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Low-level laser therapy's effect on adult male rabbits' wound healing

Abbas Ali Hussein, Thaier Alwan Abid
University of AL-Qadisiyah, College of Veterinary Medicine, Department of Surgery and Obstetrics.
Corresponding Email: Abbas.Ali@qu.edu.iq
ORCID: https://orcid.org/0000-0003-1931-4213

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Abstract
The study was designed to investigate the effect of 820 nm laser therapies on skin wound healing in rabbits. Thirty male adult New-Zealand rabbits were utilized and randomly divided into two equal groups (n 15), G1 laser therapy and G2 control groups. Two parallel (right and left) full-thickness skin incisions 2 cm lengths were made on the dorsum of each rabbit at a distance of 3cm from the spine. Incisions were closed by simple interrupted sutures using 4/0 silk suture. The right incisions in G1 were irradiated with pulsed (GaAlAs) laser therapy (820 nm, 8 J/cm^2 energy density, and 50 mW power output, for 30 sec. once a day for 3 successive days). Incisions of G2 and the left incisions of G1 were left without treatment as a control. Skin wound specimens were taken from animals of both groups after 3, 7, and 14 days' post wounding for histopathological evaluation of the healing process. Histopathological examinations demonstrated that wound healing in G1 was promoted such as fast restoration of tissue continuity, improved collagen fiber production, and the reduction of excessive inflammation, while the control group did not. Thus LLLT found to promote the healing of incisional wounds in the present rabbit model, and laser treatment has been beneficial effects during the inflammatory, proliferation, and maturation phases of a wound healing. The 8 J/cm^2 energy density of (820 nm) LLLT promote the wound healing, through prevention of excessive inflammation, increased formation of collagen fibers, and recovery in continuity of tissue.
Keywords: Wound healing, Skin, LLLT, Rabbits

Introduction
Skin is the largest organ of the body, covers the whole body surface and serves as a barrier against various infections and physical injuries (1). It also protects the body from ultraviolet rays, regulates water and electrolyte balance, controls body temperature, and is essential for maintaining life. It also plays a key role in the immune system's control of the body (2; 3). When this barrier is disrupted due to any cause such as surgery, ulcers, burns, neoplasms or trauma, the functions of the skin are not adequately performed. Therefore it is vital to restore the integrity of the skin as soon as possible (4). Wound healing is dynamic processes involving the secretion of soluble mediators types, degradation and formation of the extracellular matrix and expansion of appropriate cell types, and follows a temporal organization scheme (5). It has traditionally been divided into distinct phases; these phases take place simultaneously, and can be categorized into separate steps: The inflammatory, proliferative and remodeling phases. A clot forms and inflammatory cells debride injured tissue during the inflammatory phase. Epithelialization, fibroplasias and angiogenesis occur during the proliferative phase, meanwhile, granulation tissue forms and the wound

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begins to contract. Finally, during the remodeling phase, collagen forms tight cross–links to other collagen and with protein molecules, increasing the tensile strength of the scar (6, 7)**.** Due to the complexity of the wound healing process, a number of factors, including local and systemic ones, might prevent appropriate wound healing. Some of these factors are under control, while others are beyond control. Local factors include the presence of foreign particles or microorganisms, ischemia, tissue maceration, callus formation, pressure, mechanical stress, wound hydration, and infection whereas systemic factors comprise malnutrition, age, vascular insufficiencies, vitamins, immune suppressive medication and underlying conditions such as diabetes mellitus (8, 9). Low level laser therapy (LLLT) also known as a cold laser, soft laser, low intensity laser, biostimulation and photobiostimulation is a form of phototherapy or light therapy that involves the application of low-power monochromatic and coherent light to injuries and lesions in order to stimulate wound healing (10). It has been show increased the speed, quality and tensile strength of tissue repair, resolve inflammation and provide pain relief (11). There are still several unknowns regarding the precise LLLT mode of action. The reaction of cells happens as a result of changes in photoacceptor molecules, also known as chromophores, and the effect is photochemical rather than thermal (molecules which are able to absorb photonic energy). Many photoreceptors exist, including porphyrins. Cytochrome c-oxidase, the final enzyme in the Krebs cycle, has been identified as the most significant receptors. The enzyme cytochrome c-oxidase produces ATP (12, 13). Yet, it is understood that during laser irradiation, cells absorb photonic energy that is built into chromophores, stimulating cellular metabolism in the process (14). Chromophore is able to transfer the absorbed energy to other molecules and thus cause chemical reactions in surrounding tissue. The acceptor molecules' kinetic energy is increased, thereby activating or deactivating enzymes, which, in turn, are able to alter the physical and/or chemical properties of other macromolecules (for example DNA and RNA) in order to facilitate wound healing (15). This study was aimed to investigate the effect of Low Level Laser Therapy (LLLT) on healing of skin incision wounds in rabbits.

