetic wound healing dynamics in Balb/C mice. Streptozotocin (180 mg/kg) was applied for diabetes induction. An oval full-thickness skin wound was created aseptically with a scalpel in 100 diabetic mice and 20 non-diabetic mice on the shaved back of the animals. The study was performed using 650 nm diode laser in doses (3 J/cm^2, 4 J/cm^2, 5 J/cm^2& 6 J/cm^2) for 3 times/week. The area of wound in all mice were measured and plotted on a slope chart which revealed a significant differences ($p < 0.001$) in the percentages of wound healing acceleration (15, 20, 22.9, and 24.9) in the four doses respectively in comparison with positive and negative control groups and confirmed by histological studies which showed a highly increase in collagen fibers in sub epidermal tissue, and with intact epidermis presence of hyperplasia covering well-developed granulation tissue and demonstrated collage fibers. We can conclude that, the optimum wavelength was 650 nm, and the optimum incident dose was 6 J/cm^2 in our study.

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1. Introduction:

Wound healing and tissue repair are complex processes that involve a dynamic series of events including clotting, inflammation, granulation, tissue formation, epithelization, collagen synthesis and tissue remodeling. Chronic wounds are slow, non-healing wounds that can last for weeks despite adequate and appropriate care. Such wounds are difficult and frustrating to manage (Ivandic *et al.,* 2008). In recent years, low-intensity laser therapy has gained considerable recognition and importance among treatment modalities for various medical problems including wound repair processes, musculo-skeletal complications, and pain control (Fahey *et al.,* 1991. Clinical studies have shown low energy lasers to be effective as analgesics and to accelerate the healing of injured tissue (Mester & Koebner, 1980). Although the beneficial effects of laser photo stimulation are now generally accepted, the mechanisms by which laser light facilitates wound healing and tissue repair yet to be clearly understood (Lam *et al.,* 1986). Low-level laser therapy (LLLT) is also known as biostimulation and photobiostimulation. Photobiostimulation is a form of phototherapy that involves the application of low-power monochromatic and coherent light to injuries and lesions in order to stimulate wound healing. LLLT has been shown to increase the speed, quality and tensile strength of tissue repair, resolve inflammation and provide pain relief. (Fahey *et al.,* 1991). It is probable that applications of LLLT in animal models will be more effective if it is carried out on models that have some intrinsic disease states. Although there have been several reports concerning processes such as wound healing which are accelerated by LLLT in normal rodents, an alternative approach is to inhibit healing by inducing some specific disease state (Yaakobi *et al.,* 1996). This has been done in the case of diabetes, a disease known to significantly depress wound healing. LLLT significantly improves wound healing in both diabetic rats (Lam *et al.,* 1986) and diabetic mice (Lyons *et al.,* 1987). The beneficial effect of LLLT on wound healing can be explained by considering several basic biological mechanisms including the induction of expression of cytokines and growth factors known to be responsible for many phases of wound healing (Poon *et al.,* 2005). Yu and colleagues (1997), showed that low-level laser irradiation (an argon dye laser wavelength: 630 nm; power density: 20 mW/cm^2) improved wound healing in genetically diabetic mice and enhanced wound closure over time. Reddy *et al.* (2001), have concluded that photobiostimulation promotes tissue repair by accelerating the production of collagen and promotes overall connective tissue stability in wound healing. Open wounds have lost the barrier that protects tissues from bacterial invasion and allow for the escape of vital fluids. Wound

healing and tissue repair are complex processes that involve a dynamic series of events including clotting, inflammation, granulation tissue formation, epithelization, collagen synthesis, and tissue remodeling (Reddy, 2001). The exact pathogenesis of the delayed wound healing is not clearly understood, but evidence from studies involving both human and animal models reveal several abnormalities in the various phases of the wound healing process (Singer & Clark, 1999).Impaired wound healing is an enigmatic and debilitating complication of diabetes and poses a serious challenge in clinical practice without expeditious healing, in which infections become more frequent. Most wound complications such as wound dehiscence or skin graft loss are associated with some form of host impairments such as infection, diabetes, or chemotherapy (Goodson, 1989).A cceleration of cutaneous wound healing has always been a very important factor after surgery for rapid recovery and returning to ordinary life style. Wound healing process included multiple stages suchas infiltration of inflammatory cells, migration and proliferation of fibroblasts, synthesis of extracellular Matrix proteins (ECM) such as collagen, connective tissue and parenchymal remodeling and then collagenization in order to increase wound strength (Houreld, 2011).

