# fizjoterapia polska

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**A congenital malformation syndrome – situs inversus, esophageal atresia**

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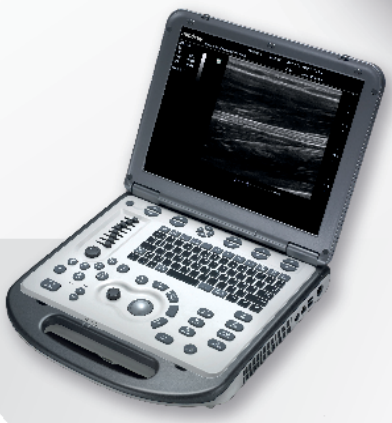


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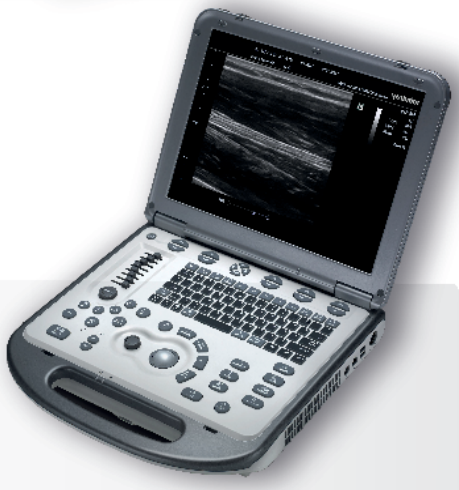


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# Immunohistochemical study of LED light photobiomodulation using two different wavelengths on surgical wound healing in mice

*Badanie immunohistochemiczne dotyczące fotobiomodulacji przy użyciu światła LED o dwóch różnych długościach fal w leczeniu ran chirurgicznych u myszy*

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## Abstract

Background. Impaired wound healing is a disastrous medical problem associated with chronic diseases and aging. To accelerate skin regeneration many techniques have been researched laser and LED has been used for these purposes. Topical phenytoin is simple to use, safe, inexpensive and readily available drug and plays a significant role in the rate of healing of wounds. Objective. This study aimed to study the effects of irradiation with light emitting diode (LED) 670 nm in comparison to irradiation with LED 830nm for wound healing. Materials and Methods. three groups 24 mice per group received treatment as follows. Group I: Wounds followed up without any treatment modality. Group II: wounds irradiated with red LED 670 nm at fluence of 5 J/cm$^2$. Group III: wounds treated by infrared LED 830 nm at fluence of 5 J/cm$^2$. All treatments started from the 1st day postoperatively and up to three consecutive weeks or till complete healing of ulcer. Results. The results showed the fastest healing in the infrared group with more deposition of collagen fibers, larger amounts of granulation tissue, less edema. The second best treatment was the red LED 670-nm the non-treated group as mice showed less evident features with less collagen fibers deposition. Conclusions. These data suggest that infrared LED 830nm combined is an effective enhancer of wound healing that stimulates the secretion of growth factors in the wound bed induce several modifications during the cutaneous healing process, especially in favoring newly-formed collagen fibers to be better organized and compacted disposed. and led more deposition of collagen fibers.