Materials and Methods

Thirty adult male New Zealand rabbits, aged (6-8) months, and weighing 1±0.300kg were used in this study. Bedding and environmental conditions were similar among all animals. The rabbits were randomly divided into two equal groups; Laser treatment (G1), and control (G2) groups. Each group was divided into 3 subgroups (n=5) according to follow up of histopathological study at 3, 7, and 14 days post wounding (PW). After anesthesia of animals by IM injection of xylazine 2% (5mg/kg B.W) and ketamine (40 mg/kg B.W) mixture (16), and aseptic preparation of the back of animals, two parallel 2 cm full thickness skin incision on the back of animals at a distance of 3cm from the vertebral spine were made in each animal. The skin incisions were closed by simple interrupted 0/4 silk suture. Right skin incisions of animals in G1 were irradiated by 8 J/cm2 energy density (17, 18) of diode laser (Gallium Aluminum Arsenide Lasers (GaAlAs) (Omega Laser Systems Limited, UK), at a wave length of (820 nm) (10), 10 KHz frequency, 50mW power output, 30 second exposure time, immediately after finishing the surgical operation and for more 3 successive days. The left incisions were left without treatment and in meantime were covered with black cover (while irradiating the right incisions), and consider as intra animal control. The probe of laser irradiation was applied closely to the wound surface, at 90° angle with the wound surface to ensure

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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maximum energy to the wound tissue. The wounds in G2 (control group) were not exposed to the laser. Full thickness skin wound biopsies (1 cm^3) were taken from each wound of subgroups animals at 3, 7, and 14 days post wounding, for histopathological evaluation. The specimens were preserved in 10% neutral puffers formalin solution and routine process were done to prepare the sections and stains with H&E stain, and examined under light microscope (19).

Ethical approval: The researchers obtained ethical approval from the research Ethical Approval Committee of the College of Veterinary Medicine, University of Al-Qadisiyah.

Results

At 3 days PW, the histopathological sections of G1 (laser treated group) demonstrate that the healing process was more notable, concerning a thin, flattened scab, thickening of the epithelial layer along the two wound margins, and the development of tongue-like epithelial cells from both sides of the incision toward the center of the wound and moreover full re-epithelialization in some animals were seen. Also presence of dense collagen fibers, more fibroblast, with less inflammatory response including less vasodilatation and discrete mononuclear inflammatory cells infiltrate more evident on the deeper part of the wound. New collagen fibers filled most of the wound center (Fig.1). During the same time period, the G2 (control group) show a thick scab upon the wound. Necrosis and destruction of the epidermis beyond the scab in the wound region, severe inflammatory cells infiltration (neutrophils and macrophages) with congestion of the blood vessels, hemorrhage in the dermal layer and severe edema (Fig.2). At 7 days PW, the histopathological sections of G1, display existence of severe hyperplasia of the epithelial layer, even became bigger than the normal adjacent tissue in some cases. The basement membrane was seen connecting the two edges of the wound. The epithelial layer saw consisting from several layers of pink hyperchromatic polygonal cells (Fig.3). There were either no or very few inflammatory cells in the dermis, and there was no edema or blood vessel congestion. The amount of collagen fibers decreased, they became more regular, and the wound's edges shrank. Close to the incision site, the sebaceous glands (secretory tubules and excretory ducts) could be seen in the surrounding tissue (Fig. 4). Through the same period (7 days PW), G2 were exposed the scabs riding the site of wound center in most of animals, the inflammatory cells (the macrophages and the polymorphoneuclear cells) were found in large numbers, edema and congestion of blood vessels still found but less than the 3 days' time, hemorrhage also was seen. The epithelization being progressed but not yet finished, discrete epithelial cells distributed in the dermis, but not connecting and closing the surface of the wound center. Massive quantities of collagen fiber were present, but it was irregular in orientation, with large numbers of fibroblasts (Fig. 5). On 14 days PW, the histopathological sections of G1, display complete reepithelization characterized by presence of highly differentiated epithelial layers resting on basement membrane, the keratin presence in the epidermal cells. The repaired dermis was easily distinguished from the original one (adjacent normal tissue) by recognizing highly arranged parallel collagen fibers has less fibroblast with the increase number of myofibroblast giving the character of mature fibrous tissue riches in collagen oriented in one direction (Fig.6). The wound regions become narrower than the 7th day time of observation. Discrete moving epidermal cells in a fresh extracellular matrix deep in the dermis were observed in the absence of edema or inflammatory cells. The epithelium in G2 at