2. Materials and Methods

A total of 120 male Balb/c mice weighting 18-20 gm were used in this study and classified into 6 groups as shown in table 1 where the negative control group (Non diabetic control) received an equivalent volume of sodium citrate buffer solution, while the diabetic groups induced by Streptozotocin (Sigma) which dissolved in a cold 0.01M citrate buffer, pH 4.5 prepared freshly for immediate use within 5 min. STZ injections administrated intraperitoneal at a dose of 180 mg/kg body weight injected groups (A,B,C,D,&E) with STZ. The blood glucose concentration estimated every week from the day of STZ injection. Only diabetic animals with blood glucose at least double the baseline value (350 mg/dl) by one touch instrument glucometer device were included in the study. The blood glucose tests diabetic animals were monitored weekly regularly from tail vein until end of the experiment (4 weeks). (Arora *et al.,* 2009) .

Table (1): The experimental design.

Group	Experimental conditions
A	Twenty Diabetic mice non treated with laser. (Positive control).
B	Twenty Diabetic mice treated with laser at dose (3 J/cm^2) for 3 times/week.
C	Twenty Diabetic mice treated with laser at dose (4 J/cm^2) for 3 times/week.
D	Twenty Diabetic mice treated with laser at dose (5 J/cm^2) for 3 times/week.
E	Twenty Diabetic mice treated with laser at dose (6 J/cm^2) for 3 times/week.
F	Twenty mice, Non diabetic , non treated (negative control)

Collection of the blood samples:

After 7 days of stabilization period, blood samples were obtained from animals fasted. About 1ml of blood was drawn into a test tube having sodium fluoride. After an hour, the sample of blood was centrifuged at 2000 rpm for about 15 minutes. After centrifugation, the supernatant serum was collected and the glucose level was estimated. The STZ-injected group with more than 200 mg% of blood glucose has been included in the study. The animals were assigned to the study and control groups, with 20 animals in each group with same weight, age, and blood glucose level.

Laser treatment parameters:

The laser used was diode laser (NEC, Japan), Laser has a given wavelength of light. Its energy density is the most important factor in determining the tissue reaction (Plaetzer *et al.,*2002) It is the output power density which determines the time required to deliver a particular energy density (Joules/cm^2) dosage that means, the output power determines the corresponding energy (Joules) which delivered during that time. Power density 150 mW/cm^2, wavelengths, 650 nm, the spot size of the laser beam was 1 cm^2 with incident doses of (3-6 Joules/cm^2). Power density measured the potential thermal effect of those photons at the treatment area (Bruce *et al.,*1992). The calibration and output of the equipment were checked before and during the experiment to maintain the accurate dosage with the help of a dosimeter. The method of irradiation was standardized before the experiment. Then on-contact method (6-mm distance from the wound surface) was found to be accurate for irradiation in wound healing, and the technique was used during the study. In the study (laser) group for excisional wound a constant spot of 1 cm^2 was irradiated for varying length of time to achieve the desired fluency or the dose (Baxter *et al.,* 1994).

Surgical procedure:

The animals were anesthetized with intravenous ketamine of 2 mg/kg body weight. The dorsal furs of the animals were shaved with an electric clipper. The

area of wound to be created was marked on the back of animals by methylene blue using circular stainless steel stencil. A full thickness of the excision wound of circular area 250 mm^2 and 2 mm depth will be created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound will be left open (Maiya *et al.,* 2009).

