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Use of 820 nm Diode Laser Conjugating With Methylene Blue to Improve Open Wound Healing in Rats

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Abstract

Objective: The study was aimed to evaluate the use of 820 nm diode laser conjugating with methylene blue (MB) to improve open wound healing in rats.

Methods: Sixty adult male Wistar rats weighing (240±20gm), aged between (7-12) weeks were divided into four equal groups (n 15); G1/Control group which not receive any treatment. G2/Laser treatment group, were treated with 8J/cm²/day for 7 successive days. G3/ MB treatment group, were treated with topical application of MB dye 0.01% for 5 min/day for 7 successive days. G4/ MB + Laser treatment group were treated with MB and laser for 7 successive days, the session include a topical application of the MB as in G3 followed by laser treatment as in G2. After general anesthesia, two open circular full-thickness (0.8 cm in diameter) skin incision on the back of the animals were made. In treated groups one incision was treated and the 2nd were left as control in the same animal. Assessment of the wound healing was done grossly and microscopically.

Results: Macroscopically were seen significant $P < 0.05$ decreased of the size of wound in treated groups in compare with control at 7, and 14 days, while the readings become near each other at 21 days. The epithelization period in treated groups were 8-10 days and 8 days in control group. Microscopically G1 show no epithelialization, new collagen fiber, new blood vessels, numerous PMNCs, and large number of fibroblasts at 7th day PW. At 14th day exhibit thin epithelial layers connecting the two edges of wound, and more collagen fibers arranged horizontally in the dermis, while at 21st day, no inflammatory cells, more collagen fiber, and less cellular field appear. G2 at 7th day PW, show severe numbers of PMNCs, new collagen fibers, plentiful numbers of fibroblast, and severe numbers of new blood vessels. At 14th day PW, thick well differentiated epithelial layers were seen, upon new collagen fibers, less numbers of PMNCs, plentiful numbers of fibroblast and myofibroblasts. At 21st day, thick even epithelial layers were seen upon mature well-arranged collagen fibers, scanty blood vessels, and myofibroblasts. Buds of hair follicles were seen also. G3 at 7th day PW, show thin epithelial layers, plenty of PMNCs, and numerous new blood vessels with more new collagen fibers. At 14th day PW moderate thickening of epidermis were seen upon deep area of granulation tissue (more collagen and less cellular). At 21st day PW, thin epidermal layers were seen upon mature granulation tissue. G4 at 7th day PW show complete well re-epithelialization. Epidermal layers were seen riding on distinct basement membrane. The dermis seen have dense cellular pinkly granulation tissue, abundant PMNCs and fibroblast and a lot of number of new blood vessels. In deep dermis, scanty sebaceous glands, solitary hair follicles in

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different stages of development were seen. At 14th day PW normal conventional appearance of the epidermis were seen. The dermis consist of mature granulation tissue with a lot of numbers of fibroblast, myofibroblasts, and little PMNCs. At 21st day PW normal thickness of epidermis were seen resting on basement membrane has many inner invaginations toward the dermis. The dermis was full with mature granulation tissue with no inflammatory cells, no new blood vessels, the collagen fibers were dense thick and regular, more hair follicles and more sebaceous glands were seen.

Conclusions: In conclusion, laser treatment with 820 nm were seen improve the healing process and accelerate the proliferation, wound contraction, maturation and remodeling phases of wound healing. Conjugating methylene blue with laser give better enhancement.

Key Words: LLLT, Photodynamic therapy, Photosensitizer, Methylene blue, Wound healing

Introduction

Skin is the largest organ in the body, compose of 15% of total adult body weight. It serves a variety of functions, including protection from external attackers, prevention of excessive water loss, and thermoregulation (1). To restore the tissue architecture after injury, skin wound healing necessitates a complex and well-coordinated interaction between different cells (2), including keratinocytes, endothelial cells, fibroblasts, inflammatory cells, and macrophages (2,3, 4). Healing is a complicated process that can be divided into at least three overlapping stages: an inflammatory response, a proliferation process leading to tissue restoration, and finally tissue remodeling (5). Open wounds that have sustained extensive tissue damage and are contaminated or infected are allowed to heal completely through contraction and epithelialization (second intention healing). Epithelization in closed wounds, can take as little as 48 hours as the epithelium migrates through the fibrin clot, while in open wound occur after a bed of granulation tissue has formed after a latent period of approximately 4 to 5 days (6,7).Low-level laser therapy (LLLT) is a type of phototherapy that is used to aid wound healing in a variety of clinical settings. At the right wavelength, intensity, and dose, LLLT can speed up tissue repair (8). It boosts cellular activity in injured skin (2), and also has been shown to be effective in open wounds, allowing faster healing and restoration of structural and functional integrity (9).Photodynamic therapy (PDT) involves the administration of a photosensitizer (PS), followed by laser irradiation of target cells, which produces reactive oxygen species (ROS), which can cause cell death and microvascular damage (10,11, 12, 13, 14). Porphyrins, chlorophylls, and dyes such as methylene blue (MB) are the three most commonly used photosensitizers (15, 16). When exposed to a specific wavelength of light, methylene blue (MB) is a photosensitizer (PS) that can cause the production of high levels of singlet oxygen (1O2), or reactive oxygen species (ROS) (17). Previous research has confirmed the effectiveness of MB as a photosensitizer for tumor cell death induction (18). Systemic PS, or those given intravenously, cause local microcirculation to deteriorate, which is one of