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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the same time (14 days) was less developed, had thin layers, and an uneven basement membrane despite covering the surface of the wound (Fig. 7).

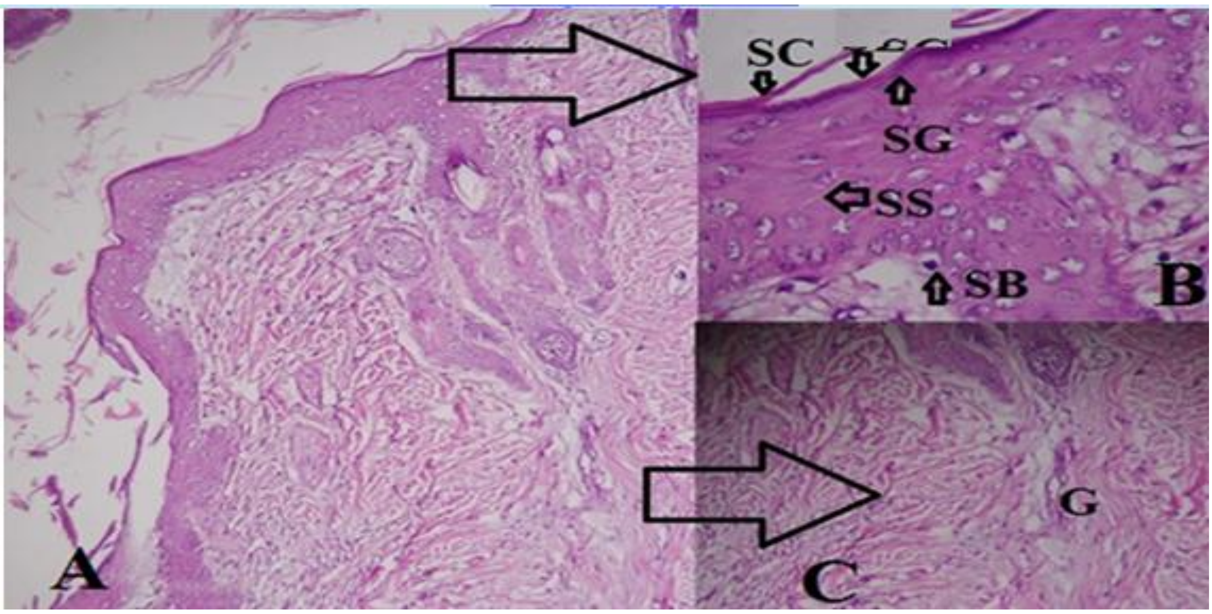

Fig.(1): Microphotograph of G1, 3 days PW, show(A) complete regenerated epidermal layer with deep extension of epithelial layer H&E.40X: (B), Stratum Corneum (SC), Stratum Granulosum (SG), Stratum spinosum (SS) and Stratum basale (SB) H&E,400X: (C)The incision line is filling with granulation tissue(G).H&E,400X.

