Effects of green laser therapy on healing of infected wound in mice.

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Abstract:
Background: Little studies focused on stimulation of healing process of infected wound in mice. The predominant pathogens that infected wound were gram positive such as *Staphylococcus aureus* and gram negative such as *P. aeruginosa* and *Acinetobactor Baumannii*. *A. baumannii* cause wide range nosocomial infections.
Objective: study the influence of green laser 532nm at constant power density (irradiance) at different exposure times (5, 15) on healing of wound infected by *Acinetobacter Baumannii* created on BALB/C mice.
Methods: An elliptical full thickness skin wound was created aseptically on a back of 45 adults' females BALB/C mice. The wounds were infected by *Acinetobacter Baumannii* and the mice were randomly divided into two sets, first one was infected (non-irradiated controls)15 animals (five in each subgroup)dividing according to the days of irradiation, second set was infected(irradiated groups)randomly divided in to two groups, first group irradiated for 5 mints and second group irradiated for 15 minutes. Both irradiated groups subdivided according to follow up period 3, 5, 10 days. Animal killed on day 3, 5, and 10 after treatment and control group killed also on 3,5,10 after contamination. Cold laser therapy (CLT) achieved by semiconductor diode laser 532nm, output power =40mw, continuous wave laser, power density =5.71 mw/cm^2 and doses 1.7J/cm^2, 5.14J/cm^2. The output of green laser fitted with beam expander to irradiate a circular area of diameter 7cm^2. CLT started after surgery and repeated for 3, 5, and 10 days. Wound healing studied by calculating the percentage of wound closure and histopathological evaluation.
Conclusion: the present study showed that green laser therapy had obvious influence on healing of infected wound especially with a dose 5.14 J/cm$^{2.}$
Keywords: Green laser therapy, infected wounds, *Acinetobacter baumannii*

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Introduction:

Cold laser therapy CLT is a type of laser irradiation or monochromatic band light on a system of living organism, which stimulates or inhibits biological functions but does not result in irreducible damage.
The CLT used in photobiostimulation is always low irradiance about 10 mw/cm^2. However, irradiance for CLT, 10^2-10^3 mw/cm^2 is of CLT if exposure time is not so long that it damages organelles cells. Today, CLT has increased considerable importance among treatment types for different medical problems including wound healing , and pain management.[1-3]
A lot of researches were reported that CLT can improve the healing process by lowering or minimize pain and inflammation, develop cell proliferation, increasing collagen synthesis, supporting immunity tensile stableness. [3-7] Considerable amount of studies show that CLT with suitable treatment parameters can stimulate in wound healing in infected wound mice. [13]

A. Baumannii, still remain a major problem regarding morbidity and mortality in both civilians and wounded military service members.[9]
Antimicrobial resistance of A.baumannii in Iraq has increased which may affect the antimicrobial resistance of this organism worldwide. [10]

Materials and Methods

Study subjects:

Forty five (45) BALB/c mice weighing (18-32) gram were enrolled in the study. Animals were kept in individual plastic cage in hygiene conditions with wood chip bedding and maintained at 22c^o in day/night light cycle and fed with standard pelted laboratory diet and had water ad libidum. The study was approved by the animal house of National center for drugs control researches committee/Iraq.

Irradiation procedure

All equipments were calibrated prior to the study to make sure they delivered an accurate dose during the study protocol. The method of irradiation was standardized before experiment. Low energy continuous wave portable green laser 532 nm from Laser scientific Ltd, UK) were used in all experiments of irradiation. The output power was measured using a laser power meter (SOLOPE Genetc-EoInc, Canada).
Cold laser therapy was started immediately after wounding and repeated for three, 5 and 10 days. The protocol was chosen because the conventional clinical approach to laser therapy for wounds in three and five exposure per week of 24 hours. [11,12]
This protocol was chosen because the conventional clinical approach to laser therapy for wound is 3and 5 exposure per week of 48 hour interval. [3]
Each laser was organized in a metal holder which is fixed the laser and beam expander in perpendicular position over the wound and a fixed distance between beam expander and the wound surface. Beam spot size for green laser 532nm was 7cm² at the wound surface, doses were 1.7J/cm² and 5.14J/cm², exposure time five and 15 minutes, power density (irradiance) =5.714 mw/cm^2, output power of green laser measured after beam expander equal to 40mW.