**Key words:**

healing, bio modulation, LLLT

## Streszczenie

Informacje wprowadzające. Ograniczone gojenie się ran jest trudnym problemem medycznym związanym z chorobami przewlekłymi i starzeniem się. Aby przyspieszyć regenerację skóry, zbadano wiele technik laserowych, wykorzystujących światło LED. Fenytoina do stosowania miejscowego jest prostym w użyciu, bezpiecznym, niedrogim i łatwo dostępnym lekiem, który odgrywa znaczącą rolę w szybkości gojenia się ran. Cel. Niniejsze badanie miało na celu zbadanie wpływu napromieniania diodą elektroluminescencyjną (LED) 670 nm w porównaniu z naświetlaniem diodą LED 830 nm na gojenie się ran. Materiały i metody. Trzy grupy po 24 myszy były poddawane następującemu leczeniu. Grupa I: Rany monitorowane bez żadnego leczenia. Grupa II: rany naświetlane czerwoną diodą LED 670 nm o strumieniu światła 5 J/cm$^2$. Grupa III: rany naświetlane podczerwoną diodą LED 830 nm o strumieniu światła 5 J/cm$^2$. Realizację zabiegów rozpoczęto od 1. dnia po operacji do trzech kolejnych tygodni lub do całkowitego wygojenia ran. Wyniki. Wyniki wykazały najszybsze gojenie w grupie naświetlanej podczerwienią, w której zaobserwowano również większe odkładanie włókien kolagenowych, większą ilością tkanki ziarninowej, mniejszy obrzęk. Drugą najlepszą metodą leczenia było stosowanie czerwonej diody LED 670 nm. W grupie nieleczonej myszy wykazywały mniej widoczne cechy z mniejszym odkładaniem włókien kolagenowych. Wnioski. Dane sugerują, że dioda LED na podczerwień 830 nm w połączeniu jest skutecznym środkiem wspomagającym gojenie ran, który stymuluje wydzielanie czynników wzrostu w ranie, wywołuje szereg modyfikacji podczas procesu gojenia skóry, szczególnie sprzyjając lepszemu uporządkowaniu i zagęszczeniu nowo utworzonych włókien kolagenowych i doprowadza do większego odkładania włókien kolagenowych.

**Słowa kluczowe**

gojenie, biomodulacja, LLLT

## Introduction

Acute wounds are an important source of morbidity and mortality worldwide, traumatic injuries and surgical procedures results in a challenging wound and their complications [1]. Advances in wound healing research will improve outcomes, particularly in the areas of wound infection, wound pain, acute wound failure and excessive scar formation [2]. Healing is a complex process that involves a series of events, including clotting, inflammation, granulation tissue formation, epithelialization, collagen synthesis, and tissue remodeling. Thus, it has been researched extensively, particularly, regarding factors that could delay or hinder the healing process [3].

The effects of laser therapy on wound healing have been extensively studied, and conflicting results have been reported. Many reports demonstrated a positive influence of several types of lasers on healing, and others have found no effect at all [4]. This is a consequence of the different protocols used and of the settings of the laser therapy [5]. Laser therapy is characterized by its ability to induce photo biological processes without significant thermal effect [6], by pain relief, as an anti-inflammatory and as an anti-edematous agent [7]. Studies using animals have been conducted to evaluate the effect of various lasers on wound healing, treatment with a 632-nm He-Ne laser at a fluence of 5 J/cm$^2$ enhanced the percentage of wound closure over time in mice [8]. Wound tensile strength increased in mice by daily irradiation with an 830-nm diode laser on of wounds. with a fluence of 5 J/cm2 [9]. based on biomechanical and biochemical findings, laser treatment enhanced the tissue repair process by accelerating collagen production and promoting connective-tissue stability in mices wounds after laser treatment with a 632.8-nm helium-neon laser at a fluence of 1 J/cm$^2$ [10].

A preliminary clinical study to evaluate the effect of a 980-nm GaAlAs diode laser applied once every 2 weeks to healing wounds on the foot or lower legs. Both diabetic and nondiabetic patients were enrolled in the study. At each visit for laser treatment, the wounds were cleaned, debrided with a scalpel to remove hyperkeratotic tissue, and then treated with the laser set at a power of 5 W. Nineteen wounds from 16 patients were included in the study. Seven of the 19 wounds, or 36.8%, were completely healed during the course of laser treatment. This included 6 of the 12 diabetic wounds, or 50%. The average time until complete wound closure was 8.3 weeks [11].

This form low level laser therapy (LLLT) is currently being used to treat various conditions based on the principles of photobiomodulation, that influences a variety of biological processes, including the acceleration of wound healing. It is important to consider that there is an optimal dose of light for any particular application, and doses higher or lower than this optimal value may have no therapeutic effect [12]. Wound healing effects are generally observed at fluences between 1 and 10 J/cm2, while photo inhibitory effects are typically observed at higher fluences [13].