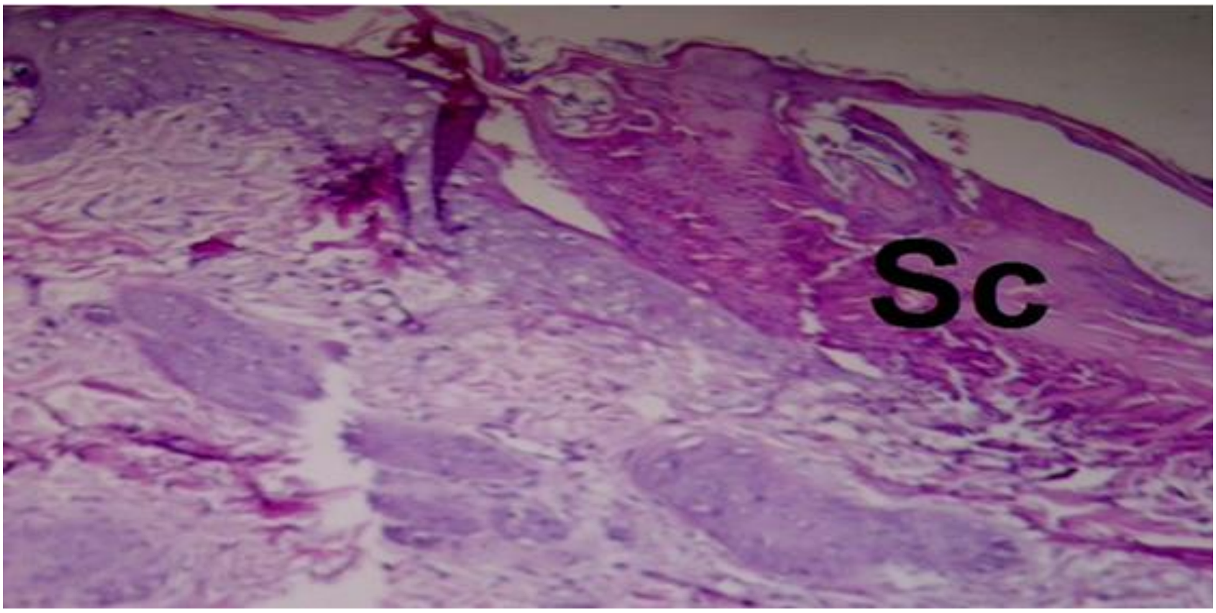

Fig.(2): Microphotograph of G2, 3 days PW, show thick scab (Sc) upon the wound with necrosis and destruction of the epidermis beyond the scab. H&E,100X.

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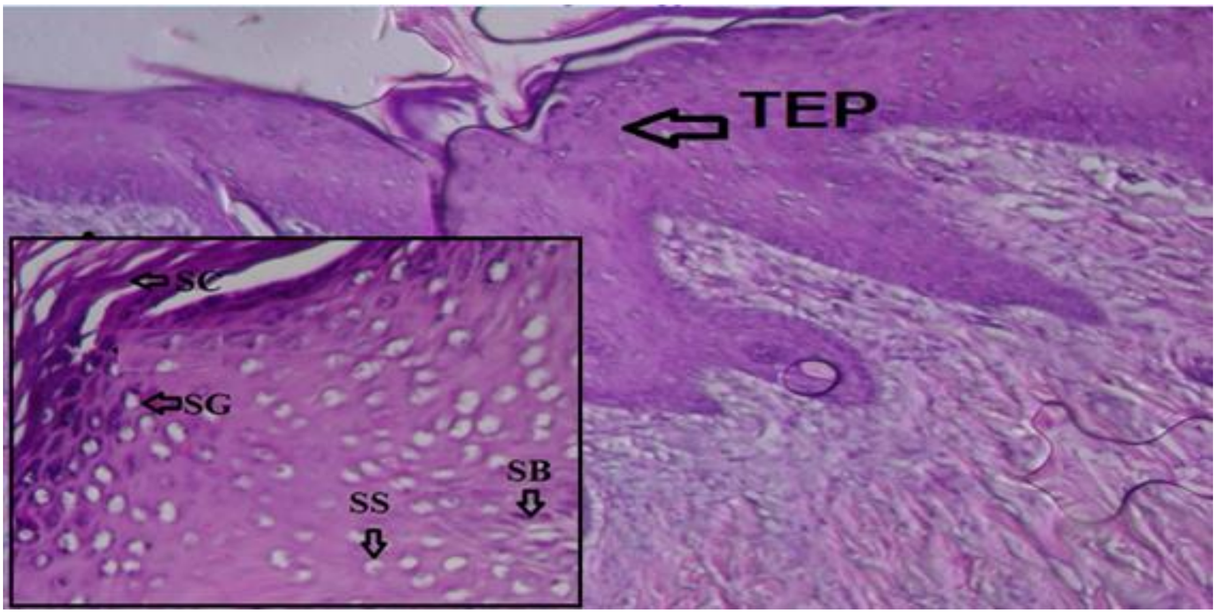