Sample collection and analysis:

On fifth and final, the granulation tissue of approximately 1 cm wide and 6 cm length was collected from each animal for analysis. The tissue was kept in an oven at 60°C and its dry weight was noted. Then 0.4 ml of deionized water, 1.0 ml of 2.5 N NaOH, 1.0 ml of 0.1 M $CuSO_4$ and 1.0 ml of 6% hydrogen peroxide were added to 1 ml of hydrolysate. The tubes were covered and boiled for 15 minutes at 80°C, and absorbance was read at 540 nm using spectrophotometer to note the hydroxyl proline level (Lyons *et al.,*1987).

Data analysis:

The areas of wound in all mice were measured using a caliper daily for 5 days/week and plotted on a slope chart of wound healing for 4 weeks (Fig. l). A trend-line was applied on the slope chart, and the slope value (mm^2/day) of the wound healing in all mice was computed using the linear type and set intercept option. Mean slope value of wound healing was computed in every group. (Farrouk *et al.,*2007). The percentage of relative wound healing (RWH) was calculated as follows:

% of RWH = (slope value in diabetic control or treatment
- slope value in non-diabetic control) / slope value in non-diabetic control x 100 (%)

The percentage of wound healing acceleration (WHA) was calculated as follows:

% of WHA = %RWH in diabetic treatment group
- To RWH in diabetic control group
%Relative W.H. = (Slope in Treatment - Slope in Control) / Slope in Control x 100
*Slope Values means closure of wound area (mm^2)/ day

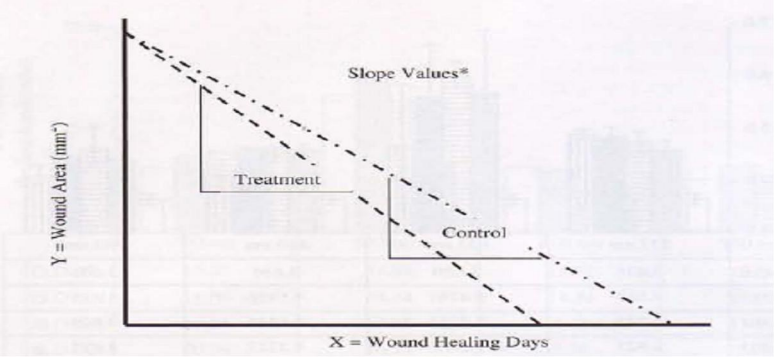

Figure (1): Calculation method of slope values and percentage of relative wound healing in mice (Farrouk *et al.,* 2007).

Histopathological studies:

At the end of the experiment all mice were sacrificed , tissue samples were excised from the experimental animals of each group and placed in 10% neutral buffered formalin for 48 hrs .They were then processed for paraffin. The sections were taken at 5µm thickness using microtome, processed in xylene alcohol- series and stained with alum-haematoxylin and eosin. The sections were examined microscopically for the evaluation of histopathological changes (Galigher & Koyloff, 1971).

Method of statistical analysis:

The experimental observation parameters (mean wound healing time) between treated and control groups were analyzed by independent *.t.* test and the SPSS-9 software package was used for statistical analysis. Results are presented as mean $\pm$ S.D.

3.Results:

1-Effect of mean slope value of wound healing on non-diabetic control mice and diabetic control mice:

Mean slope values of wound healing were 6.077 (mm^2/day) in non-diabetic control mice and 3.00 (mm^2/day) on diabetic control mice. There was a significant difference ($p < 0.005$) in the mean slope values of wound healing between non-diabetic (negative control) mice and diabetic (positive control) mice (Fig. 2).