Photobiomodulation may induce a decisive impact on the course of biological events that take place during wound healing, as it enhances collagen synthesis in the wound area with gradual fibroblastic proliferation and the amount of collagen being synthesized can be particularly affected during tissue regeneration [14]. The basic biological mechanism behind the effects of LLLT is thought to be through the absorption of red and NIR light by chromophores, in particular cytochrome c oxidase (CCO), which is unit in the respiratory chain located within the mitochondria [15]. The increased mitochondrial respirateion and adenosine triphosphate (ATP) synthesis, leading to the enhancement of enzyme activity, increased electron transport, oxygen consumption, [16]. It is assumed that this absorption of light energy may cause photo dissociation of the inhibitory nitric oxide, as well as alter the mitochondrial or cellular redox state, inducing the activation of numerous intracellular signaling pathways and altering the affinity of transcription factors concerned with cellular migration, proliferation, survival, tissue repair, and regeneration [17-19].

Many light based systems had effects on wound healing; these outcomes were noted irrespective of whether a laser, light emitting diode (LED) or broadband polarized light source used as a method of wound healing [20]. The use of LED devices promises huge savings in energy consumption, and based on the use of visible light to cause photo stimulation of cellular chromophores and subsequent biologic effects including proliferation, cellular migration speed, and myofibroblast differentiation [21]. Several commercially available systems have now appeared based on arrays of LEDs, designed to use the potential efficacy of LEDs heads emitting at near infrared (830 nm) and visible red (633 nm) in accelerating wound healing and controlling side effects such as pain, exudation, crusting, oedema and erythema [22]. When lasers come into contact with cells, they act on the mitochondria to increase adenosine triphosphate (ATP) production. In turn, that increased ATP production can lead to faster production of collagen, vascular structures, DNA, RNA and other materials essential to the healing process. In contrast, LEDs emit incoherent light in a broader range of wavelengths. Their power output is significantly lower than that of lasers, which tends to make them less invasive. Still, they exert the same end effect on ATP production and healing as lasers [23]. Likewise, LEDs have clear benefits for wound care, burns and skin conditions, and the treatment of muscle damage and inflammation. For therapists who work with active populations, LEDs can even enhance muscles' contractile ability and post-exercise recovery – a significant performance benefit for hard-training athletes [24].

The purpose of this study was to evaluate the effect of a diode LED 670 nm in comparison to irradiation with LED 830 nm on healing of wounds, using a controlled animal model. Specifically, the study examined the imunohistochmechal changes that occurred during wound healing.

## Materials and Methods

### Animals

After the Research Ethics Committee of Cairo University granted a total of 75 mice between 6 and 9 weeks of age and weighing 18–22 g, were used in this study. The animals were housed one per cage (to prevent cage-mate attacks on wounds) and had access to food and water ad-libitum. The mice were maintained on a 12-hour light/dark cycle.

**Wound model**

All surgical procedures were carried out by the same operator under aseptic conditions, mice were anesthetized by an intraperitoneal injection of a ketamine–xylazine cocktail (90 mg/kg ketamine and 10 mg/kg xylazine) before wounding the dorsum of the anesthetized mice were shaved using an electric fur clipper. The anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil, to ensure same wound size in all the mice. A full thickness excision wound diameter 12 mm, the wound was left uncovered during the whole period of experiments. To prevent post-surgical infection, antibiotics were administered until 5 days following surgery

**Light source**

The Phototherapy unit used in the study was the LED-based phototherapy system (made by the laser technology center, NILES, Cairo University) which consists of a base unit with two handles one with LED emitting at 670 nm in the visible red whereas The other handle with LED emitting at 830 nm in the infrared spectrum.