Fig.(3): Microphotograph of G1, 7 days PW, show hyperplasia of the epithelial layer even become thicker than normal. H&E, 100X. Black box; show complete regeneration of 4 layers of epidermis : Stratum Corneum (SC), Stratum Granulosum (SG), Stratum spinosum (SS) and Stratum basale (SB). H&E, 400X.

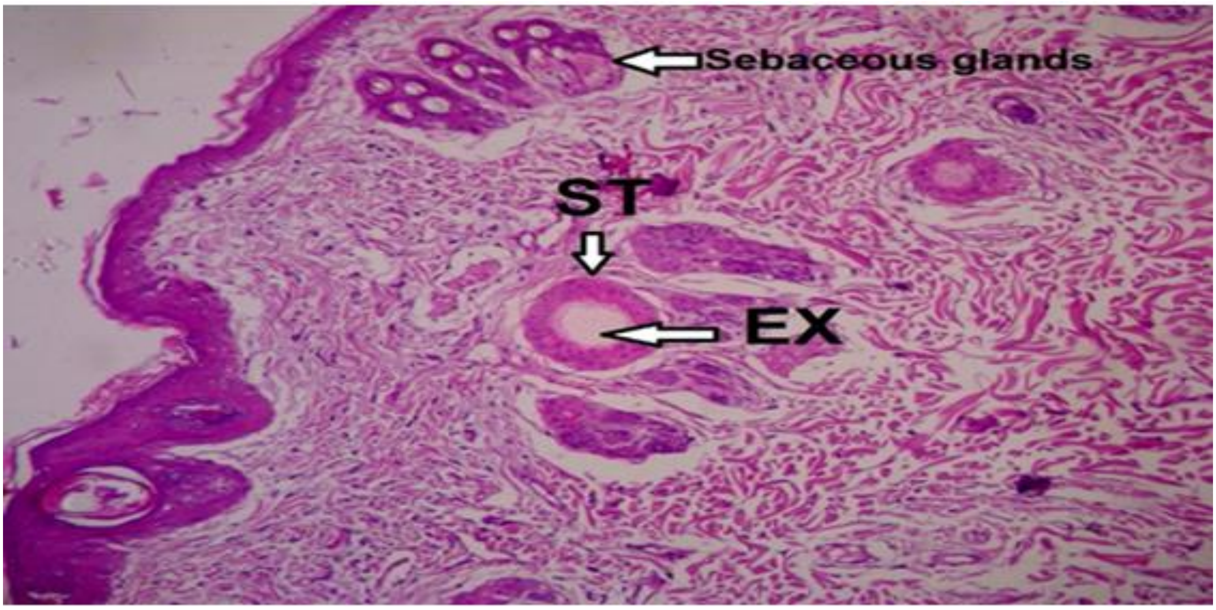

Fig.(4): Microphotograph of G1, 7 days PW, show complete regeneration of epidermis, healing of dermis, though sebaceous glands (secretory tubules and excretory ducts) were seen in the adjacent tissue close to the site of incision. H&E, 40X.