Wound model:

On day zero, the day of wounding inoculation, mice were anaesthetized with injection of ketamine at 130mg/kg and xylazine at 10mg/kg was given via injection for pain management. Hair was clipped

from the cervical to mid-lumber dorsum. The operative site was prepared aseptically with alcohol 70% and an elliptic full thickness skin wound (incision wound) was created aseptically with scalpel in all mice on the shaved back of the animal skin defect overlying the thoracic spinal column and adjacent musculature [3,4]. Figure Each wound measured approximately (1.4-2.5 cm), the wound was left uncovered during whole period of experiments. Figure 1.

Figure 1: Incision wound

Percentage of wound closure:

At 3, 5, and 10 days after wounding, the area of wounds of all mice were recorded. The wound area of all mice was measured at regular intervals with a caliper. The wound area for all ellipses was calculated as follows:

Area=L/2×W/2×π (cm^2). Where L and W are the length and width of wound respectively. Percentage of wound closure was calculated using the following Formula: [13].

(Area of day one - Area of x days)/ Area of day one) ×100%

Inoculation preparation:

Swab samples were taken from wound area of patients whose wounds suspected to be infected by *A.baumannii* (using sterile swabs in transport media). These samples were collected from patients hospitalized at AL-Yermook teaching hospital in the Baghdad during period from February 2015 to March 2015. *A. baumannii* is isolated and identified using microscopic, cultural characteristic, biochemical test, and API system. One isolate of *A. Baumannii* was selected according to the resistance test to several antibiotics .Standard of suspension of bacterial growth with dilution of 10^6 CFU/ml (viable cells/ml) was chosen from the other serial dilution from *Acinetobacter Baumannii.*

Immediately after the creation of wound, a bacterial suspension containing 10^6 CFU/ml in 100µl sterile normal saline was inoculated on the surface of each wound with a pipette tip and then was smeared on to the wound surface with an inoculating loop. [14]

Study design

Prior to surgery, an inoculation of the wound with bacterial suspension was done. Animals were divided into two principle groups, irradiated group (30animals) and control infected non irradiated group (15animals). Wounds were irradiated for 3, 5 and 10 days beginning on day 1 immediately post inoculation. This regimen was chosen because the common clinical approach to laser therapy for wounds is three and five exposures a week at 48 hours intervals [11, 12, 15].Animals were euthanized immediately after completion of exposure of the wound on day 3, 5 and 10. The distribution of animal groups shown in Table (1).

Histopathological Evaluation:

At 3, 5, and 10 days after wounding, five mice were selected from each group randomly and killed by ether inhalation. The tissue specimens were stained with hematoxylin and eosin examined with a semi-quantities method [16] to evaluate following histopathological parameters: polymorph-nuclear leucocytes (PMNL), re-epithelialization, fibroblasts, angiogenesis, granulation tissue formation and collagen fibers deposition. [11,12].The section were examined by two trained observers and assessed on a scale of 0-3. [16].Glass slides were prepared and evaluated by two pathologists who were not aware of the sample codes. By using light microscope (Olympus, Japan), sections were graded for wound healing according to seven parameters related to acute inflammatory response and repair: polymorph nuclear leucocytes, granulation tissue, fibroblasts , collagen deposition, and evidence of epithelialization. Each feature was semi-quantitatively evaluated (from 0=absent or no evidence, to 3 = prominent or marked) based on well defined and reproducible histological feature as described by [16].