The photo irradiation performed daily for three weeks, starting after 6 hours postoperatively and for up to three consecutive weeks or till complete healing of ulcer as denoted by its complete closure. The LED for both handles applied in continuous mode and above the wound surface. The output power of LED was 40 mW with fluence 5 J/cm$^2$ and fluence rate (12 mW/cm$^2$). Each wound area measured approximately 12×12 mm hence the irradiation Time was (6.6 min).

**Experimental design**

Mice were randomly divided into three groups 25 mice per group. The groups were divided into three groups that receive treatment in form of LED as follows. Group I (Control Group): Wounds was followed up without any treatment modality. Group II LED (670 nm): Wounds was subjected to LED (red spectrum) Group III (LED (830nm): Wounds was subjected LED (infrared spectrum) at the same conditions and parameters.

**Methods of Assessments**

***Wound area measurement***

The two maximum perpendicular diameters of each wound were measured with Vernier's digital caliber on the day of wounding and at days (0–7–14–21) The measurements were determined primarily by the same person individual (a veterinary assistant) throughout the course of the experiment to reduce variability in data collection. The relative change in the surface area of wounds with respect to the initial surface area was determined.

Moreover, the healing process was observed and recorded till the timings of complete closure of wounds

Wounds were photographed using a high definition digital camera images were obtained from the first day then subsequent images were captured till complete closure of the wound.

***Histological Evaluations***

*Immunohistochemically Analysis*

Animals were randomly sacrificed from all groups at each evaluation time by using an intraperitoneal overdose of ketamine at (0–7–14–21) day's postoperatively. Standardized rectangular specimens were harvested across the wound using a double-blade cutting instrument then preserved in 10% formalin and then embedded in paraffin. Serial tissue sections of 4-μm thickness were prepared, stained with hematoxylin and eosin (H&E), and observed for histological changes under a light microscope to assess epithelialization, epidermis thickness, inflammatory cell infiltration, and blood vessel proliferation.

Staining with Methyl trichrom on paraffin sections done to determine the amount of collagen at the wound the most representative findings was documented by photomicrography

Basic fibroblast growth factor vascular endothelial growth factor (VEGF) and tumor necrosis factor was investigated the Glass slides were pretreated with organosilane adhesive (3-aminopropyltriethoxysilane, SIGMA, St. Louis, Missouri). All the antibodies used in the study were standardized (Anti-CD31/1:1000, SC-1506, Santa Cruz, Biotech and Anti- VEGF/ 1:50, Clone VG1, Neomarkers). Peroxidase was used as the immunostaining method. Control sections in which the primary antibody was either omitted or replaced by normal mice serum was used as negative controls. For positive controls, a section of the mice granulation tissue was used.

The histological sections were de-waxed in xylol and rehydrated with absolute alcohol and then with water at room temperature. Antigen retrieval was performed using a 10 mm Citratee Buffer, pH 6.0 in a 96°C water bath, and blocking of the endogenous peroxidase using the Dako Dual Enzyme Block (Dako, Carpinteria, California). Removal of non-specific binding was performed with Protein Block Serum Free (Dako), and thus, the cuts were incubated at 4°C with the primary antibodies in a wet chamber overnight. The secondary antibody was Polymer Dako Envision Peroxidase (Dako), incubated at room temperature for 30 minutes.

A semi-quantitative graduation standard was used to indicate the intensity of the immunohistochemically reaction, in which 0 described absence of reactivity, + (moderate), and strong.

**Statistical Analysis**

Data was presented as percentage. One–way analysis of variance (ANOVA) test was used to compare changes of wound area among three groups. Values of $P \leq 0.05$ was considered statistically significant.

## Results

**Results of changes of Wound area from all study groups**

In this study, the effect of red LED wavelength at 670 nm compared with near infrared LED wavelength both were delivered at a constant fluence (5 J/cm$^2$) and fluence rate (12 mW/cm$^2$) showed that showed that wound areas decreased in all groups compared to the initial wound area measured after surgery.