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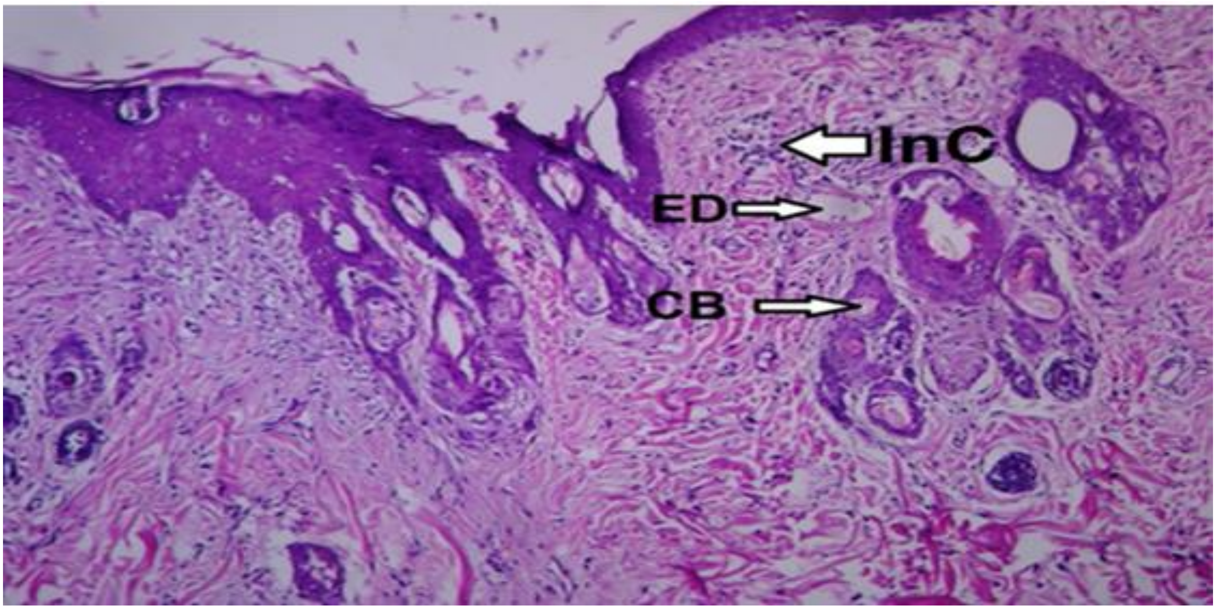

Fig.(5): Microphotograph of G2, 7 days PW, show large number of inflammatory cells, edema, congested of blood vessels and collagen fibers. H&E, 100X.