Statistical analysis:

Statistical analysis and reporting of obtained data were carried out by using the computerized database structure; statistical package for social science (SPSS V.20, computer software was used for this

purpose). Frequency distribution was done for the study variables. Data were reported and presented as mean ±SD and or (95% confidence interval) for the normally distributed variables. The bootstrapping was done for small groups to the 1000 sample size and the statistical significance of difference between mean of a normally distributed continuous parametric variables of two groups was assessed using the independent samples students' t-test; and the Analysis of variance (ANOVA) were used to compare continuous parametric variables between more than two groups. Statistical tests were approved by assuming a null hypothesis of no difference between mean of variable, a P value ≤0.05 and ≤0.005 was considered statistically significant. Histopathological parameters were compared via Chi-square test. The association between two categorical variables was assessed by Chi-square test of independence.

Results:

The wound inoculated with *A. Baumannii* shows after 48 hours of inoculation a clinical signs of infection which includes skin inflammation, redness and swelling at a site of infection with few pus spots on the skin.

Percentage of wound closure of infected wound groups irradiated by green laser 532nm

After 3 days of treatment with green laser, mean percentage of wound closure was (21.2±9.1) for group exposed to 5 minutes at dose of (1.7J/cm²) and (19.1±5.9) for group exposed to 15 minutes at dose of (5.14J/cm²),while the percentage of wound closure for infected un-irradiated control group was (20.0±7.0).The mean differences between irradiated groups and control group was not significant, (P_{ANOVA}= 0.9) Table 2, Fig 2. After 5 days of treatment, mean percentage of wound closure was (46.7±25.8) for group exposed to 5 minutes at dose of 1.7J/cm² and (52.9±24.1) for group exposed for 15 minutes at dose of (5.14J/cm²). Both percentages were higher than that of control group (34.7±5.8), but the difference in means between infected irradiated groups and control group were not significant, (P_{ANOVA} =0.4). Table 2, Fig 2. After 10 days of treatment, irradiated infected groups and control group showed significant difference in means of percentage of wound closure,(P_{ANOVA}= 0.0005).Table 2, Fig 2

Table (1) Distribution of Animal groups

Infected control groups	**No. of animals**	**Infected and irradiated Groups**	**No. of animals**
Group for 3 days	5	Irradiated with **5** minutes for **3** days	5
Group for 5 days	5	Irradiated with **5** minutes for **5** days	5
Group for 10 days	5	Irradiated with **5** minutes for **10** days	5
		Irradiated with **15** minutes for**3** days	5
		Irradiated with **15** minutes for**5** days	5
		Irradiated **15** minutes for **10** days	5
Total No. of animals =**30**+**15**=**45** animals			

Table (2) Percentage of wound closure for infected wounds irradiated for 5 and 15 minutes with green laser at day 3, 5 and 10 days after treatment.

Follow up period	Exposure time (min)	Percentage of wound closure of Infected wounds for green 532nm laser			
		Infected wound (M±SD)	Control (M±SD)	Calculated t-test (P-value)	ANOVA* F value (P-value)
Day3 (No =10)	5 (No:5)	21.2±9.1	20.0±7.0	0.249 (0.81)	0.1046 (0.90)
	15(No: 5)	19.1±5.9		-0.211 (0.84)	
Day5 (No = 10)	5 (No: 5)	46.7±25.8	34.7±5.8	1.02 (0.34)	0.9975 (0.40)
	15(No: 5)	52.9±24.1		1.64 (0.14)	
Day10 (No =10)	5 (No: 5)	57.9±2.0	48.2±18	1.19 (0.27)	15.17 (0.0005)***
	15(No: 5)	84.7±4.9		4.37 (0.0024)**	

**Bootstraping was done for the independent samples up to the sample size 1000.*

***=t-test; statistically significant at level of significance of 0.05, 0.005, compare between irradiated group and control group.*

****= one way ANOVA; statistically significant at level of significance of 0.05, 0.005, this statistical test compare between two irradiated groups and control group included in this experiment.*

Dose =1.7 J/cm² for 5 minutes exposure time, Dose=5.14 J/cm² for 15 minutes exposure time, No: number of subjects.