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the main mechanisms for treating tumors, as opposed to topical PS, which has a minor effect on blood vessels. However, maintaining local vascularization is critical for wound healing, which is why most studies recommended using topical PS (13).High doses of PS or high doses of light, for example, tend to cause extensive necrotic cell death, whereas less intense protocols appear to favor apoptosis cell death. A biostimulatory effect in cells and tissue could be achieved by reducing the laser dose commonly used in basic PDT protocols. Further to the presence of low amounts of photosensitizer compounds in the cells, they can proliferate better after application of laser at the right wavelength and precise window of time, as the low concentration of generated ROS initiates a cascade of events leading to a proliferative cellular pathway, as the low concentration of generated ROS initiates a cascade of events leading to a proliferative cellular pathway (19). Previous research has shown that the PDT improved wound healing in humans, such as wound healing and scarring in human skin (20), and in animals, such as skin wounds healing in rats (12), the healing of cutaneous third-degree burn in rats (21), mediated by methylene blue in rat wound healing (22), revealing that PDT itself as a promising therapeutic modality, stimulating wound healing and remodeling (23). For that the study was aimed to evaluate the use of 820 nm diode lasers conjugating with photosensitizer; methylene blue (MB) to improve open wound healing in rats.

Materials and Methods

Sixty (60) adult male Wistar rats weighing (240±20gm) and aged between (7 - 12) weeks were utilized in this study. After general anesthesia with a mixture of ketamine (50 mg/kg. B.W.) and xylazine (10mg /kg. B.W), IM (24). and preparing the back of animals for aseptic surgery, two open parallel circular full-thickness (0.8 cm in diameter) skin incision on the back of the animals were made by using punch machine (25. 26) (Fig.3-1a,b,c). One incision was treated and the 2nd incision were left without treatment as control in the same animal. Animals individually were kept in plastic cages with a metal top in same normal laboratory conditions (room temperature 20-24 C° and humidity 60%) and were fed a solid diet and water were given ad libitum. Animals were randomly divided into four groups (n15). G1; Served as control group and did not receive any treatment with MB or laser and the wound left to heal by second intension. G2; were treated with single dose 8J/ cm^2/ day of (820 nm) (GaALAs) diode laser (Omega Laser System Limited UK) for 7 successive days. The laser probe is placed closed perpendicularly in direct contact with the center of wounds. G3; were treated with topical application of the 0.01% methylene blue (MB) dye (22) by cotton swab, for 5 min/day for 7 successive days, involving the wound and the near periphery. Animals were kept away from direct sun light through these 7 days.G4; Were treated with both methylene blue and laser for 7 successive days: The session include a topical application of the methylene blue dye 0.01% for 5 min/day as in G3 followed by exposing to the laser treatment as in G2. Also animals were kept away from direct light through these 7 days. The study was

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conducted according to the national guidelines for the Care and Use of Laboratory Animals. All protocols were approved by the High Committee for Review and Approval of Research Proposals of the Faculty in the University of Al-Qadisiyah College of Veterinary Medicine.To evaluate the progress of wound healing morphometric and histological assessment of the wound healing were assessed.Morphometric assessment include; wound surface area (wound size), and percentage of wound contraction were performed by direct measurement of wound dimensions at 7th, 14th, and the 21st days post wounding (PW) using a millimeter graduated ruler. When the wound is circular; Circular wound surface area = (half diameter)^2 x 3.14. When the shape of wound changed; the wound surface area = Length x Width. Percentage of wound contraction = (wound area on day 0 - wound area on day n / wound area on day 0 x 100) (27, 28). Complete epithelialization period was calculated as the number of days required for falling of dead tissue remnants without any residual raw wound (Falling of scab leaving no raw wound behind) (27, 28, 29, 30). Morphometric data were statistically analyzed, using ANOVA test, and Least Significant Difference (LSD) to find the significance between groups under the level of P<0.05.Specimens of healed skin (1 cm^3) were taken after (7, 14, and 21) days (PW) from all animals for histological assessment. The specimens were preserved in 10% buffer formalin solution and send for histopathological examination after sectioning into 5 μm and staining with Hematoxylin and Eosin stain, to evaluate the progress of healing process (26, 31).

Ethical approval

The study protocol was approved by the College of Veterinary Medicine, University of Al-Qadisiyah, Iraq.

Results

clinically all animals throughout 21 days PW were seen healthy, and active. No infection was developed in all wounds and no deaths were recorded. Primarily all wound areas increased in size within 4 hours after creation of wound and continued in swelling and increase in size for the next 24 hrs. post wounding (PW) with exaggeration of the inflammatory signs. The whole wound seen swollen, and the edges of the wounds were elevated, red in color, and from the second day PW show a thick scab upon the wound persisting more than the 7th day PW. The wound gradually decreased in size till the 21st day were become small scar tissue as circular or liner in shape.

Surface area and wound contraction

The initial wound surface area (size of wound) was (50.24 mm^2) on day zero in control and treated animals. It was decreased significantly P < 0.05 in treated groups in compare with control group and control reading of same animal at 7, and 14 days, while the readings become near each other at 21 days (Table 1) (Fig. 1). The epithelization period in treated groups were seen between (8-10) days which it was more than that of control group (8) days (Table 2). The percentage of wound contraction was significantly increased (P < 0.05) in treated groups at 7th and 14th days as compared with control group. G2 (laser treated

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group) recorded the least significant decrease of wound size (24.61, and 3 mm^2 respectively), and the highest significant percentage of wound contraction (51.01%, and 94.02%) at 7 and 14 days respectively (Table 1) (Fig. 2).

