Assessment to the Effects of Low Power Diode Laser on Wound Healing in Diabetic Rats

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Abstract: Objective: Evaluation of the effect of low level laser therapy (LLLT) using diode laser 808 nm on wound healing in diabetic rats as experimental animal model. **Back ground data:** Delayed wound healing is reported by several medical care units as changing cases. One of the causes for chronic wounds and delayed wound healing is diabetes which sometime associated with suppuration, gangrene and may be ended by amputation. This is encountered in different medical specialities. **Methods:** 40 male albino rats, each weighed 200-220 gm. Diabetes was chemically induced using streptozotocin, 40 mg/kg, dissolved in citrate buffer solution (pH 4.3) and administered as tail vein injection in all experimental rats groups expect control group . Seven days after streptozotocin injection, blood glucose levels were measured by using a glucometer and test strips. **Results:** Gross examination showed faster wound closure in the laser exposed groups with minimal scar tissue formation in comparison with non-laser treated group. The clinical findings were confirmed by the histopathological study using Haematoxylin and Eosin and Masson's Trichrome stain that revealed moderate inflammatory reaction in the laser group versus severe suppurative inflammatory reaction with keloid formation in the control non-treated group. Laser group also showed earlier granulation tissue formation and re-epithelization than non-treated control group. **Conclusion:** Low level laser therapy using diode laser 808 nm can be applied as an efficient method to accelerate wound healing in diabetic patient. Low level laser therapy has anti- inflammatory and antiseptic effects in addition to minimal scar tissue formation.

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1. Introduction

Diabetes is a complex metabolic disorder affecting many body organs and systems with devastate the lives of affected individuals **[1]**. The global incidence of diabetes gets to reach 6.6% (285 million people) in the end of 2010, and the number of people with diabetes will have increased to 438 million or 7.8% of the world's population by 2030 **[2].** Impairment wound healing is a very common complication of diabetes and a serious problem in clinical practice **[3]**. As many as 15% of people with diabetes will develop foot ulceration and wounds, on the other side 3% will have a lower limb amputation **[4].**

Wound healing is a complex and dynamic process which considered as a natural restorative response to tissue injury. Healing is the interaction of complex cascades of cellular events that associate with resurfacing, reconstitution, and restoration of the tensile strength of injured skin. Wound healing is a complex process involving various processes, as coagulation, inflammation, matrix synthesis, angiogenesis, fibroplasia, epithelization, contraction and remodeling. Growth factors are polypeptides that control the growth and differentiation of cells which regulate the process of tissue repair **[5]**.

Healing is a systematic process, explained in three classic phases: inflammation, proliferation, and maturation. A clot formed phagocytic cells debride injured tissue during the inflammatory phase. Epithelialization, fibroplasias, and angiogenesis occur during the proliferative phase. Meanwhile, mature granulation tissue forms and the wound begin to contract **[6].** Finally, during the maturation phase, collagen forms tight links to other collagen and with protein molecules that increasing scar tissue strength. An incision made through a full thickness of skin leads to a disruption of the microvasculature and immediate hemorrhage **[7]**.

The vasoconstriction period is followed by long persistent period of vasodilatation which is responsible for the redness, edema and heat observed after tissue injury. Vasodilatation is an important means by which the wound can be exposed to increased blood flow, associated with the necessary inflammatory cells and factors that fight infection and debride the wound of devitalized tissue. The cellular aspect of the inflammatory phase occurs within hours of injury. Neutrophils are the most common cell type within the first 48 hours after injury. Macrophages are essential cells to wound healing through phagocytosis of debris and bacteria. Several defined

peptide growth factors, including epidermal growth factor; platelet derived growth factor, fibroblast growth factor and transforming growth factor-beta, has been shown to stimulate cellular proliferation and synthesis of the extracellular matrix. In addition an important cytokine that stimulates the chemotaxis and proliferation of fibroblasts and smooth muscle cells**[8]**. Finally, macrophages secrete substances that attract endothelial cells to the wound and stimulate their proliferation to promote angiogenesis**[9]**.