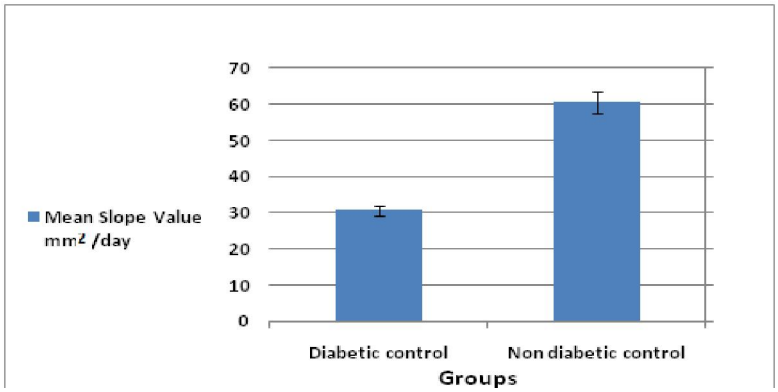

Figure (2): Mean slope values of wound healing on non-diabetic control mice and diabetic control mice.

2-Effects of continuous exposure to 650 nm of Laser beam on mean slope value of wound healing in mice:

There were significant differences ($p < 0.001$) in the mean slope value of wound healing on diabetic mice between control and treatment groups using several doses (Fig. 3).

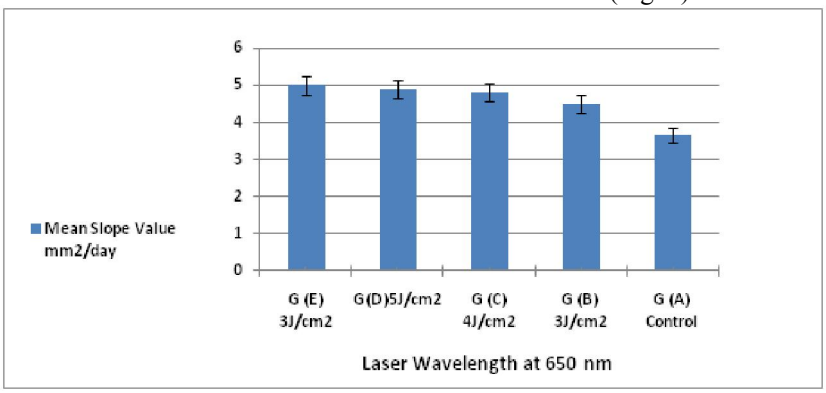

Figure (3): Mean slope values of wound healing on diabetic mice in control group and treatment groups using different doses from 650 nm of Laser beam.

3-Effects of continuous exposure to 650 nm Laser on percentage relative wound healing diabetic control mice and diabetic treatment mice:

There were significant differences ($p < 0.001$) in the mean slope value of relative wound healing on diabetic mice between control and treatment groups using several doses (Fig. 4). There were significant differences ($p < 0.001$) in the mean slope value of percentage of wound healing acceleration in diabetic mice treatment groups using several doses (Fig. 5).

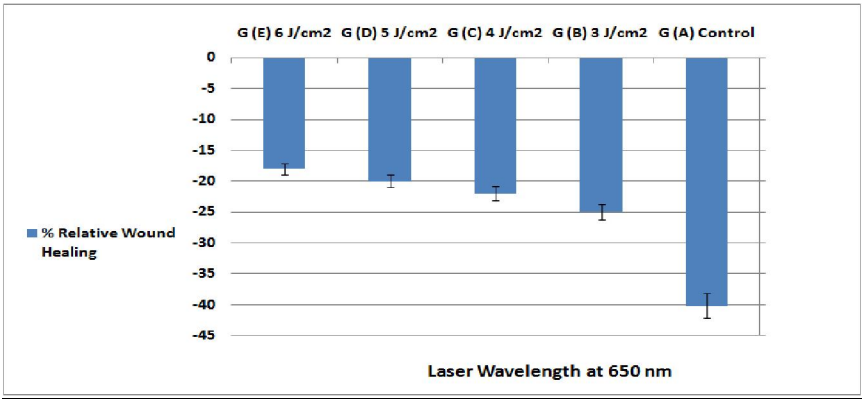

Figure (4): The percentage of relative wound healing (RWH) in the diabetic mice after 650 nm of Laser beam therapy compared with non-diabetic control.