Macroscopic assessment of the wound healing

The wounds of G1 at 7th day show no signs of inflammation, little decrease in the size of wound in compare with day 0, and the wound was covered with thin scab. On day 14th the scab was not found, more decrease in the size of wound were occur, and scar tissue was developed. On 21 day PW, scar tissue were seen on wound site, and more decrease in the size of wound but it still large and obvious. Treated wounds of G2 at the 7th day PW exhibit marked reduction in size in compare with same time of control group and control wounds in the same animal, although still seen covered with scab. On day 14th no scab was seen, the wound exhibit complete epithelialization, and scar tissue formation, with marked reduction in size of the treated one in compare with control. On day 21st the areas of both wounds (treated and control) become small and fad with tiny scar tissue taking liner shape. It was difficult to distinguish from the unwounded normal skin. Treated wounds of G3, at the 7th day PW show little reduction in size in compare with control in same animal, and control group at 7th day. The scab was seen still cover the incisions. On the 14th day PW, more reduction in size in compare with 7th day were seen, while both incisions (control and treated) appears in same size. Complete epithelialization, scar tissue formation, and absence of scab were seen. At the 21st day PW, more reduction in size occur in compare with 14th day, where the both incisions (control and treated) appears in same size. In G4, the treated wounds at the 7th day PW show little reduction in size in compare with incisions of control in same animal, while the scab still cover the both incisions. On 14th day PW the treated incisions show more reduction in size in compare with control incisions, also there was complete epithelialization, scar tissue formation, and no scab cover the incisions. On the 21st day PW more reduction in size in compare with control of same animal was seen. The shape of incisions sometimes remain circular and other times change to liner (Fig. 3)

Histopathological assessment of the wound healing

Wounds of G1 7 days PW exhibit thin amorphous (not rested on base membrane) epithelial layers connecting the two edges of wound found under thick scab. There was thickening of the epidermis at the periphery of wound. Numerous polymorphonuclear cells (PMNCs) and a large number of multi oriented neovascularization (severe) with more collagen fibers. The field appeared dominant with new collagen fiber, new blood vessels, numerous PMNC, and fibroblasts. A large number of fibroblast was seen, proliferation of collagen, fibrous tissue and capillaries in the dermis. At 14 days PW, no scab was found, the epithelial layers were thin composed from 4 layers rested upon unclear basement membrane, tinny and thin keratinized layer was seen. Discrete inflammatory signs were seen, including disseminated PMNCs, moderate new blood vessels arranged horizontally with the surface of the wound, and more collagen fibers arranged horizontally. At 21 days PW, the wound edges, and scabs were disappeared. Thin even regular epidermal layers composed of 4 layers

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(including thin keratin layer) was seen rise on basement membrane. Also more collagen, less cellular, no inflammatory cells were seen. The superficial and deep dermis seen regular, and has numerous hair follicles in different stages of development (Fig. 4).

Treated wounds of G2 7 days PW. exhibit cracked splintered thick scab found upon an irregular complete epithelial layers composed of many layers of cells. A basophilic hyper chromic, hyper cellular field was gotten; The field were infiltrated with severe abundant numbers of PMNCs, new pinkly collagen fibers, plentiful numbers of fibroblast, and severe numbers of new blood vessels vertically oriented and not congested, many vacuoles of adipose tissue also were found. At 14 days PW, there were thick well differentiated epithelial layers (epidermis) consist from 4 layers riding on well distinguished basement membrane, including well differentiated keratin layer was seen. The epidermal layers were hyper cellular taking deep basophilic stain. Below the epidermis, immature scar tissue was seen composed from immature collagen fibers, myofibroblasts, and scant small blood vessels. No hair follicles, no sebaceous glands, no adipose tissue, no panniculus carnosus The field were dominated with new pinkly collagen fibers, less numbers of PMNCs, plentiful numbers of fibroblast and myofibroblasts, and less numbers of new blood vessels horizontally oriented and not congested. At 21 days PW thick even epithelial layers (epidermis) composed from 4 layers riding on faint well-arranged basement membrane. Scar tissue was seen underneath the epidermis, it composed from mature well-arranged horizontally oriented collagen fibers, scanty blood vessels, and myofibroblasts. Buds of hair follicles were seen, no sebaceous glands, no adipose tissue, no panniculus carnosus (Fig. 5).Treated wounds of G3 7 days PW show thin epithelial layers connecting the two edges of wound found underneath thick scab, they consist from disseminated epithelial cells in the center of wound and a thickening of the epidermis at the periphery. A tongue of epithelial cells at the periphery was directed towards the center of wound. No edema and no congested blood vessels were seen. Plenty PMNCs, and numerous multi oriented new blood vessels with more new collagen fibers was seen. Field was dominated with pink fresh new collagen fiber, new blood vessels, numerous PMNCs, and large number of fibroblasts. Huge numbers of vacuoles in the dermis, and more collagen fibers also was seen. At 14 days PW, moderate thickening of basophilic hyper chromic epithelial layers consist from 4 layers resting on well distinguished basement membrane has many inner invaginations toward the dermis was seen covering the surface of the wound. Epidermis found upon deep area of granulation tissue has an abundant fibroblasts and myofibroblasts with immature collagen fibers, moderate new blood vessels and moderate PMNCs. An abundant mass of immature collagens fibers seen inside the dermis, type I and type III collagen fibers. The field seen more collagen and less cellular. At 21 days PW connecting thin epidermal layers composed from 4 layers resting on even basement membrane, the granulosum cells were large and have large nucleus. The dermis consist of mature granulation tissue has more collagen fibers, less fibroblasts and more myofibroblasts. Fewer blood vessels and no inflammatory cells.

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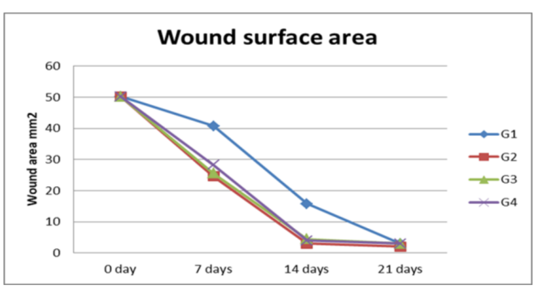
Deep dermis contains solitary hair follicles in different stages of development. The field seems increase in density of collagen fibers and myofibroblasts (Fig. 6).