Compared with mice in the control group, mice in the groups treated with the LED light showed a statistically significant accelerated wound healing ratios of wounds with $P < 0.001$; and mice treated with near infra-red LED light at 830 nm showed a statistically significant accelerated wound healing ratios of wounds $P < 0.05$; compared to wounds treated with red LED light 670 nm. No significant difference could be detected re-

garding the degree of granulation between all groups. Wounds treated with different wavelengths exhibited wound contraction from day 1 until observation of day 21 after wounding. However, non-treated control wounds showed no contraction (fig 1, table 1).

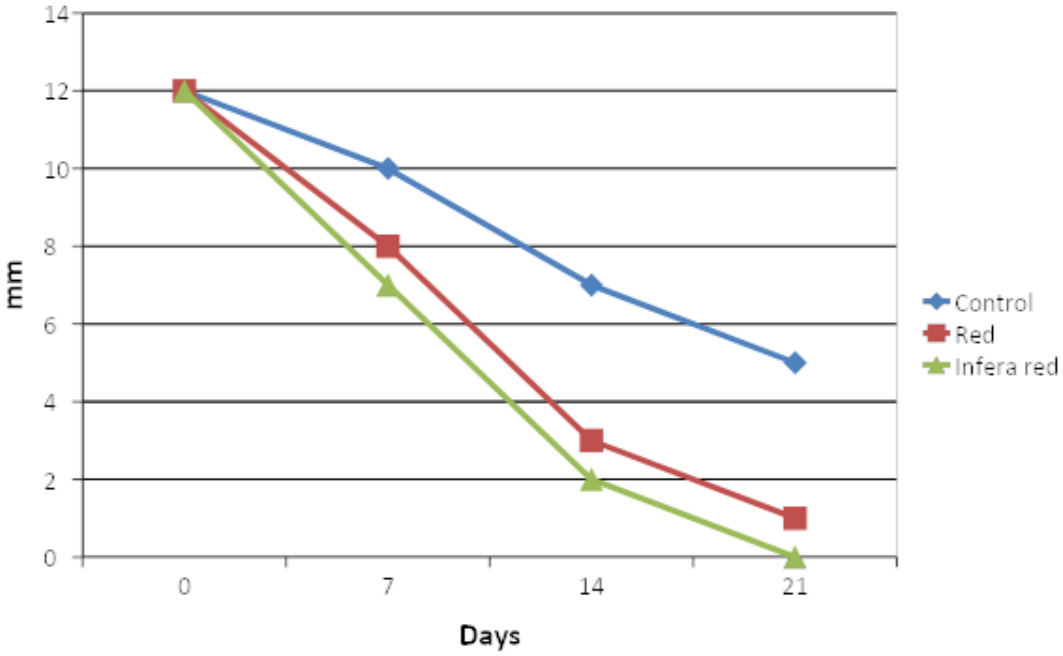


**Figure 1. Wound area changes in the three groups**

**Table 1. Morphological changes in wounds in the three groups**

| Group | Day 0 | Day 7 | Day 14 | Day 21 |
|---|---|---|---|---|
| Control | 12 | 10 | 7 | 5 |
| Red | 12 | 8 | 3 | 1 |
| Infera red | 12 | 7 | 2 | 0 |

**Histological Evaluations**

Multiple cross-sections of H&E-stained sections of wound tissues obtained from control group and treated groups showed necrosis associated with oedema and inflammatory infiltrate at day 7 showed necrosis associated with oedema and massive dermal inflammatory infiltrate, at day 14 ill developed granulation tissue appeared, at 21 days' granulation tissue formation with inflammatory cells infiltration and congested newly formed blood capillaries were noticed. But less necrosis, oedema and inflammatory cells infiltration and no haemorrhage is seen with the group treated with red LED wavelength at 670 and at day 21 showed epithelization granulation tissue formation collagen fibers. infra-red LED light at 830 nm showed less dermal inflammatory infiltrate and congested dermal blood vessel at day 21 it showed epithelization and granulation tissue formation.