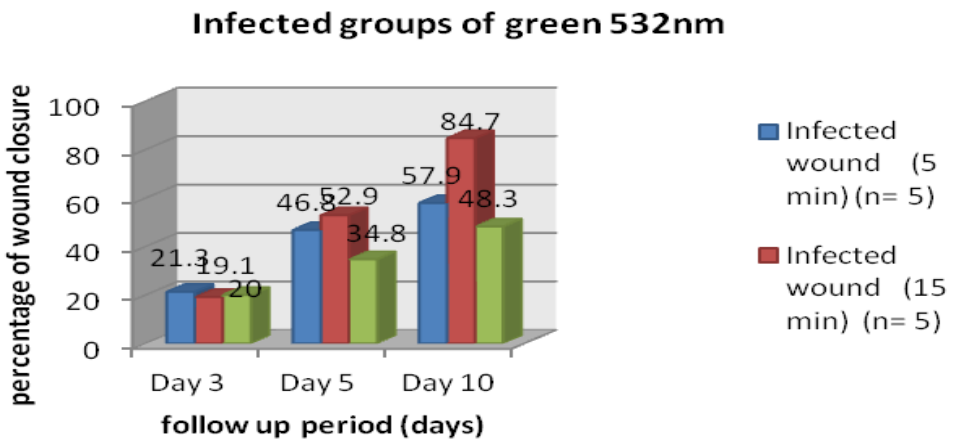

Figure 2: Percentage of wound closure after 3, 5 and 10 days of treatment with green laser compared control group stratified by exposure time(dose) and bacterial wound inoculation status.

Other cellular parameters associated with wound closure: Wound healing assessed by using qualitative variables (healing parameters).These includes re-epithelialization, granulation tissue formation (fibroblast and angiogenesis), and inflammatory reaction (leucocytes and macrophages). Keratinocytes, an essential cellular complex of epidermis are responsible for renew the epidermis after injury through Epithelialization process .Epidermis and dermis supply the perfect cover for any wound**.** Collagen deposition and remodeling share in the increase of strength and scar formation of the wound as a support for the injured tissue and myofibroblast seems to have a restricted role in the re-epithelialization process and associated more with increase extracellular matrix secretion.

Epithelialization is an important structure of wound healing and used as a defining parameter of its success, and the wound cannot be considered healed in the absences of re-epithelialization. So, for these reasons , this study concentrate on re-epithelialization as a corner stone parameter for Histopathological evaluation of wound healing in addition to granulation tissue, inflammatory cells infiltrate and collagen deposition. [18]

This study designed to evaluate the effect of exposure time on healing parameters, for each type of laser and for a combination of two wavelengths by beam combiner. Also for two inoculation status, and on follow up periods (3, 5 and 10 days).

Chi-square test used for study the effect of exposure time of laser on histopathological parameters, to study whether healing parameters of irradiated subjects and control subjects had a significant difference.

The bar graph is a way to visually represent a set of histopathological data to observe and summarize difference in response between groups.

Outcome results of wound healing process described as:

Complete healing, incomplete healing and no healing response. [18]

- **Complete healing:**

Complete healing characterized by complete re-epithelialization, moderate to marked granulation tissue formation, presence of collagen fibers and scattered to mild inflammatory cell infiltration.

- **Incomplete healing:** Characterized by (incomplete re-epithelialization, mild to moderate granulation tissue formation, presence of collagen fibers, mild to moderate inflammatory cell infiltration.
- **No healing response:** Characterized by (no evidence of re-epithelialization, no evidence to mild granulation tissue formation, absence of collagen fibers and moderate to marked inflammatory cell infiltration.

Histological assessment of wounds of control groups: Histopathological results for all selection with hematoxylin and eosin were summarized according to day of euthanasized and whether wounds are infected or non-infected.

Nearly all commonly reported results for control groups are tabulated in table 3 All un-irradiated wounds were incompletely re-epithelialized with the inflammatory response of extremely ranging from moderate to marked; nearly all had granulation tissue developed mainly at the wound edges and ranging from no evidence to moderate.