Treated wounds of G4 7 days PW show complete well re-epithelialization were occur representing by complete thick dense deep basophilic hyper chromic epithelial layers composed of well differentiated 4 layers including the well differentiated keratinized layer, and dominant large dark basophilic granulosum cells. Epidermis layers were seen riding on clear distinct basement membrane, and no scab upon were seen. The superficial and deep dermis underneath the epidermis were seen with dense cellular young pinkly granulation tissue composed from a bundles of young collagen fibers arranged transversely with the surface of wound, with an abundant numbers of PMNC and fibroblast and a lot of number of multi oriented new blood vessels. In the deep dermis, scanty sebaceous glands seen, solitary hair follicles in different stages of development, and many adipocytes were seen. The panniculus adiposus and carnosus were disappear. At 14 days PW normal conventional appearance of the epidermis demonstrating 4 epithelial layers (including thin Keratinized layers) taking deep basophilic stain, resting on even well distinguished basement membrane. The dermis underneath the epidermis, enclose mature fibrous tissue consist from heavy bundles of mature collagen fibers with a lot of numbers of fibroblast, myofibroblasts, and little PMNCs, with scanty blood vessels. Panniculus adiposa and carnesus not seen. At 21 days PW, ordinary thickness of epidermis were seen, it consist from 4 well differentiated layers (including thin keratinized layer) resting on basement membrane has many inner invaginations toward the dermis, the epidermis taken deep basophilic stain with prominent granulocyte cells. The dermis, underneath the epidermis, was full with mature granulation tissue with no inflammatory cells, no new blood vessels, the collagen fibers were dense thick and regular, there was no enlargement in thickness of dermis, more hair follicles and more sebaceous glands were seen (Fig. 7).



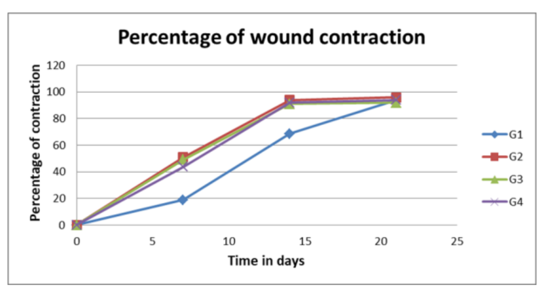


Fig. 1: Wound surface area (size of wound) was decreased significantly $P < 0.05$ in treated groups in compare with control group and control reading of same animal at 7, and 14 days, while the readings become near each other at 21 days.

Fig. 2: Percentage of wound contraction; Show the highest significant percentage of wound contraction in G2 (51.01%, and 94.02% respectively) at 7 and 14 days.

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Table (1); Surface area (mm^2) and wound contraction %.

Periods	Groups	Surface Area (mm^2)		Wound Contraction %	
		Control	Treated	Control	Treated
0 day	G1, G2, G3, G4	50.24 Aa	50.24 Aa	0 Ja	0 Ga
7 days	G1	40.69 Ba	40.69 Ba	19 Ia	19 Fa
	G2	41.83 BCa	24.61 Cb	16.73 Hb	51.01 Da
	G3	42.98 Ca	25.50 Cb	14.45 Gb	49.24 Da
	G4	38.46 Da	28.26 Db	23.52 Fb	43.80 Ea
14 days	G1	15.75 Ea	15.75 Ea	68.65 Ea	68.65 Ca
	G2	8 Ga	3F Gb	84.07 Cb	94.02 Aa
	G3	12 Fa	4.5 Fb	76.11 Db	91.04 Ba
	G4	15 Ea	4 Fb	70.14 Eb	92.02 ABa
21 days	G1	3 Ia	3 FGa	94.02 Aa	94.02 Aa
	G2	3 Ia	2 Ga	94.02 Aa	96.01 Aa
	G3	6 Ha	3 FGb	88.05 Bb	94.02 Aa
	G4	9 Ga	3 FGb	82.08 Cb	94.02 Aa
$LSD_{0.05}$		1.68		2.24	

- Capital letters refers to the vertical statistical comparison, whereas small letters refer to the horizontal statistical comparison.
- Different letters denote to the significant difference at $P<0.05$, whereas similar letters refer to the no significant difference.

Table (2); Epithelization period (per days) in control and treated groups.