Formation of granulation tissue is a central event. During the proliferative phase, the inflammatory cells, fibroblasts, and newly formed capillaries in a matrix consisted of fibronectin, collagen, glycosaminoglycans and proteoglycans were seen**[10].**Granulation tissue formation occurs within 3-5 days following injury and overlaps with the preceding inflammatory phase. The new blood vessel formation is a complex process that relies on several angiogenic factors such as vascular endothelial growth factor, angiogenin, and angiotropin. The fibroblast is a critical component of granulation tissue. Fibroblasts grow in the wound as the number of inflammatory cells decrease. Fibroplasia begins 3-5 days after injury and may last as long as 14 days. Contraction considered as the centripetal movement of wound edges that facilitate closure of the wound defect, is maximal 5-15 days after injury **[11].**

Epithelialization is the formation of epithelium over a denuded surface. This epithelial layer provides a seal between the underlying wound and surrounding environment. The process begins within hours after tissue injury. Epidermal cells at the wound lips undergo structural changes, allowing them to detach from their connections to other epidermal cells and their basement membrane. Collagen remodeling occurred during the maturation phase depends on continued collagen synthesis. During remodeling, collagen becomes organized and fibronectin, hyaluronic acid and glycosaminoglycans are gradually disappears and replaced by proteoglycans. These events allow collagen fibers to cross-link closer together and decreasing scar thickness **[11]**

Chronic wounds are a major source of disability for many people in the entire world. They are very difficult to treat with standard protocols. Often chronic wounds occur on the feet and lower limbs of the diabetic patient as a result of poor circulation. Diabetes and atherosclerosis are common contributing factors for producing chronic unhealed ulcers. Laser therapy has been proven to speed healing and resolution of skin ulcers and chronic wounds [**9].**

Low-intensity laser treatment is a method accepted by Food and Drug Administration (FDA) as an effective chemical treatment for tissue repair; since then it has already been widely studied**[12].**

In recent years, photobiomodulation has gained considerable recognition and importance among treatment modalities for some serious medical problems including chronic wound repair, and pain control. Many literatures were reported that, the photobiomodulation can enhance the healing process by reducing pain, inflammation, promoting cells proliferation, facilitating collagen synthesis[**13]**.

Low level laser therapy is a medical procedure used to treat pain and speed up wound repair. Low-level laser therapy works; it is thought to decrease inflammatory reaction by reducing the number of cellular chemicals and enzymes linked to pain and inflammatory process. On the other side, Low-level laser therapy may be related to the stimulation of the phagocytic cells to produce certain enzymes which affect cell proliferation. The effects of low-level laser therapy appear to be linked to the specific wavelength of the laser treatment itself. The ideal wavelength duration and location of treatment is specific to each wound. The effective clinical dosage of low-level laser therapy varies by manufacturer and remains a subject of continued research **[14]**.

Low Intensity Laser Therapy speeds up the recovery process of wound healing by delivering light energy (photons) to the cells. The cells use this energy in the natural healing process and known as photobiostimulation. Low intensity laser is painless and has been use for over 20 years with no known side effects [**15]**.

Several studies used many types of lasers with different wavelengths for enhance the wound healing such as Heluim neon 632 nm[**15]**, Argon laser 488-514, krypton laser **[16],** Copper vapor laser 578 nm **[17]** diode lasers (685,904,830,670,980,815 and 809 nm) **[18 -21]**, Nd-YAG 1046 nm **[22]**. Others used polarized light, light emitting diodes which were polychromaticor near infrared LED **[23].**

Photobiomodulation has been applied clinically in the treatment of soft tissue damages and acceleration of wound healing. The mechanism of photobiomodulation appeared as activation of mitochondria, resulting in initiation of a signaling cascade that enhances cellular proliferation and cytoprotection.Photostimulation induces a cascade of signaling events initiated by absorption of light by cytochrome oxidase **[24]**.

Several reports on the use of laser therapy have shown that the healing process is positively affected with Low level laser therapy depending mainly on wave length and intensity **[25].** Laser therapy reduced the inflammatory reaction; induce increased collage

deposition and greater proliferation of myofibroblasts in experimental cutaneous wounds**[26]**.

The aims of our paper were to study the effects of low-intensity laser irradiation (LIL) on the wound healing of male Wistar rats with STZ-induced diabetes. With more detailed, we tried to gain better insight into the healing process by using of low level laser therapy.