- **Results of control infected group:** Three days after wounding showed no evidence of healing characterized by no evidence of re-epithelialization, no evidence of granulation tissue formation, moderate inflammatory reaction, absent collagen fibers and presence of necrosis. Five days after wounding ,it showed no evidence of healing characterized by no evidence of re-epithelialization ,no evidence of granulation tissue formation ,marked to moderate

inflammatory reaction, absent collagen fiber and, presence of necrosis.

Ten days after wounding, it showed incomplete healing response expressed by incomplete re-epithelialization, mild granulation tissue formation, and moderate inflammatory reaction, presence of collagen fibers and absent necrosis tissue. Figure (3).

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Table 3: Main histological aspects of infected wound control non-irradiated groups.

Infected wounds groups
Day 3
Epith: No evidence Granul: No evidence Inflamm: Moderate Collagen: Absent Necrosis: Present
Day 5
Epith: No evidence Granul: No evidence Inflamm: Marked Collagen: Absent Necrosis: Present
Day 10
Epith: Incomplete Granul: Mild Inflamm: Moderate Collagen: Present Necrosis: Absent

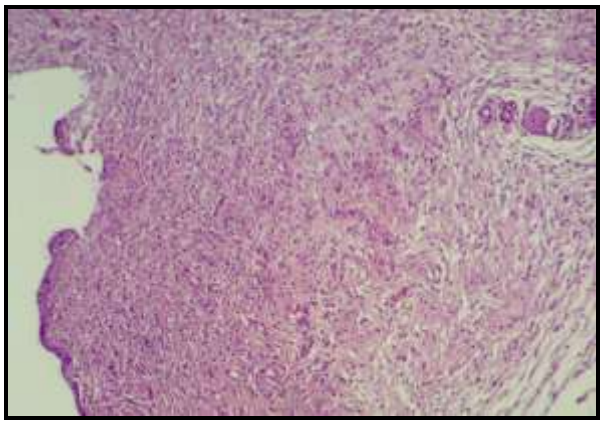


Figure 3: Photography of specimen of infected control at 10 days. Incomplete healing, incomplete epitheliazation.

Histological evaluation for groups irradiated with green laser 532nm

Irradiated groups classified into groups according to laser dose and bacterial inoculation status:

Group 1: infected wounds group irradiated for 5 minutes exposure time at dose 1.7J/cm².

Group 2: infected wounds group irradiated for 15 minutes exposure time at dose 5.14J/cm².

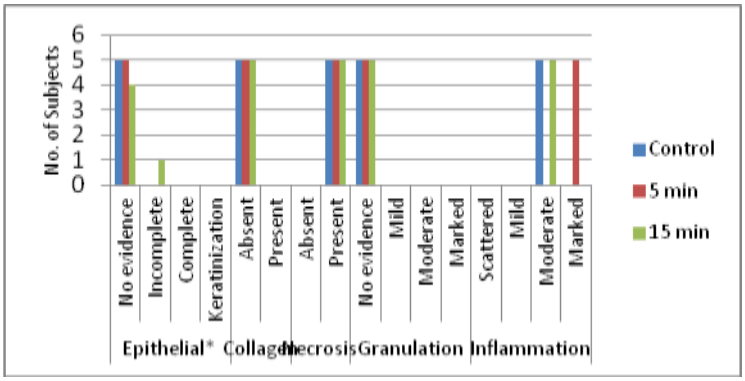
- **Histological assessment at day 3 after treatment by green laser**: Infected wounds irradiated groups showed no evidence of healing compared to control group .Figure4.
- **Histological assessment at day 5 after treatment by green laser:** Comparing infected groups exposed to 5 minutes at 1.7J/cm² to control infected group showed no evidence of wound healing, while the irradiated groups exposed to 15 minutes at 5.14J/cm² showed incomplete response of healing (incomplete re-epithelialization, mild granulation tissue formation, presence of collagen fibers and moderate inflammatory reaction. Figure 5, Figure 7

Significant difference in increase of re-epithelialization (P=0.001), granulation tissue formation (p=0.001) and inflammatory response (p=0.024) compared to control infected group Figure 5.