Groups	Epithelialization / day	
	Control	Treated
G1	8	-
G2	8	9
G3	9	10
G4	8	9

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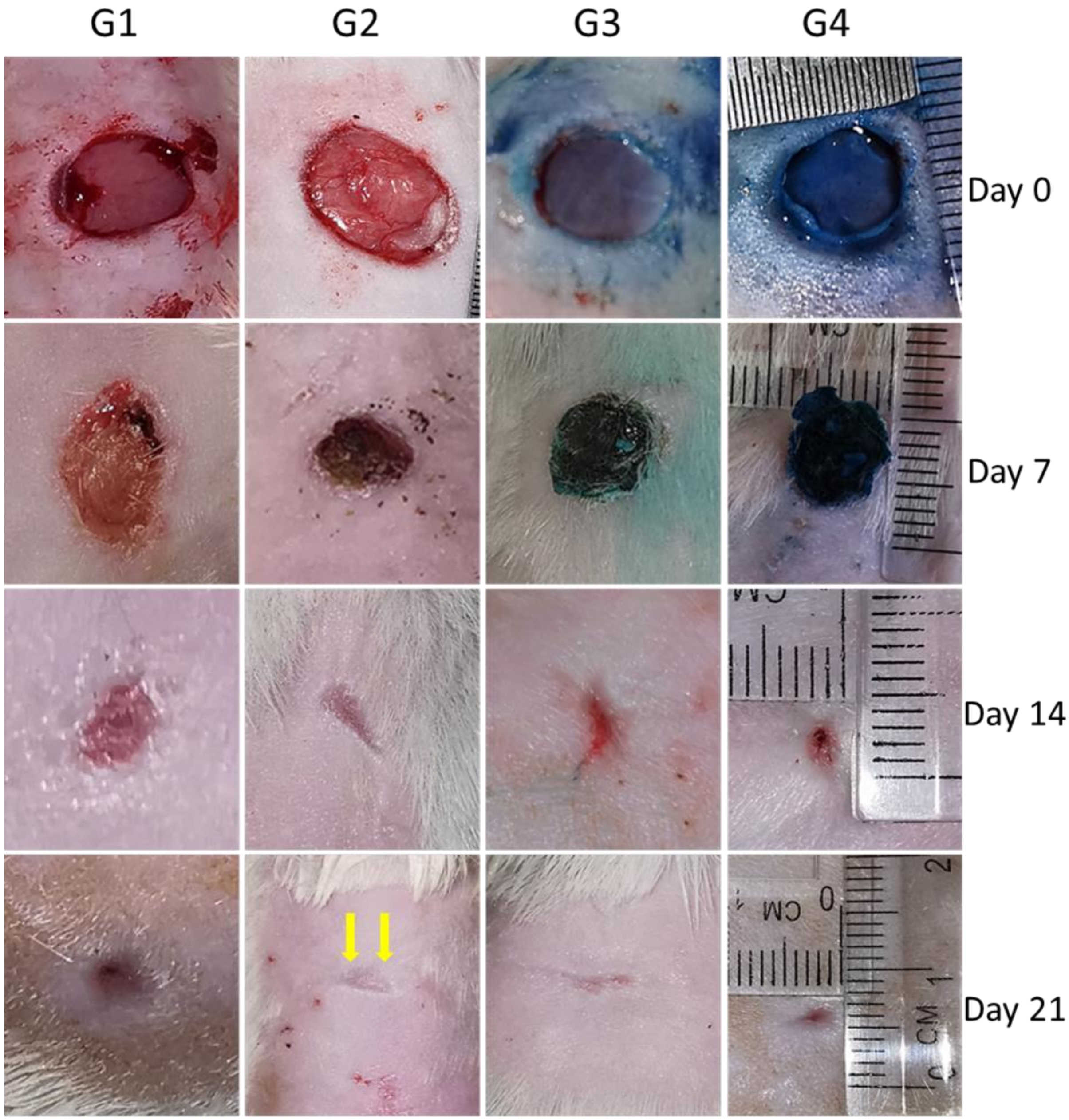

Fig. (3): Photographs of control (G1), and treated (G2, G3, G4) open circular full-thickness skin incision of Wistar rats showing various phases of wound healing at 7, 14, and 21days. On day 0 see the sharp edges of 8 mm circular wound, On 7th day the wounds still covered with scab, and see there was more reduction in size than the 0 day in treated groups (G2, G3, G4) than the control (G1), while the G2 laser treated show significant decrease in size at this time. At 14th day G4 show significant decrease in size in compare with other groups and control group, complete epithelialization was occur, more scar tissue, and the shape of wound was changed to liner in G2 and G3. At 21st day more reduction in wound size in the treated groups in compare with control. Complete epithelialization was occurring, and less scar tissue was seen. In G2 difficult to distinguish the healed wound from the normal skin (yellow arrow).

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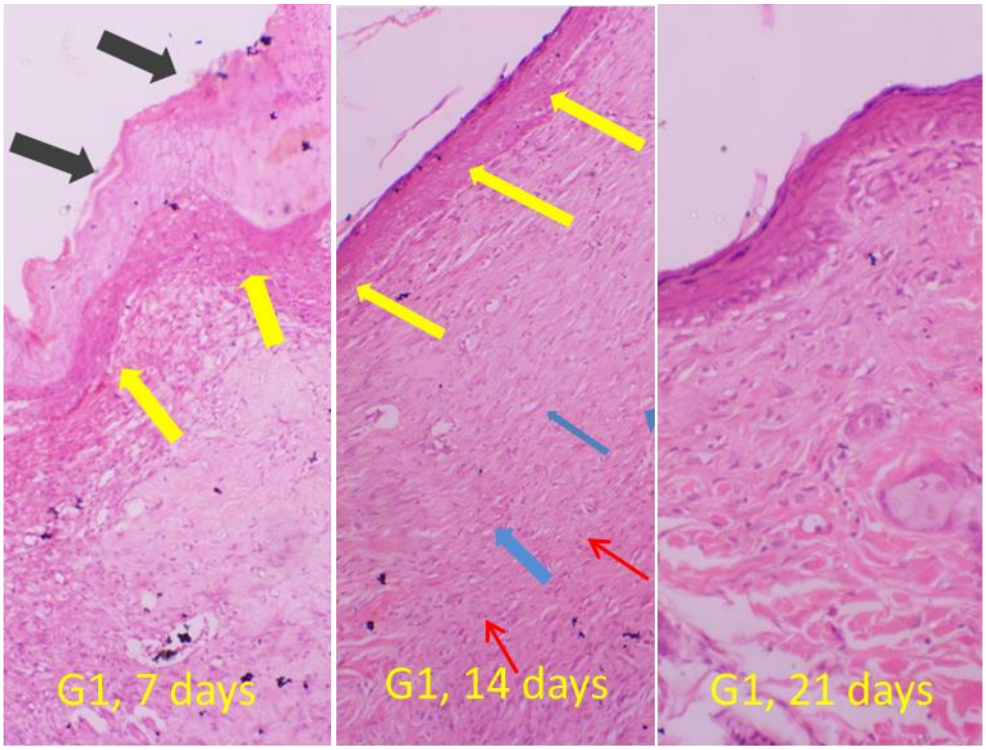

Fig. (4): Control group (G1); At 7 days PW show thin amorphous epithelial layers connecting the two edges of wound (yellow arrows) found under thick scab (black arrows). H&E stain 10X. At 14 days PW, show no scab, thin epithelial layers (yellow arrows) rested upon unclear basement membrane. Discrete PMNC (red arrows), moderate non-congested new blood vessels arranged horizontally (blue arrows) and more collagen fibers arranged horizontally, H&E stain 10X. At 21 days PW, show more collagen, less cellular, and no inflammatory cells were seen. H&E stain 20X.