2. Material and methods

2.1. Animals:-

A 40 male albino rats, each weighed 200-225 gm. They were housed in special cages (5-rats/cage) and maintained under controlled environmental conditions (12-hours light/dark cycle, temperature 27°C), and provided with standard laboratory food and water *ad libitum*.

2.2. Induction of Diabetes

Diabetes was chemically induced using streptozotocin (Sigma Co., USA), 40 mg/kg, dissolved in citrate buffer (pH 4.3) and administered as single dose tail vein injection of rats.

2.3. Blood Glucose Level

Before surgery and 7 days after wounding, the blood glucose level of each rat in both treated and non-treated group was checked. Seven days after streptozotocin injection, blood glucose levels were measured by using a glucometer and test strips.

2.4. Wound Surgery

Before surgery, the blood glucose level of each rat was checked again. Each rat was anesthetized with 10% chloral hydras (300 mg/kg) intra-peritoneal. The hair on the dorsum of all rats was shaved using manual clipper. The operative site was prepared aseptically. Surgical incision was made by using sharp sterile scalpel to produce deep wound on the dorsum of each rat about 2 cm lengths and 3 mm depth.

2.5. Groups of Experiment and Treatment Parameters

This work was designed to evaluate the wound healing in both treated and non-treated animal model. Therefore the animals were classified into two main groups each one consisted of 20 rats.

The first group consisted of 20 rats considered as control diabetic group with dorsum wound without treatment. The second group was consisted of 20 rats all of them diabetic with dorsum wound and treated by exposure to 100mW for 240 seconds in defocused mode one inch away from laser aperture for three doses with three days interval time using **Diode 808nm laser(Luis Grees, Australia, and Laser Medics)**. Five rats from each group were sacrificed at 3, 5, 7 and 10 days the end of the experiment.

2.6. Pathological examination:-

Gross examination of dorsum induced wound of each rat in both treated and non-treated group was performed to evaluate the degree of inflammatory reaction and scar tissue formation.

Skin tissue samples were taken from the surgical induced wound of all experimental animals at 3, 5, 7 and 10 days of both groups. The samples were trimmed, washed and dehydrated in ascending grades of alcohol, cleaned in xylene, embedded in paraffin then sectioned at (4-6 micron) and stained with haematoxylin and eosin as well as Masson's trichrome stain for detection of collagen fibrous according to [27].

3. Results

3.1. Blood Glucose Level

All streptozotocin - injected rats, blood glucose level revealed (16.5 mmol/L) or more were seen in all rats a long of experimental period.

3.2. Pathological findings:

Five animals of each diabetic non-treated and treated group were sacrificed after **3 days** of surgical incision. The area of incision in non-treated group covered with grayish white layer of necrotic tissues mixed with creamy yellow exudates as well as widening of wound lips [plate (1-a)]. On the other side, the treated group with one dose of Diode 808nm laser revealed congestion of both lips of the wound with narrow gap and minimal necrotic tissue [plate (1-b)].

Microscopically, the epidermal layer at the site of incision showed necrotic tissues mixed with desquamated epithelial cells and exposure of sub-epithelial connective tissues. The dermal layer revealed severe inflammatory reaction characterized by leukocytic infiltration mainly neutrophils and macrophages [plate (1-c)]. These findings were seen in the non-treated. The animals of treated group showed highly vascular granulation tissue fill the dermal gape with mild degree of inflammatory reaction [plate (A-d)].

Five animals from treated and non-treated group were sacrificed after **five days** of surgical incision. The wound surrounded by swollen and hyperemic zone. Thick, viscous yellow exudates oozed from the wound. The gross findings in this group were relatively the same after 3 days of surgical incision. Moreover, the wound of treated group showed raised edges and gap between the two ends contained soft and granular bright red granulation tissues, oozing clear watery exudates under pressure. Some tissue debris was also detected at wound edges.

Microscopically, each lip of the non-treated wound showed necrosis of epidermal layer. The dermal layer revealed ill-developed granulation tissue

infiltrated with inflammatory cells mainly macrophages and plasma cells, also some cases showed micro-abscess [plate (1-e)]. On the other hand, the base of wound of treated group contained well developed granulation tissue which consisted of fibroblasts, collagen fibers and newly formed capillaries [plate (1-f)].