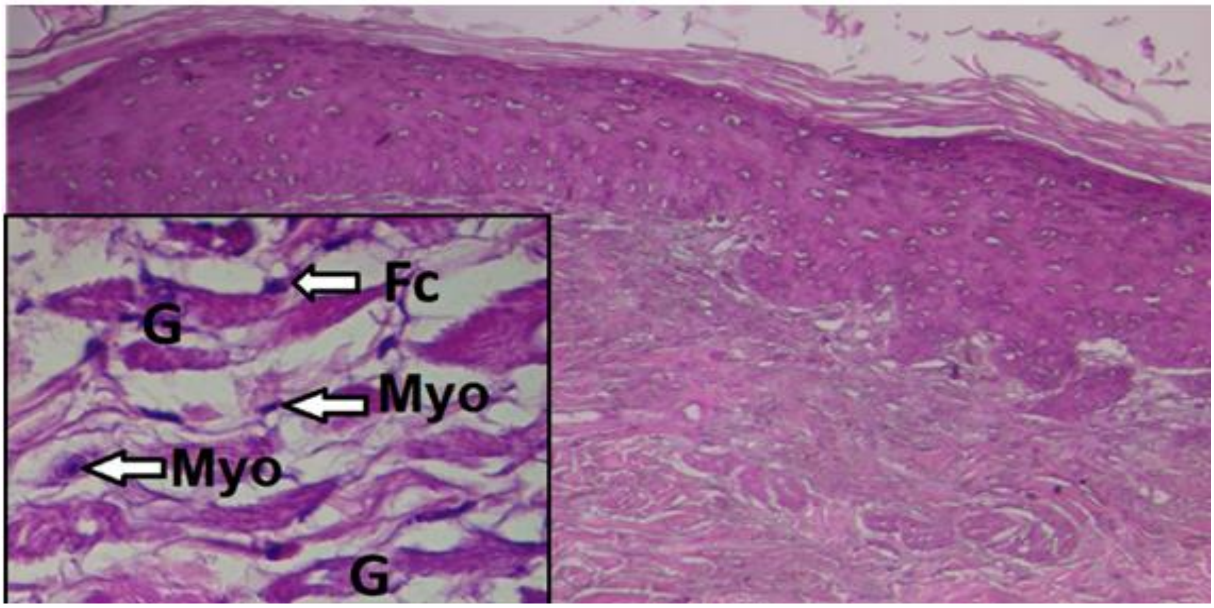

Fig.(6): Microphotograph of G1, 14 days PW show thick well regenerated epithelial layers with presence of keratin in the epidermal cells. H&E, 100X. Black box display mature fibrous tissue riches in collagen oriented in one direction(G), less fibroblasts (Fc) with the increase number of myofibroblast (Myo) H&E, 400X.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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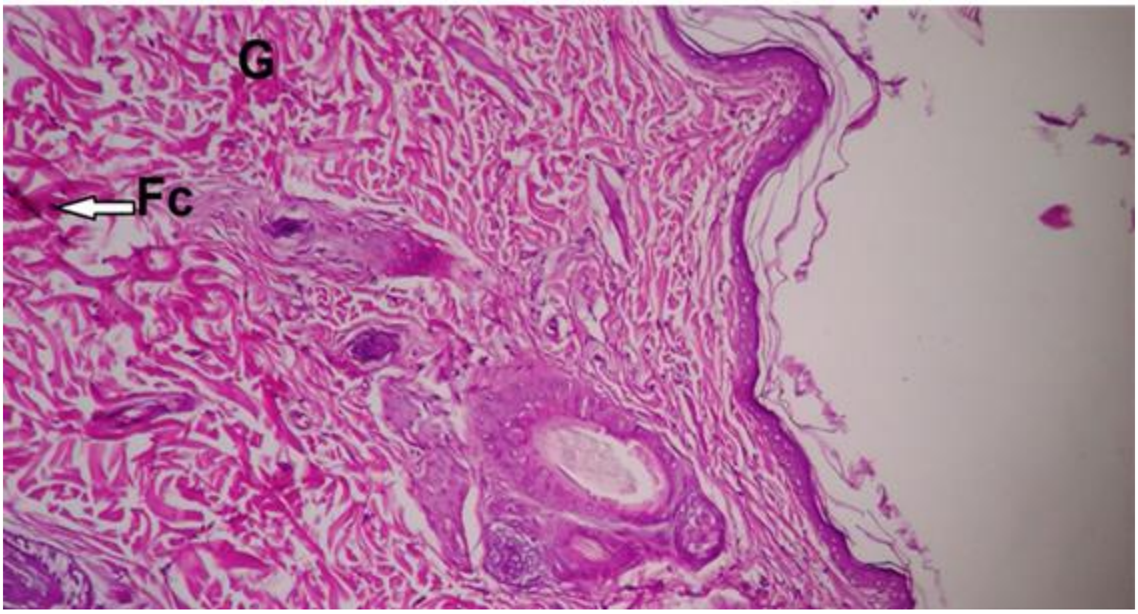

Fig.(7): Microphotograph of G2, 14 days PW, show thin epithelium resting on uneven basement membrane, Intense collagen deposition (G), enriched fibroblasts(Fc), and discrete inflammatory cells filling the wound center were still present, with active excretory duct and secretory tubules adjacent to the site of wound. H&E, 40X

Discussion

This study was designed to investigate the effects of Low Level Laser Therapy (LLLT) on healing of incisional wounds in the skin of rabbits.The healing process was seen more distinguished in laser treated group at 3 days post wounding. This result is accorded with (20, 21, 22, 23) who found the inflammation was less intense and more advanced in laser irradiated wound than in control group, due to the early onset of the inflammatory response when shorter wave lengths are used. More fibroblast attending with dense collagen fibers were seen involved in the laser treated wound, this due to the increase in fibroblast migration and cell proliferation (24, 25). The PDGF,TGF-β and ECM molecules stimulate peri-wound fibroblasts to proliferate and migrate into the area of wound, Once the fibroblast phenotype changes from proliferative to contractile and collagen synthetic (26, 27). It found that the epithelial layer adjacent the two edges of the wound were thickened accompanied with the formation of epithelial cells tongues from both sides of the incision toward center of the wound. This result is harmonized with (28, 29) whom found the laser has the capacity to induce epithelial cells mitotic activity and this faster the epithelialization process, which may be due to light induced increasing of ATP synthesis and building of a proton gradient lead to an increasing mitotic activity (30, 31, 32).Severe hyperplasia of the epithelial layer at seven days was found in our result, even if became bigger than the normal adjacent tissue, this agrees with the findings of (33), who discovered an increase in mitotic and epithelial hyperplasia near the wound borders. The basement membrane is seen connecting the two edges of the wound at 7 days PW in the laser treated group and this confirmed with (23) who declare that irradiated epidermis is totally restored, due to the more rapid resolving of the acute inflammation and the proliferative phase of healing is begin earlier and this may be related to the activation of chemotactic factor by irradiation (34, 35). Scarce inflammatory

