Effects of cold laser therapy on infected wound healing in mice

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Abstract:

Despite a lot of researchers were suggested that cold laser therapy can enhance healing by reducing inflammation, increase cells proliferation, enhanced collagen synthesis, its use still controversial. The optimum parameters have been still not defined. We aimed to study the effect of cold laser therapy at a constant power density (Irradiance) at different illumination time (5,15 minutes) on healing of noninfected and infected wounds. Ninety animals with cutaneous incisional wound were divided into 12 irradiation groups, six groups for each noninfected and infected animal, and 30 animals as nonirradiated controls divided into noninfected and infected groups. All groups irradiated with red Laser light 635 nm with constant irradiance 9.0 mw/cm², power= 60 mw, continuous wave started immediately after surgery and repeated every day for 3,5,10 days using lens expander to cover the wound area (2.7J/cm²) for 5 minutes and (8 J/cm²) for 15 minutes. Wound healing was studied by calculating the percentage of wound closure and histological evaluation. The animals were killed either at 3, 5, or 10 days after irradiation. Specimen were taken, embedded in paraffin and sectioned, stained for histological analysis. Cold laser therapy clearly stimulates wound contraction, granulation tissue, collagen formation and reduces inflammation in both irradiated groups (infected and noninfected) groups. Polymorph nuclear infiltrate was lower in both noninfected irradiated groups at (2.7J/cm²), 8.0J/cm² compared with the control. The synthesis of collagen was enhanced in both noninfected groups compared with noninfected controls. Significant difference in the newly formed granulation tissue in the irradiated groups was recorded on five days after injury compared to noninfected controls. On five days, the response of noninfected wounds at (8.0J/cm²) was more than the group exposed at (2.7J/cm²). On day 10, good response was noted in both irradiated noninfected groups compared to controls. On day 5, the irradiated groups were more responsive at (8.0J/cm²) compared to infected controls. At day 10, infected irradiated groups had complete healing compared to incomplete healing in infected control group. We concluded that the most important parameter in determining the optimal light delivering regimen is irradiated or illuminated time.

Key words: cold Laser therapy, infected wounds.

I. INTRODUCTION

Cold laser therapy (CLT) is related to a group of recent experimental process used in wound healing therapy [1]. CLT as therapeutic tools was introduced by the research of Mesterand Colleagues, who noted an enhancement in wound healing with application of low energy ruby laser. [2, 3]. Wound healing consists of three distinctive phases: inflammation, tissue formation, and tissue remodeling[4]. The skin is one of the most important and biggest organ in the body achieved a lot of vital functions, such as immunologic functions, neurosensory function, fluidhomeostasis, and providing essential protection against infection by acting as physical boundary when the barrier is injured, the pathogen can infiltrate the body, preceding, infection [5].

The primary pathogens that infected the wound are gram positive bacteria such as S. aureus and gram negative bacteria such as klebsiellapneumonia, P. aeruginosina, and A. Baumannii of primary pathogen in wound infections[6]. A. baumannii cause wide range nosocomial infections. Including Ventilation associated infection, urinary tract infections essentially, bacteremia by multidrug resistance, A. baumannii cause ≥50-60% mortality rate even with antibiotic treatment[7, 8].

Many therapeutic approaches have been recommended of being effective in enhancement wound healing including the use of many light sources such as Laser. Earlier studies have shown that the use of accepted protocols may enhance tissue response may traumatic agents or to either local or systemic conditions [9, 10]. Cold laser therapy at a certain wavelengths may give a positive photobiological effect and accelerate wound healing process. Since addition of its action to promote healing process. Cold laser produce its effect at a cellular level by decrease inflammatory cell response and wound repair [11, 12].

The main mechanism of action of cold laser therapy in wound healing is still not completely accepted, so in this study we planned to examine the effect of different doses of CLT on infected skin wound with Acinetobacter Baumannii in mice using histological evaluation. The aim of study was to explore the effects of cold laser therapy with different doses on wound healing in an animal model.
II. MATERIALS AND METHODS

2.1 Study subjects:
Ninety females 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 22°C in day/night light cycle and fed with standard pelleted laboratory diet and had water ad libidum. The study was approved by the animal house of National center for drugs control researches committee/Iraq.