Tissue section stained with Masson's trichrome showed complete separation of epidermal layer and ill developed granulation tissue formation at the base of wound characterized by present of weak blood capillaries appeared in form of interstitial hemorrhages. The wound of treated group showed mature collagen fibers and well developed capillaries occupying most of the wound gap most of the wound gap.

Five animals were sacrificed from treated and non-treated group after seven days of skin incision. Wound gap was filled with gelatinous granulation tissue mixed with blood and thick yellow exudates in the non-treated group. The wound surface appeared smooth with minimal elevation of its edges from surrounding area as well as disappearance of wound gap after treatment of the second dose of diode 808nm laser.

Microscopically, the dermis and subcutaneous tissues were repaired by granulation tissue. Angiogenesis was observed at the wound margins which producing newly formed capillaries some of them lacking of basement membrane were also seen. Long, spindle-shaped fibroblasts proliferated and migrated into the wound. The collagen fibers lied across the incision line to unite the cut edges of the wound. On the other side the severity of inflammatory reaction remain with the same degree and characterized by massive infiltration with neutrophils. The epidermal layer of treated group showed re- epithelization with non-keratinized stratified squamous epithelial cells. The dermal layer was repaired by mature granulation tissue.

Tissue section stained with Masson's trichrome from non-treated group revealed congestion of dermal blood capillaries with accumulation of clotted blood [plate (2-a)]. On the other side, treated group showed newly formed blood capillaries with prominent collagen fibers [plate (2-b)].

The last five animals of both treated and non-treated group were sacrificed after **ten days** of surgical incision. The wound surface appeared smooth, hairless with grayish white crusts. The edges of the wound were still elevated edges from surrounding skin with prominent scar tissue formation in the non-treated animals [plate (2-c)]. In treated group, the surface of wound appeared smooth and resembles the surrounding area. Hair growth was more rapid in comparison with non- laser managed group [plate (2-d)].

Microscopically, the granulation tissue appeared a vascular and shrinkage. The dermal layer showed dense fibrous tissue at the site of incision consisted of collagen fibers and few numbers of fibroblasts connected with basal epithelial layer. Re-epithelization of epidermal cell layer with clear intact basement membrane was noticed. The epithelial surface consisted of non-keratinized stratified squamous epithelial cells [plate (2-e)]. The epidermal layer of treated group revealed well-developed stratified keratinized squamous epithelial cells. The dermal layer showed small area of shrinkaged collagen fibers with few numbers of fibroblasts. The epidermal and dermal layers showed complete healing with minimal scar tissue formation in comparison with previous group [plate (2-e)].