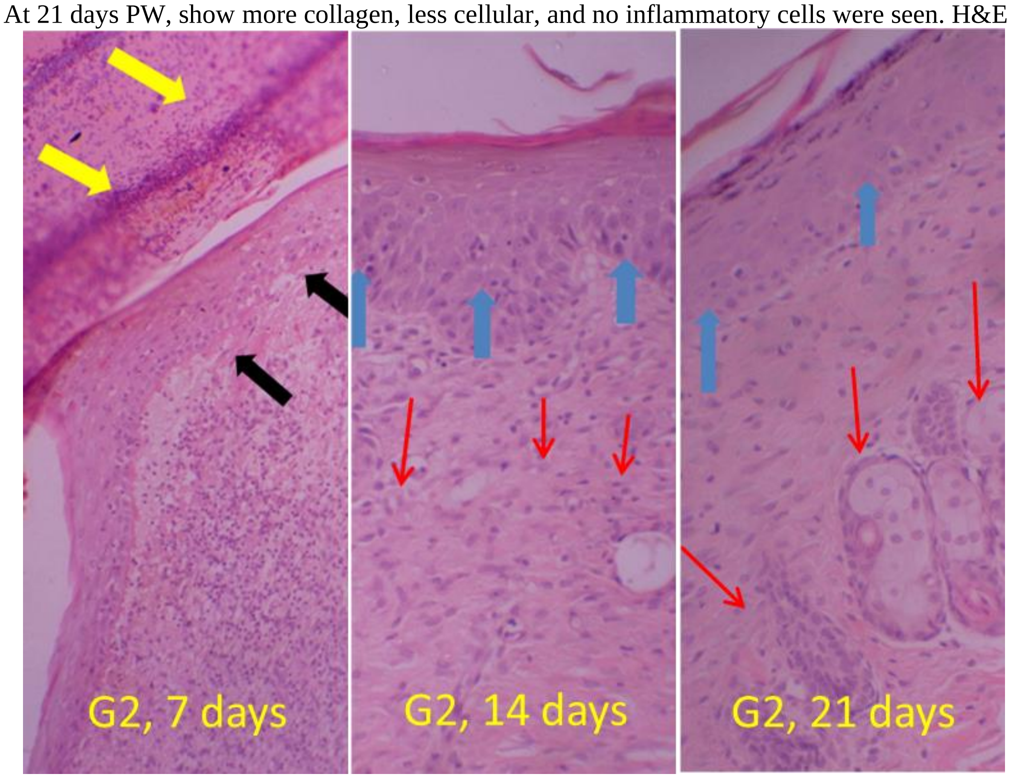

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Fig. (5): G2 Treated wounds 7 days PW, show detached scab (yellow arrows) upon connected epithelial layers (black arrows), and cellular dermis. H&E stain 10X. At 14 days PW, show the 4 epidermal layers were hyper cellular taking deep basophilic stain (blue arrows). The dermis, contain immature collagen fibers (red arrows), myofibroblasts, scant not congested horizontally oriented blood vessels. No hair follicles, no sebaceous glands, no adipose tissue, no panniculus carnosus. H&E stain 20X. At 21 days PW, show thick even epidermis composed from 4 layers riding on faint well-arranged basement membrane (blue arrows). Scar tissue was seen underneath the epidermis. Buds of hair follicles were seen (red arrows), no sebaceous glands, no adipose tissue, and no panniculus carnosus. H&E stain 20X.