***Results of immune histochemical study of the Inflammatory Mediators***

*Tumor necrosis factor TNF*

Immunohistochemically staining of TNF in skin of control group showing strong positive expression of TNF in skin of mice not treated whereas the group treated with red LED light at 670 nm showing moderate positive expression of TNF in the mice skin. The group treated with infrared LED wavelength at 830 nm showing no expression of TNF (negative immunoreactions for TNF) (Fig 2).

*Vascular endothelial growth factor (VEGF)*

Immunohistochemically staining of VEGF in skin of control mice showing weak positive expression of VEGF Whereas the skin of mice treated with red LED wavelength at 670 showing moderate positive expression of VEGF. The skin of mice treated with infra-red LED light at 830 nm showing strong positive expression of VEGF (fig 3).

*Transforming growth factor (TGF)*

Immunohistochemically staining of TGF in skin of mice from control group showing no expression of TGF. In skin of mice treated with red LED wavelength at 670 showing positive expression of TGF. In skin of mice treated with infra-red LED light at 830 nm showing strong positive expression of TGF (fig 4).

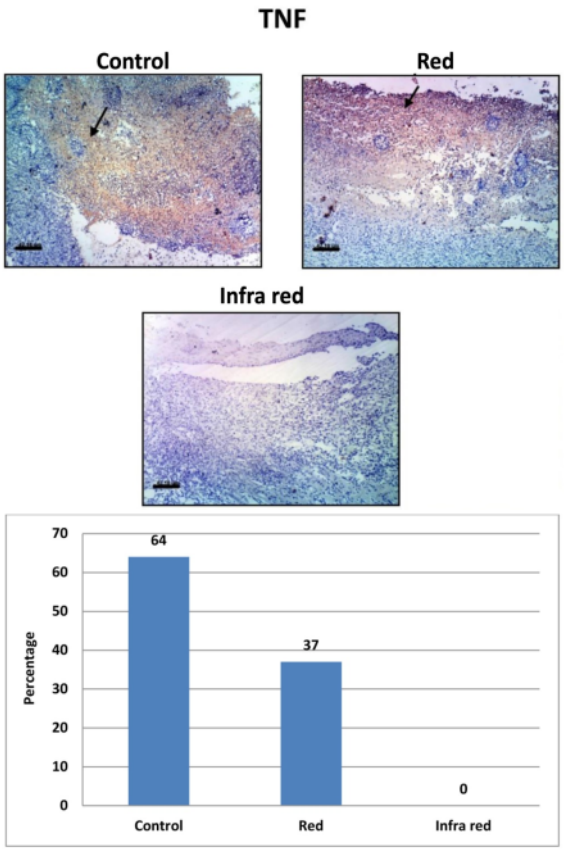


Figure 2. Immunohistochemically staining of TNF in skin at three groups at 14 days

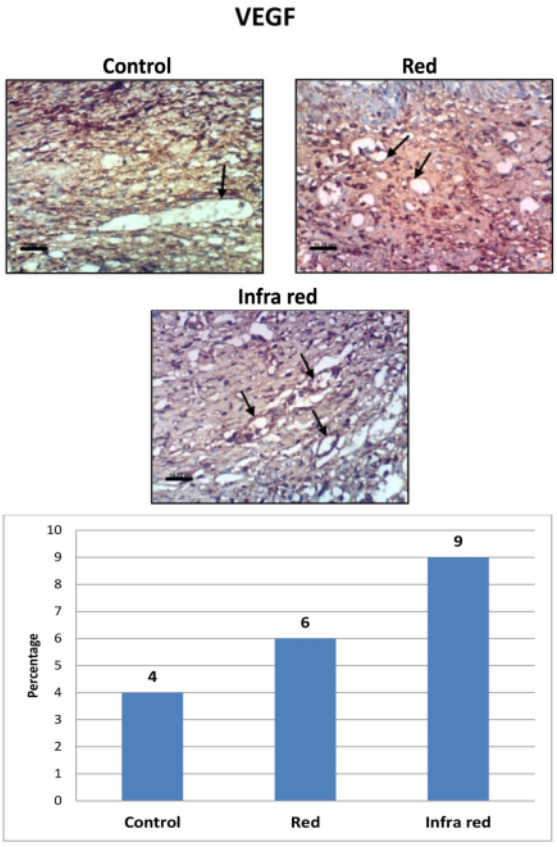


Figure 3. Immunohistochemically staining of VEGF in skin at three groups at 14 days