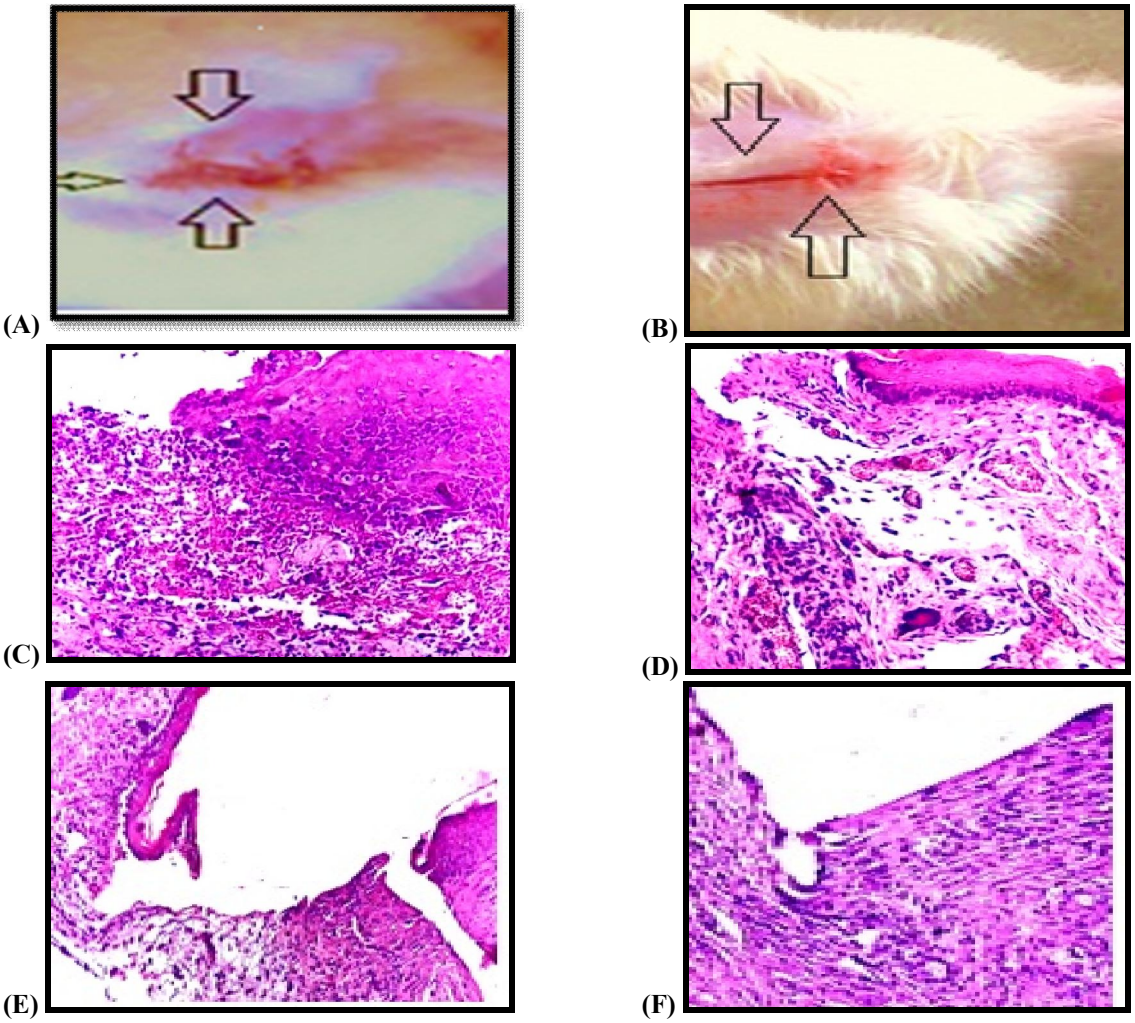

Plate: (1): Fig. (a) Skin wound showing severe inflammatory reaction of dermal layer (3days) (H&Ex100). (Non-treated group)
Fig.(b) Rat showing swollen and hyperemic wound (3days after incision). (Treated Group)
Fig.(c) Rat showing shrinkage of the wound lips with granulation tissue formation (3days after incision with laser therapy).
Fig.(d) Skin wound showing highly vascular granulation tissue (3 days) (H&E x100)
Fig.(e) Skin wound showing ill developed granulation tissue with numerous number of neutrophils (5days) (H&Ex100).
Fig.(f) Skin wound showing well developed granulation tissue (5days) (H&E x100)