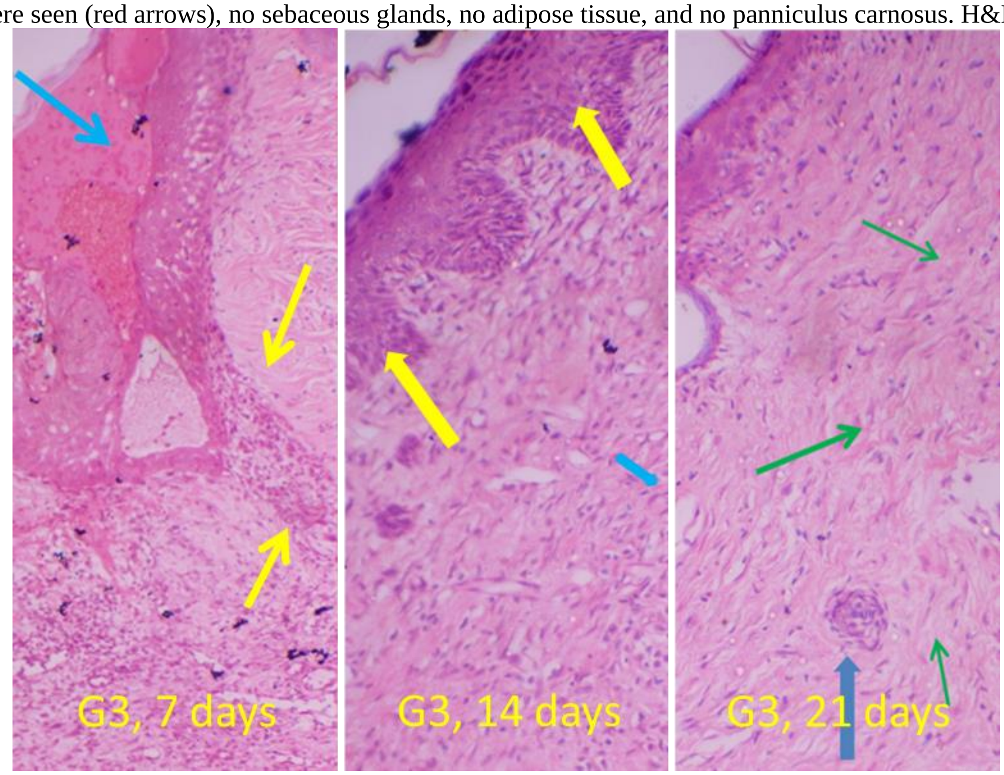

Fig. (6): G3 Treated wounds 7 days PW, show thick scab (blue arrow) and, a tongue of epithelial cells at the periphery of wound was directed towards the center of wound (yellow arrows). Plenty PMNC, numerous non- congested multi oriented new blood vessels with more new collagen fibers was seen. H&E stain 10X. At 14 days PW, show moderate thickening of basophilic hyper chromic epithelial layers resting on well distinguished basement membrane has many inner invaginations toward the dermis (yellow arrows). Dermis consists of an abundant fibroblasts and myofibroblasts with immature collagen fibers (blue arrows), moderate new blood vessels and moderate PMNC inflammatory cells. The field saw more collagen and less cellular. H&E stain 20X. At 21 days PW, show the dermis consist of mature granulation tissue, the field seem increase in density of collagen fibers and myofibroblasts (green arrows). Deep dermis contains solitary hair follicles in different stages of development (blue arrows). H&E stain 20X.

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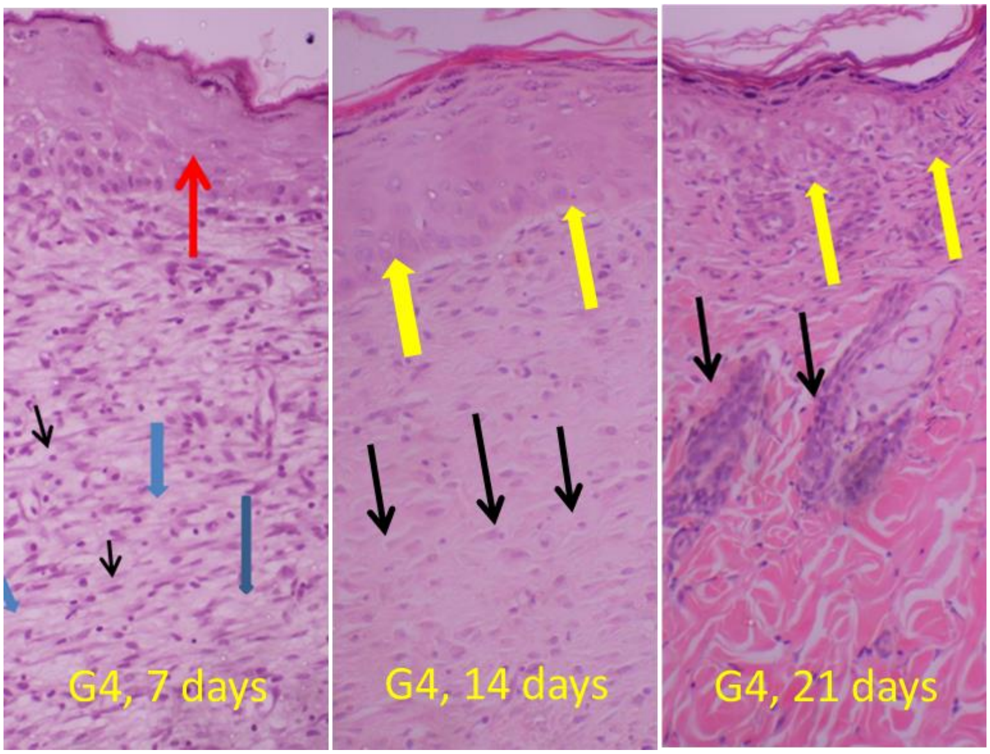

Fig. (7):G4 Treated wounds 7 days PW, show complete thick dense deep basophilic hyper chromic 4 epithelial layers (including the well differentiated keratinized layer and dominant large dark basophilic granulosum cells) riding on clear distinct basement membrane (red arrows). The dermis involved with dense cellular young pinkly granulation tissue (blue arrows), PMNC (black arrows). H&E stain 20X. At 14 days PW, show the 4 layers of epidermis taking deep basophilic stain, resting on even well distinguished basement membrane (yellow arrows). The dermis enfolds mature fibrous tissue (black arrows). H&E stain 20X. At 21 days PW, show usual thickness of epidermis consist from 4 well differentiated layers with prominent granulocyte cells, resting on invaginator basement membrane(yellow arrows). The dermis filled with mature granulation tissue (black arrows), more hair follicles (white arrows) and more sebaceous glands were seen. H&E stain 20X.

Discussion

Macroscopic Findings

The sizes of the wounds related to the treated groups showed significant decreases with no inflammation or infections at all the time of monitoring which reveals important effects of the 880-nm diode laser treatment. Such time-based decreases in the sizes of wounds were not noticed in the control groups; however, the control group animals declared such reductions in their wound sizes after a longer period of time when compared to that in the laser treated groups. This fact agrees with (32) who also examined the effects of laser on skin wound healing, in which they randomly categorized experimental groups: laser therapy at 808 nm of laser directly after wounding, 24hrs after the wound inductions, and 72hrs after the wound occurrence and detected that the wound size contraction levels of the treated groups showed a substantial improvement as compared to the control subjects throughout all sampling time points, confirming the biostimulative impact of the high-power pulsing light in the present research.