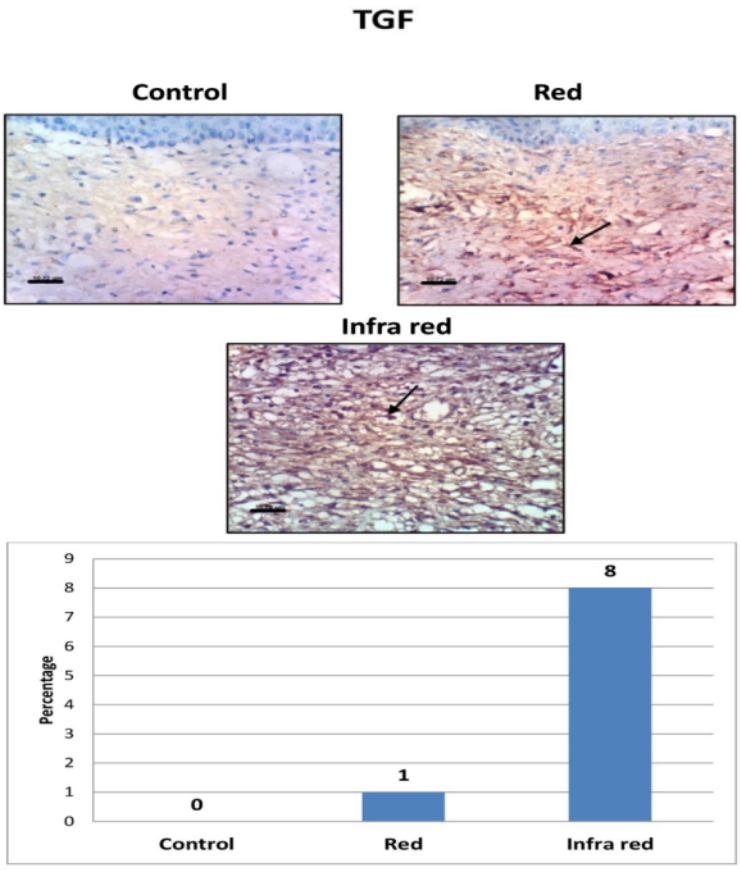


Figure 4. Immunohistochemically staining of TGF in skin at three groups at 14 days

## Discussion

Photo-biomodulation has been used for a variety of medical therapies such as wound healing through irradiation at low energy levels that can generate significant bio-effects which are manifested in biochemical, physiological, and proliferative phenomena in various enzymes, cells, and tissues [25]. The beneficial effects of coherent light such as LASER and non-coherent LED light for healing wounds in experimental animals and clinically are already documented [26]. Light emitting diodes LED discovered in the early 1900s as a bright Light sources that are fast reacting with Less Maintenance required, LEDs had high Lifetime, large spot size displays can be made Laser light has been shown to have similar effects on cells and tissues, but lasers are heavy, costlier and do not offer the the large spot size that is needed for large ulcer and wounds [27].

The specially designed near-infrared LED has a wavelength than laser light that penetrates deeper without damaging the skin and it is three times brighter than laser, hence the LED is very safe and easy to use, as well as portable. For wound healing, the LED is housed in a 3.5" by 4.5" flat array from which it emits up to three wavelengths to affect various cell. [28].

LEDs stimulate cytochromes in the body that increase the energy metabolism of cells. Cytochromes are part of the "electron transport chain" that converts sugar into instant energy required by the body to perform all of its actions, such as healing a wound. [29].