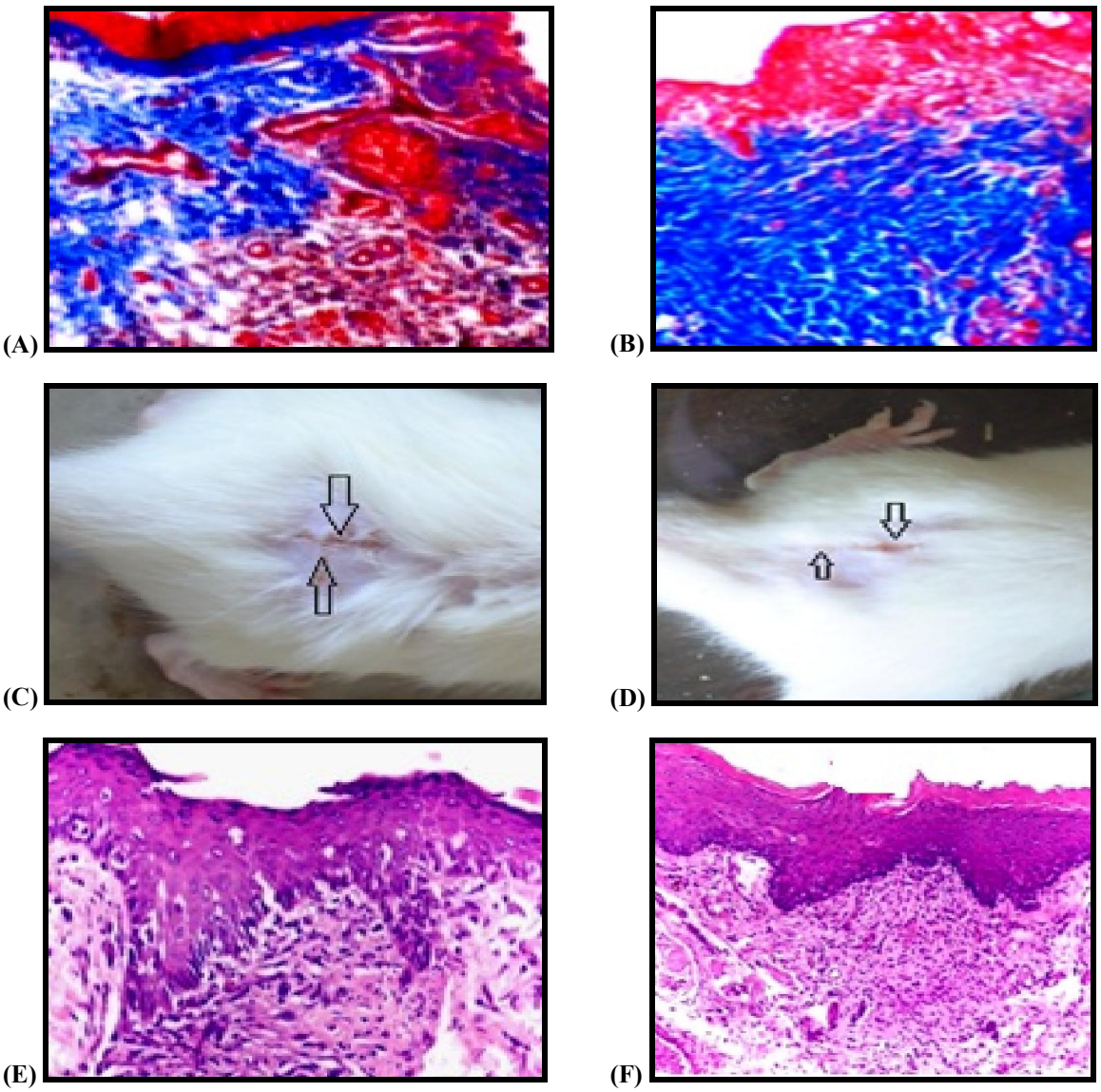

Fig. (a) Skin wound showing clotted blood, necrosed tissues (Masson's trichrome x100)
Fig.(b) Skin wound showing mature granulation tissue with prominent collagen (Masson's trichrome x100)
Fig.(c) Rat showing healed area with prominent scar tissue (10days after incision).
Fig.(d) Rat showing healed area with minimal scar tissue formation (10days with laser therapy).
Fig,(e) Skin wound showing regeneration of keratinocytes with prominent scar tissue (10 days) (H&Ex100).
Fig.(f) Skin wound showing complete regeneration with minimal scar tissue (10days) (H&E x100)

4. Discussion

Diabetes is a multisystem disorder that affects the wound healing process. Pathological changes in tissues and cells may delay healing in the diabetic patient. Therefore, the present study showed effects of diode 808nmlaseron tissue repair process, particularly regarding its influence on the modulation of certain type of cells in wound healing. None healing, chronic wounds or delayed wounds are a challenge to both health care professionals and systems.

Several factors affect wound healing negatively, as infection and devitalized tissue impairs healing, hypoxia from arterial occlusive diseases. Systemic diseases as diabetes mellitus, malnutrition and steroid therapy interfered with hydroxylation reaction in collagen synthesis and lead to delayed wound repair**[6]**.

Low level laser therapy emits energy densities that are too low to cause temperature increase beyond 0.5 ºc in the target tissue .Thus the effect on tissue is not attributed to thermal effect. Low level laser therapy affects cellular metabolism, extra cellular matrix, tissue repair and immune functions of the cells**[28]**.