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Our results also detected no signs of inflammation or infection in the laser treated sites with almost full recovery that contains the restoring of epithelia, and these outcomes go in line with the findings by (33) to show high rates of similarity.When the incision sites were treated with methylene blue, the regaining of epithelization and the reduction in the wound size were not significantly different when compared these features with those of the control subjects. It is not like the results by the laser treatment. This result agree with those by (22) who also identified that the use of methylene blue as a photodynamic therapy did not appear to add significant positive impact in accelerating the healing process, in which they recognized that the size of the incisions they made were large especially when compared with those from the laser therapy they used plus these incisions have restored epithelization in 14 days while it was 5-7 days in the laser therapy treated incisions (22).However, research on photodynamic therapy impact on wound healing has produced opposing findings. Other authors demonstrated that photodynamic therapy increases the development of granulation tissue, accelerates re-epithelialization, and reduces muscle fiber necrosis, all of which aid in wound healing. However, in a subsequent animal research, photodynamic therapy seems to reduce wound recovery and wound tensile strength, as well as the epidermal necrosis seen in the photodynamic therapy-treated wounds. Despite generating no scars or persistent regions of epidermal necrosis on the photodynamic therapy sample after 21 days, these characteristics were noticed within the starting few days (34).Our results indicated that the use of methylene blue in combination with the laser therapy was give better result than laser alone but it is not strong enough. This section is in an agreement with the findings by (22) who also have found that using the dye in conjunction with the laser did not quicken the healing procedure as compared with those from the utilization of the laser therapy alone. Also in accordance with previous reports, the photodynamic therapy did not induce any tissue suppression in the healing process, and the connective tissue reconstruction was greatly noticed in the photodynamic therapy cohort. However, in this research, we found little difference in the rate of wound healing between the dye and control groups. Furthermore, laser phototherapy, in conjunction with a photosensitive dye, was shown to have an antibacterial effect that may enhance tissue regeneration (35). This organic cationic dye is antibacterial and specifically targets dead cells. Because of its selective action, methylene blue can be quite effective against gram negative bacteria and fungus, and it can adhere to protein-rich exudate and infectious waste. Methylene blue is water-soluble, can be applied easily, and dries fast; it becomes much more effective when light is applied (35).

Microscopic Findings

At day seven, the results showed the occurrence of thick epithelial layers that connect the wound edges were seen covered by a large scab with the presence of epidermal thickening at the edges of the wound. The wound parts also contained no edema and no congestion, with presence of high numbers of inflammatory cells and more collagen fibers, new vascularization, and numerous fibroblasts.

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These findings indicate a well-established healing process. Our results agree with those by (32) who have found that, at day seven, there were aggregation of inflammatory cells including macrophages, neutrophils, and lymphocytes in the dermal layer of the skin of rats. Moreover, infiltration by fibroblasts with the presence of papillary dermis were also recorded. A few numbers of fibroblasts, new capillaries, and deposition of collagen were also noticed. However, they recorded the occurrence of newly initiated collagen deposition that missing hair follicles in the deep dermal layer of the skin. Newly generated muscle fibers were also seen due to the transformation of connective tissue with presence of high numbers of lymphocytes, fibroblasts, and macrophages (32). Although this low-level laser therapy is said to help increase mitochondrial activity, thereby increasing ATP, vasodilation, protein synthesis, the reduction in the anti-inflammatory prostaglandins, cell mitosis, and relocation and multiplication of keratinocytes, there are also claims that it stimulates the creation of new capillaries (36). (22) have also found that laser therapy had produced significant effects in promoting a god healing process, in which they recognized collagen matrix replacing the granulation tissues. At this time, they found that the wound sites had less inflammatory reactions when compared to the control and the methylene blue subjects. Moreover, re-epithelization was highly recorded in the laser group than that in the methylene and control individuals (22). These findings agree with our results in the case of the laser, methylene, and control groups.At day 14, the current study results indicated the presence of no scabs and wound-edge connecting thin and condensed epithelial layers. Less numbers of inflammatory signs such as inflammatory cells were seen. Mild newly vascularization process was seen with no congestions and the presence of more immature collagen depositions. These results come in an agreement with those by (22) who have detected the presence of residual granulation and higher levels of young fibroblasts in the methylene and control subjects with re-epithelialization in a complete way in all study groups.The increased collagen synthesis is via the use of light to stimulate fibroblast development, resulting in cellular multiplication. The mitochondria could absorb such energy better, and this may have led to an increase in ATP and DNA synthesis, with the outcome being an improvement in collagen formation, epithelial healing, and granulation tissue development (37).Our results was in agreement with (32) have also found, epithelial proliferation over tissue of granulation. These findings agreement with our results detected at day 14 of the experiment.At day 21 of the experiment, the current work investigation found that thick epithelial layers, mature collagen, scanty blood vessels, and hair follicle buds were seen in the laser treated group. Our results agree with those by (33) who have detected the presence of a fibrosis zone which is cell-rich, the occurrence of discrete-looking vessels, and the development of dense connective tissue, and complete re-epithelization was seen.Laser therapy can act in improving wound healing by reduce inflammation, pain relief, reduction in the edema production, and keeping the adjacent tissue safe. These features can be provided by using laser wavelength at 600 and 1000nm and

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power strength from 1mW-5W/cm^2 (38). data of the current study, methylene blue alone has less improve healing time as compared with that from the data of the laser treated group. This is also in agreement with data by (22) who have detected no improvement in the shortening the time of wound healing.

Conclusions

The treatment of skin open wounds in rats using 820 nm diode laser in a dose of 8J/cm^2 (energy density) were seen improve the healing process by increase the proliferation of the epithelial cells and activation of re-epithelization, and accelerate the proliferation, wound contraction, maturation and remodeling phases of wound healing. Treatment with methylene blue alone gives better healing process than control, but less than laser treatment, while conjugating of methylene blue with diode laser in the mentioned dose give better enhancement of wound healing.

Conflict of interest

The study has no conflict of interest

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