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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www.qu.edu.iq/journalvm

cells or absent were observed in the dermis, with no edema or congestion of blood vessels at 7 days PW, means earlier ending of the inflammatory phase and this compatible with (24, 36) who discovered that after 7 days, an irradiation wound displays edema regression and a decrease in the number of inflammatory cells and this may due to the effect of laser make enlarge of the lymphatic vessels which may promote better oxygenation of the tissue under repair and therefore reduce edema. Also at 7 days PW, it found the collagen fibers reduced in number and became more regular also the edges of the wound become narrow. This was confirmed with (24, 35) whom found the reduction and shrinking in wound size are observed in the irradiated group, with complete close of wound and less scar formation. In the 7th days, the sebaceous glands (secretory tubules and excretory ducts) were seen in the adjacent tissue close to the site of incision. Re-epithelialization before time was seen entire in the laser treated group characterized by presence of highly differentiated epithelial layers resting on clear visible basement membrane as well as keratin presence in the epidermal cells. This result is compatible with (10, 23, 37, and 38), they discovered that the application of LLLT increased and advanced re-epithelialization as well as many components of the healing process, such as the degree of inflammatory creation and collagen organization, new vascularization, and epithelialization.Our result at 14 days PW, the laser treated group's show the repaired dermis was easily distinguished from the original one and this accordance with (23) who found the superficial and deep dermis presented a moderate number of fibroblasts with parallel orientation with the wound surface and blood vessels. The result also recognizing highly arranged parallel collagen fibers and mature fibrous tissue rich in collagen oriented in one direction. This fact was harmonized with (23, 34, 35,and 39) whom found the increase of the amount of thin collagen fibers arranged in a parallel alignment with the epithelial surface in radiated tissue as well as find more evident remodeling (maturation phase) with better organizing tissue. This may due to the effect of LLLT which may prevents the harmful and severe inflammatory reactions and hence lead to the increased formation of collagen fibers (36). The laser treated groups showed less fibroblasts with the increase number of myofibroblast giving the character of mature fibrous tissue at 14 days PW, and this concurred with (35, 36,and 40) whom distinguished the fibroblasts and myofibroblasts in the same period. The He-Ne irradiation produce a massive transformation of fibroblasts in to myofibroblasts and this may because of the myofibroblasts are directly involved in granulation tissue contraction and the irradiation cause increased numbers of which lead to facilitated wound contraction.The laser irradiated group in our result at 14 days PW show discreet migrating epidermal cells were observed a new extracellular matrix deep in the dermis, with no edema or inflammatory cells and this fully confirmed with (36, 41, 42) who discovered that the irradiated group's migrating epidermis could be seen deep in the dermis, and also a newly formed ECM is presented with the reduction of inflammatory cells as well as a diminish in degree of edema.Our study showed that the laser treatment in the present dose (8J/cm^2) have beneficial effects in the inflammatory, proliferation, and maturation phases of wound healing, compared with their control groups. It can be used successfully to promote wounds healing. However, laser treatment was more effective in the first two phases of wound healing. It is in accordance with (10, 43).The study shows that the wound healing in the LLLT was promoted, showing improvement in tissue continuity, decreased creation of excessive collagen fibers, and prevention of excessive

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inflammation. The treated group had good wound healing in contrast to the control group which demonstrates intermediate wound healing. Consequently, it is discovered that LLLT helped the rabbits repair incisional wounds (44, 45).

Conclusion

The 8 J/cm2 energy density of (820 nm) LLLT promotes wound healing by reducing excessive inflammation, promoting the production of collagen fibers, and restoring tissue continuity.

Conflict of Interest: there is no conflict of interest.

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