The bottom of the water absorption curve is between 820 nm and 840 nm, giving 830 nm the deepest penetration of any wavelength in living human tissue. The deep penetration of both the 670-nm and 830-nm wavelengths, as well as their absorption characteristics, should ensure a clinically useful photon density in fibroblasts and other skin cells in the papillary and upper- and mid-reticular layers of the dermis. the clinical results following 670-nm/830-nm LED irradiation shows both the profilometric results and electron microscopic findings that confirm the benefits of this LED treatment [30].

Several studies have emphasized the anti-inflammatory properties of LLLT and shows a reduction in prostaglandin-E2 and the pro-inflammatory cytokine, TNFa also showed a reduced inflammatory cell migration [31].

This study showed that wounded mice responded in a dose and, more significantly, a wavelength dependent manner to LED light so at 830 nm and a fluence of 5 J/cm2 cells were protected from apoptosis; there was complete wound closure more than the group exposed to LED light at 670 nm showed incomplete wound closure and increased apoptosis [32].

It was interesting that the improvement in wound closure following LED light irradiation was apparently dependent of the wavelength used. However, there was a trend for a more pronounced increase in growth rate when the infrared [830 nm] was used [33].

Cells have adapted and responded to the LED light, hence cellular repair mechanisms to 670 nm wavelength showed the least amount of wound closure and cellular proliferation [VEGF], at a fluence of 5J/cm2 used in this study showed that not only is it important to choose the correct fluence, but it is also important in choosing the correct wavelength. Although studies have shown 670 nm to be simulative in phototherapy [34]., this study found the opposite.

Our results showed that the secreted VEGF and TGF and their productions would be significantly enhanced after LED irradiation for both 830 nm and 670nm at the energy density of 5 J/cm2. These results are consistent with previous reports in which growth factors secretion from various cell types was enhanced by LED irradiation [35]. A more direct action by reduction of TNF-a level seems to be a more plausible explanation for the beneficial LED irradiation effect demonstrated in the present analysis. In any case, it is probable that LED irradiation has an ability to increase the amount of cAMP when the cells were incubated with TNF-a and stimulated with isoproterenol. The LED irradiation was efficient in attenuating as cholinergic hyper reactivity either b-adrenergic hypo responsiveness of cells after TNF-a exposure. Considering the cellular signaling mechanism, this leads to exaggerated TNF-a reduction response, implying that the results should be interpreted with attention.

Moreover, higher concentrations of TGF demonstrated an ability to form more fibroblast LED irradiation also significantly increased fibroblast formation at both wavelengths used in this study.

The wound milieu is a dynamically active compilation of cells, biophysical, and biochemical factors. TGF-b plays a predominant role in a variety of cellular, immune, and matrix organizational functions [36]. Several interventional therapies for faster and better healing of wounds have been used in clinical practice to reduce morbidity and often mortality [37]. Previous studies demonstrate the ability of LED irradiation to activate and "prime" the latent TGF in vitro, providing one possible molecular mechanisms of its ability to modulate wound healing outcomes. [38]. In this study, Further, we tested this hypothesis in an in vivo rat wound-healing model, thus confirming this correlation and suggesting that the TGF-b pathway might be a central signal transduction pathway affected by LED irradiation. The immunostaining for TGF detects the total pool in the tissues analyzed including both the active and the latent forms. Therefore, the increase we observed in TGF staining in post LED irradiation in vivo is not as dramatic as those observed by routine histochemical staining [38]. Further, the activation process with LED irradiation show an all-or-none effect, suggesting the possibility to be elucidated whether such effects of LED irradiation are seen with other similar latent protein complexes, especially other growth factors [like TGF, VEGF] that have been shown to be activated by similar integrin-mediated mechanisms. There are also studies demonstrating the effects of LED irradiation lasers on subcellular organelles, [39].

## Conclusions

LED light 830 nm was considered more effective compared to LED irradiation 670 nm in terms of fibroblast proliferation and collagen fiber density. However, further randomized clinical studies are required to determine the effect of combination of these two modalities of therapy on wound healing in humans.

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