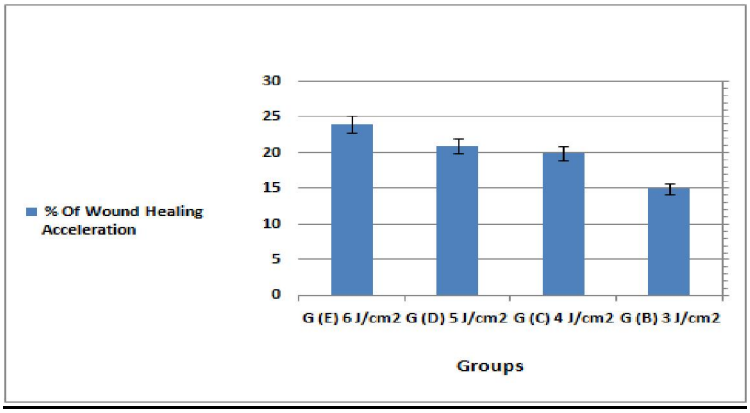

Figure (5): The percentage o f wound healing acceleration (WHA) in the diabetic mice after laser therapy using different laser doses at wavelength 650 nm.

4-Effects of continuous exposure to 650 nm of Laser beam on progress of wound healing in mice:

The closure of skin defects was practically complete in all animals of the study group but varied in mean healing days in different dose of laser treatment. The wound healing process was significantly accelerated in groups exposed to low level laser irradiation of 3-6 J/cm^2 per day in the diabetic wound healing study. The significance was very high in the group irradiated with 3-6 J/cm^2. The photo stimulatory effect was found in the dose between 3 and 6 J/cm^2 in all parameters like experimental observation and hydroxyproline level findings. In the present study, the influence of laser treatment on the healing process was most pronounced in biochemical findings. The results showed that the production of collagen in diabetic wounds can be modulated by laser treatment. The content of the total collagen was significantly increased in laser-treated wounds as compared to the control group. The total content of the collagen in the laser groups were significantly more than that of the control group on day 5 and on healing in the group with 3-6 J/cm^2 dose with $P < 0.001$ [Table 2].

Table (2): Effect of 3-6 J/cm^2 per day and 5 days a week dose of Diode laser radiation on diabetic wound healing (hydroxyproline).

Dose (J/cm^2)	N	Study		Control		t-test	*p*-value
		Mean	±S.D	Mean	±S.D		
3	20	12.99	±1.49	7.88	±1.44	11.66	0.001
4	20	12.99	±2.09	6.89	±1.31	12.99	0.001
5	20	15.36	±1.87	8.10	±0.71	19.33	0.001
6	20	14.33	±1.33	7.33	±0.89	18.33	0.001

- **S.D**: Standard Deviation , P : < 0.001 highly significant
- P : ≤ 0.05 significant , P : > 0.05 non-significant.

Histological results:

Diabetic mice tissue sample showed a thin epithelium with minimal collagen in Submucosa, Figure (6). While animals treated with laser dose (3 J/cm^2) showed a minimal in collagen fibers in sub epidermal wide area of ulceration, fibrinonecrotic material on granulation tissue and sub epithelial tissue (Fig. 7). Cutaneous sample of a animals treated with laser dose (4 J/cm^2) showed middle increase in collagen fibers in sub epidermal tissue, fibrinonecrotic material on granulation tissue and sub epithelia tissue (Fig. 8).While Diabetic mice tissue treated with laser dose (5 J/cm^2) showed a moderate increase in collagen fibers in sub epidermal tissue, fibrinonecrotic material on granulation tissue and sub epithelia tissue (Fig. 9). Figure (10), Diabetic mice tissue treated with laser dose (6 J/cm^2) showed a highly increase in collagen fibers in sub epidermal tissue, fibrinonecrotic material in granulation tissue and sub epithelia tissue and showing intact epidermis with presence of hyperplasia covering well-developed granulation tissue and demonstrate collage fibers. All these results were compared with Negative control mice tissue.Cutaneous sample which exhibited a wide area of ulceration, fibrinonecrotic material on granulation tissue and sub epithelia Figure (11).