- **Histological assessment at day 10 after treatment by green laser**

Infected group irradiated for 5 minutes at 1.7J/cm²and control infected group showed no evidence of healing response, while group exposed to 15 minutes at (5.14J/cm²) showed complete healing response. Figure 6, Figure 8

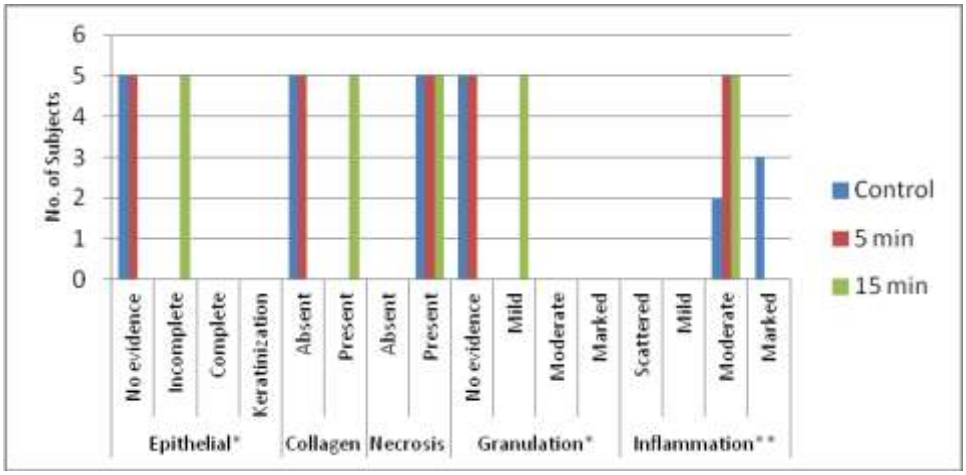
Infected groups 3 days after treatment(green532nm laser)



* Significant (Chi-square=15, df=2, P=0.001)

Figure 4: The Semi quantitative histopathological evaluation 3 days after treatment by green laser.

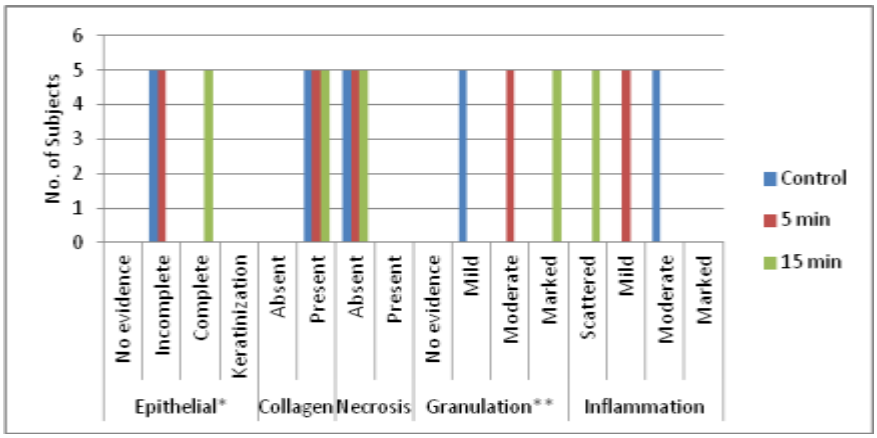
Infected groups 5 days after treatment(green532nm laser)



** Significant (Chi-square=15, df=2, P=0.001) ** Significant (Chi-square=7.5, df=2, P=0.024)*
** Significant (Chi-square=15, df=2, P=0.001)*

Figure 5: The Semiquantitative histopathological evaluation 5 days after treatment by Green laser

Infected group 10 days after treatment(green532nm laser)



** Significant (Chi-square=15, df=2, P=0.001) ** Significant (Chi-square=30, df=4, P=0.005)*

Figure 6: The Semi quantitative histopathological evaluation 10 days after treatment by green laser.