The wound-healing process consists of four highly integrated and overlapping phases: inflammation, proliferation, and scar tissue remodeling. It is widely accepted that chronic healing of diabetes wounds always accompanied with prolonged inflammation and decreased matrix accumulation **[13].**

Low-level laser therapy has thermodynamic effect on biological tissues and produces beneficial clinical effects in the treatment of soft tissue injuries. Low level laser therapy delivers light energy, penetrating the layers of skin and reaching targeted internal tissues to produce a specific, non-thermal effect on tissues. **[2].**

In the present study we tried to evaluate the effect of diode laser 808nm on the tissue healing process in diabetic rats. Gross examination of the wounds in the laser group on the day 3 showed only congestion in comparison to the control group which showed clotted blood, necrotized tissue with yellowish oozing exudates which points to the anti-inflammatory action of laser therapy. These findings coincided with that reported by **[9]**. This was also confirmed by the histopathological examination in the same stage which showed moderate inflammatory reaction in the laser group while the control group showed severe inflammatory response.

The second group that was sacrificed on day 5 showed grossly soft granular red tissue with oozing of clear exudates only under pressure in comparison to swollen tissue with hyperemic zone of inflammation in control group. This was explained by the histopathological examination which revealed the start of granulation tissue formation which consisted of fibroblasts, collagen fibers and newly formed capillaries. The control group which still showed inflammatory reaction; points out that there was delay in the wound healing in the diabetic untreated control group. On the other hand the LLLT treated group showed starting of granulation tissue formation and returning to the normal sequence of events in the healing process, with abolishing of the effects of steroids on tissues. These findings agreed with that reported by Basford. **[28].**

The results at this stage also confirm the biomodulatory effect of LLLT which was proved in different animal studies, as there was decreased edema, pointing to increased lymphatic flow**.** These finding agreed with Lyons *et al.* **[29].** The granulation tissue formation was enhanced by stimulation of fibroblasts leading to new collagen formation and also stimulation of angiogenesis as demonstrated by new capillary formation at this early stage, incomparison to the control group. These findings were come in parallel with that recorded by Halcin and Uitto **[30].**

In the third collection on the day 7 the laser group appeared with smooth lesion, and minimal elevations with disappearance of the wound gap versus thick hyperemic wound about to start granulation tissue in the control group .This denotes more rapid healing of wound in the laser group with the naked eye .

At this stage, histopathological study found that attenuated inflammation, greater re-epithelization, mature granulation tissue (fibroblasts), and extensive collagen deposition observed in diode laser 808nm treated groups at 7 days after wounding. At the same time, the control group had more inflammatory exudates and immature granulation tissue. These findings was agreed with some previous published studies [**29,30**] that established the Low level laser therapy (LLLT) are able to promote wound healing by reducing inflammation without compromising the proliferation of fibroblasts and keratinocytes. It also played a vital role in regulating the synthesis and deposition of various extra-cellular components, during re-epithelization, and promoting the migration of fibroblasts and collagenase production **[10].**

The last group after 10 days showed almost apparently normal tissue resembling the surrounding tissue while the control group showed a hyperemic wound still demarcated from the surrounding. Microscopically LLLT group showed epidermal and dermal complete healing with minimal fibrosis, while the control group showed vascular granulation tissue

with dense fibrous tissue at the site of incision. This also points to the biomodulatory effect of LLLT leading to nearly normal tissue. These findings were come in parallel with that recorded by Lyons *et al*.**[29].**

According to the obtained results it can be deduce that diode laser 808nm LLLT enhance wound healing process as a result of stimulation of fibroblasts and inflammatory cells as macrophages with their mentioned role in healing process and tissue repair even in immunosuppressed animal model. Also the rapid healing and decreased inflammation lead to decreased incidence of protrusion ofscar tissue.

The study of fetal wound healing is intriguing and may result in the discovery of an optimal method that would allow wounds to heal without scar formation **[31]**. Wounds occurring in fetuses of early gestational age can heal without any scar. Scarless healing in fetal wounds is resulted from fewer neutrophils and more monocytes during the inflammatory period in contrast, to adult wounds. These findings are similar to the effect of low level laser therapy on the adult wound healing **[32]**. Which can explain scarless healing observed in the laser group at the final stage of healing.

Conclusions:

LLLT using diode laser 808nm accelerated wound healing even in diabetic animal model with minimal scar tissue formation. LLLT leads to acceleration in resolution of the acute inflammatory stage of wound healing and the earlier begging of the proliferative phase with more organized granulation tissue which leads to nearly normal tissue healing.

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