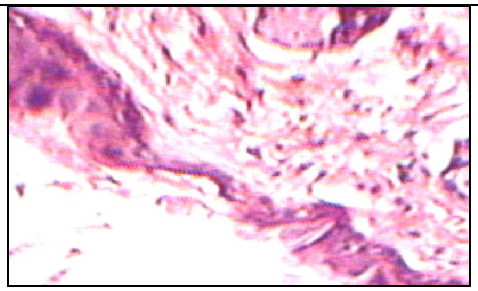

Figure (6): Diabetic mice (positive control) tissue.

(Hematoxylin-eosin stain, total magnification100 x)

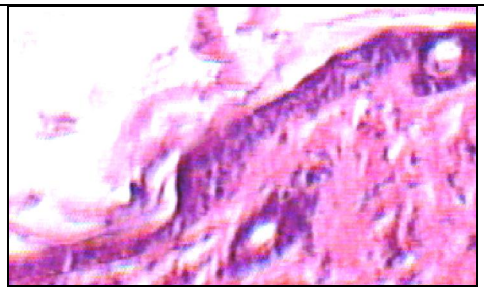

Figure (7): Diabetic mice tissue treated with laser dose (3 J/cm^2)
(Hematoxylin-eosin stain, total magnification100 x).

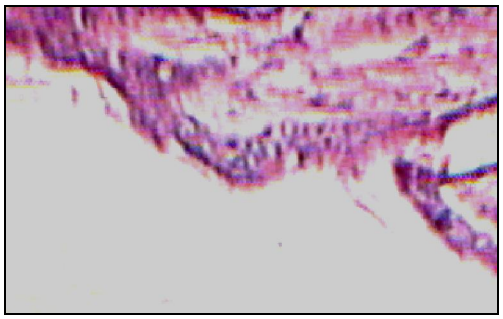

Figure (8): Diabetic mice tissue treated with laser dose (4 J/cm^2)
(Hematoxylin-eosin stain, total magnification100 x).

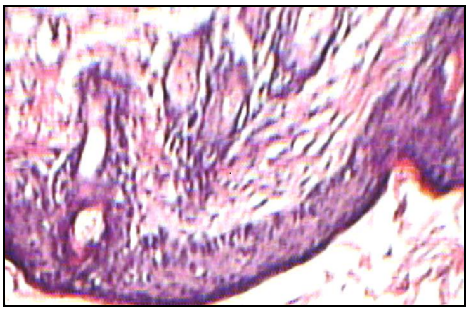

Figure (9): Diabetic mice tissue treated with laser dose (5 J/cm^2)
(Hematoxylin-eosin stain, total magnification100 x).

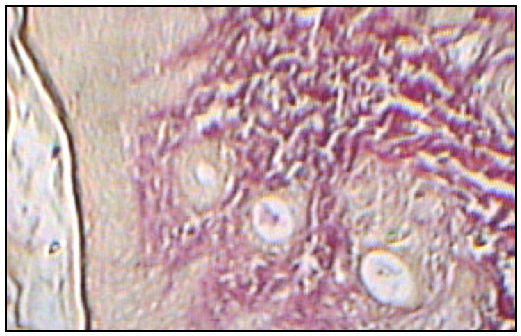

Figure (10): Diabetic mice tissue treated with laser dose (6 J/cm^2)
(Hematoxylin-eosin stain, total magnification100 x).