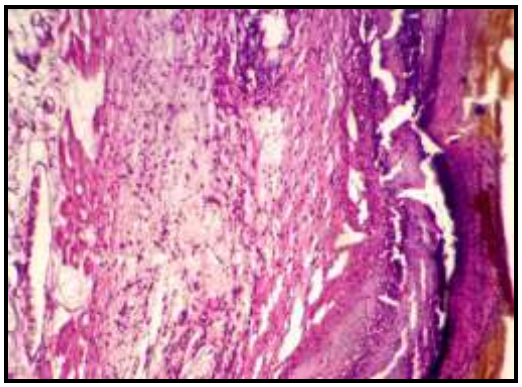


Figure 6: The Semi quantitative histopathological evaluation 10 days after treatment by green laser.

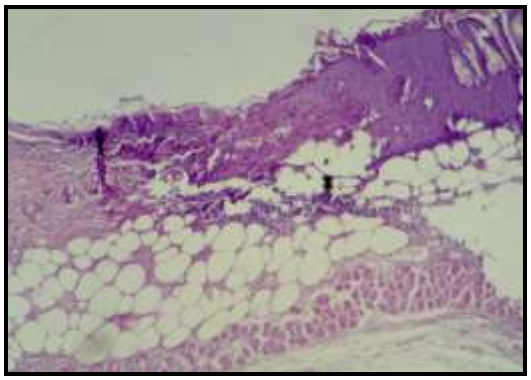


Figure 7: Photography of specimen of the green 532 nm laser (1.7J/cm²), for infected group at day 5 (5min exposure) showing no evidence of healing.

Discussion

The results showed that wound healing on control non-irradiated mice with infected wound by A.baumannii was slower than on irradiated groups by 532 nm laser. There was a significant difference (P=0.0005) in the percentage of wound closure between two irradiated groups , at two doses (1.7J/cm^2 ,5.14 J/cm^2)and control group at 10 days after treatment.

The percentage of wound closure and histopathological parameters of wound closure and histopathological parameters of wound healing accelerated after green laser therapy showed that the optimum incident dose was 5.14 J/cm^2 at exposure time equal to 15 minutes. Histopathological evaluation showed complete response of healing at 10 days after treatment, while control infected group showed incomplete healing response at 10 days after wounding.

The reason for the effectual stimulation of wound healing on mice with infected wound using cold laser was that may be the absorption of laser light with specific wavelength by target tissue resulted in the improvement of fibroblast proliferation and the progress of collagen metabolism and granulation tissue formation in mice with infected wound**.** [19]

The healing process of infected wounds is a complicated and interrelated one and is begin by various series of events that include chemo attraction, growth factor pathways, complement generation, the energy- poor environment established by low oxygen tensions, low PH. [16] Macrophages attracted to such circumstances and generate effective growth factors, resulting in energetic angiogenesis and augmentation of fibroblast at wound margins. The principle activities of the fibroblasts are the generation of elements of extracellular matrix and wound reduction or narrowing. In wound field the fibroblasts correlated to the formation of granulation tissue, and the fibroblasts generate collagen, which is the most important protein within extracellular matrix and responsible for the tensile strength of wounds. [21]

Some studies has shown that the defects that occur in infected wound healing may be caused by altered collagen metabolism and abnormal granulation tissue formation.[22] Al-Watban etal [4] proposed that using 532 nm green as a cold laser with output power 143mw,spot size (7cm^2) ,power density= 20.4 mw/cm^2,and incident doses (5,10,20,30) J/cm^2 used to enhances wound healing in diabetic rats , with treatment schedule 3 times/week. He concluded that response of healing appeared in all doses used. This in agreement with results of this research where the best response was seen at a dose 5.14J/cm^2. [4]

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