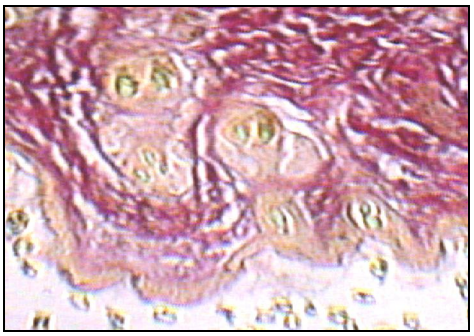

Figure (11): Negative control mice tissue.
(Hematoxylin-eosin stain, total magnification100 x)

4.Discussion

A total of 100 Balb/C diabetic mice induced by STZ were used in the study additionally, 20 non diabetic mice as control were used for comparison with diabetic control mice. Our results showed that wound healing on control mice with diabetes was slower than on control mice without diabetes there was a significant difference ($P < 0.001$) in the mean slope values of wound healing between diabetic control mice and non-diabetic control mice (Fig. 2). Impairment of diabetic wound healing is well recognized. However, the exact mechanism is still not completely known. (Reenstra *et al.,* 2001) . The course of diabetic wound healing appears to be hindered by many factors, including specific metabolic deficiencies and impaired physiological responses-for example, the altered metabolism of carbohydrates fats and proteins resulting from the absence or deficiency of insulin, hyperglycemia leading to non enzymatic glycosylation, osmotic diuresis leading to decreased perfusion and oxygenation, and free radical production (Pecoraro *et*

*al.,*1991). in our animal study, the wound healing was delayed in diabetic mice. A more recent study (Martens *et al.,* 1992), involving a non-lesion fibroblasts, showed that diabetic fibroblasts have a decreased proliferation response to growth factors, caused by a deficiency in growth factor receptor expression, compared with non-diabetic fibroblasts from sibling control. (Pecoraro *et al.,*1991), Research has shown that the defects that occur in diabetic wound healing may be caused by altered collagen metabolism and abnormal granulation tissue formation. (Spanheimer, 1988), showed that Diabetes can result in the development of several complications, including diabetic foot wounds that can potentially lead to lower limb amputation. The poor wound healing in diabetes is a barrier in medicine. Our study indicated that the in addition of low power lasers at appropriate treatment parameters can accelerate the wound healing on diabetic rats. Mean slope values of wound healing on diabetic mice in treatment groups using different laser doses and wavelengths were bigger than control groups (Fig. 3). There were significant differences ($p < 0.001$) in the mean slope value of wound healing in diabetic mice between control and treatment groups using several doses (Table 2). The percentage of relative wound healing and the percentage of wound healing acceleration after laser therapy showed that the optimum wavelength and incident dose was 650nm and 6 J/cm^2 in the study (Figs. 4 and 5). The research revealed also that the irradiation of visible laser light was better than invisible laser light in the treatment of wound healing in diabetic mice. The reason for the effective acceleration of wound healing in diabetic mice using low-power lasers was that perhaps the absorption of laser light with specific wavelength by target tissue resulted in the enhancement of fibroblast proliferation and the promotion of collagen metabolism and granulation tissue formation in the diabetic wound .The healing process of diabetic wound is a complicate done and is initiated by a complex series of events that include chemotaxis, growth factor pathways, complement generation, and the energy-poor environments created by low oxygen tensions, low pH and high lactate concentrations (Knighton *et al.,*1990). Our study showed the doses range between 3 and 6 J/ cm^2 were very effective in facilitating the wound healing process. Many studies (Abergel *et al.,*1987) suggest that laser bio-stimulation occurs at fluences between 0.05 and 10 J/cm^2, whereas fluencies above 10 J/cm^2 have inhibitory effects in the impaired wound healing process. Many of the chronic complication of diabetes involve defects in connective tissue such as poor wound healing. Goodson & Hunt, (1989) represented that, the wound healing abnormalities of diabetes results from several causes, when carbohydrates are unavailable to cells for normal aerobic metabolism, oxidation of amino acids for caloric needs results in amino acids and protein depletion. When glycolgenolysis and gluconeogenesis fail to provide glucose to meet the energy requirements for fibroblasts and leucocytes, they become dysfunctional and impaired wound healing results. The poor wound healing of diabetic has been shown to be associated with a decreased amount of collagen fibrils and collagen production. (Cukjati *et al.,* 2000) demonstrated that low energy laser enhances wound healing in diabetic rats as evidenced by experimental observation, area measurement, and biochemical and histopathological analyses of the study group and control group. In our study, we found that 3-6 J/ cm^2 was a bio-inhibition dose in diabetic wounds, and the influence of laser treatment on the healing process was most pronounced in biochemical findings. (Neda Nasirian *et al.,*2012) showed that the effect of red light laser 630 nm on cutaneous surgical wound in hamster and to compare outcomes such as angiogenesis, number of fibroblasts and collagen formation in intervention and control group. The results of the study showed that the production of collagen in diabetic wounds can be modulated by laser treatment. The content of the total collagen was significantly increased in laser-treated wounds as compared to the control group. The total content of the collagen in the laser group was significantly more than that of the control group on day 5 and on healing in the group with 3-6 J/cm2 dose with $P < 0.001$ [Table 2]**.**The low energy at wavelength of 650 nm can modulate the cell proliferation and the release of growth factors from fibroblasts. This in agreement with Dyson & Young (1986) where they showed that positive effect of laser photostimulation on wound healing may involve the enhancement of growth factor release, which in turn promotes extracellular matrix production and degradation. In our study, we found that the main biological effect of laser is due to the major absorbing structures. The major absorbing structures for the red visible laser wavelengths are the proteins; however, the identity of the photo receptors responsible for the biological effects of low energy laser therapy (LELT) has not been resolved. Several studies have suggested that either elements in the mitochondrial cytochrome system or endogenous porphyrins in the cells are the energy-absorbing chromophores in LELT (Cukjati *et al.,*2000). Consequently their selective permeability for sodium, potassium, and calcium ions or by the increasing the activity of certain enzymes such as cytochrome oxidize and adenosine tri phosphatase. It also increases DNA synthesis, collagen, and pro collagen production and may increase cell proliferation or alter

locomotors characteristic of the cells. This finding in our study was also supported by a similar finding of the previous report (Labbe *et al.,*1990). The results of this study and the possible biomechanics involved were discussed in the context of other experimental findings of increased cell counts following (Cecilia *et al.,*1998). The present study suggests that photoenergy of 632.8 nm wavelength at the given parameters possibly induced the fibroblasts to secrete the growth factors that probably acted in an autocrine manner to increase their rate of mitosis and or reduce cell death. The response of low energy laser on cells may be dose dependent as well as wavelength dependent (Karu & Yearly, 1990) Therefore, we strongly suggested that correct energy density with an appropriate wavelength can be absorbed by the targeted tissues. Therefore, employing the correct power density, exposure time, and energy density are important parameters to achieve the bio-stimulation effect in wound healing. In our study, we found that the reason for the bio-inhibition mechanism with increasing doses was due to in adequate incident photo energy or had exceeded the stimulatory range for inducting stronger biological activities in the cells. The inhibition with higher dose was probably caused by formation of dry harder scar. For this effect, absorption of light energy in the tissue in the form of heat, i.e. without wavelength specificity, seems to be of importance. However, the higher dose leading to direct thermal destruction of tissues was described earlier after long-term irradiation (Hallman *et al.,*1988).Thus, the excessive cumulative effects of daily exposure to low energy laser for several days may have reversed any initial beneficial effects of red light irradiation that also occurred in few previous studies (Surinchak *et al.,*1983)**.**

Conclusion:

Therefore, we concluded that low level laser energy .650 nm diode laser irradiation at certain dose has a significant beneficial effect on wound healing. The present study highlights the possible utility of 650 nm diode laser with appropriate energy density as an adjunctive modality for diabetic wound healing in clinical practice. Our study suggests that low-level laser therapy 650 nm can accelerate and promote surgical wound healing in mice.

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