2.2 Study design:
Animals were divided into two main groups, sixty animals used as irradiated groups (30 non infected, 30 infected), and thirty Animals used as non irradiated groups. Each irradiated group subdivided into 30 non infected and 30 infected animals exposed to 3, 5, 10 days (frequency of irradiation) with illumination time (5 and 15 minutes). Non irradiated control group divided into 15 non infected and 15 infected groups and each subdivided into 3 subgroups contain 5 animals.

2.3 Methods:
2.4 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 Al-Ga-Inp (Aluminum gallium indium phosphate 635nm) from Laser scientific Ltd, UK) were used in all experiments of irradiation. The output power was measured using a laser power meter (SOLOPEGenetc-EoInc, Canada). The laser treated parameter listed in Table (1). Cold laser therapy was started immediately after surgery and repeated 3, 5, 10 days. This protocol was chosen because the conventional clinical approach to laser therapy for wound is three and five exposures per week of 48 hour interval [13, 14]. Laser was fitted with a beam expander at the distal end to irradiate a circular area of diameter 3cm, (area = 7cm², Irradiance 9.0 mw/cm²), which incorporated the wound and some surrounding intact skin, Table (2). The laser was organized in metalholder which fixed the laser perpendicular to and at a fixed distance from the wound surface. The red laser was studied at radiantexposure of (2.7 J/cm²), and (8.0)cm² (5 mint, 300 sec and 15 mint, 900 sec exposure time respectively).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Power density (mw/cm²)</th>
<th>Irradiation time (min)</th>
<th>Energy density (J/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>9.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Non infected group(5min)</td>
<td>9.0</td>
<td>5</td>
<td>2.742</td>
</tr>
<tr>
<td>Infected group(5min)</td>
<td>9.0</td>
<td>15</td>
<td>8.0</td>
</tr>
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</table>

TABLE 2

<table>
<thead>
<tr>
<th>IRRADIATION PARAMETERS</th>
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<tbody>
<tr>
<td>Wavelength</td>
</tr>
<tr>
<td>Model</td>
</tr>
<tr>
<td>Irradiance</td>
</tr>
<tr>
<td>Output power(mw)</td>
</tr>
<tr>
<td>Time of irradiation/day (min)</td>
</tr>
<tr>
<td>Spot size on mouse(cm²)</td>
</tr>
<tr>
<td>Number of animals</td>
</tr>
</tbody>
</table>

2.5 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 ellipticfull thickness skin 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[14-17]. Each wound measured approximately (1.4-2.0 cm), the wound was left uncovered during whole period of experiments.

2.6 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:

\[ \text{Area} = \frac{L \times \pi}{2} \times \frac{W}{2} \times \pi \text{ cm}^2 \]

Where \( L \) and \( W \) are the length and width respectively. \[16,17\]. Percentage of wound closure was calculated using the following Formula: \[16\].

\[ \left( \frac{\text{Area of 1 day} - \text{Area of x days}}{\text{Area of 1 day}} \right) \times 100\% \]

### 2.7 Bacterial strain and inoculation preparation:

Swab sample were taken from wound areas of patients whose wound infection with \( A.\ baumannii \) suspected (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. \( A.\ baumannii \) 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 \( 10^{-6} \) viable cells/ml was chosen from the other serial dilution from \( A.\ baumannii \) \[18\].

Immediately after the creation of wound, a bacterial suspension containing \( 10^8 \) cells in 50 µl 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. \[18\]

### 2.8 Antibiotic susceptibility test:

The susceptibility of ten isolates of \( Acinetobacter Baumannii \) different antimicrobials was determined by Kirby-Bauer disk diffusion method on Muller Hinton agar. Eight different antibiotics: Amikacin (30 µg), Imipenem (10 µg), Ceftazidime (30 µg), Gentamycin (10 µg), Tobramycin (10 µg), Meropenem (10 µg), Cefepime (30 µg), Ceftaxime (30 µg). Inhibition zones developed around the discs were measured by millimeter (mm) using a metric ruler according to clinical laboratories standards Institute [20].

### 2.9 Procedures

Prior to surgery on day 1, animals were assigned to one of 12 possible irradiation groups (5 animals per group) or to control group (30 animals in total). Wounds were re-inoculated for 3, 5, and 10 days beginning on day 1 immediately post inoculation. Animals were euthanized immediately after completion of exposure of the wound on day 3, 5, and 10.

#### Infected control group

- Group for 3 days (No: 5)
- Group for 5 days (No: 5)
- Group for 10 days (No: 5)

#### Total No. of subjects in control infected group = 15 subjects

#### No = number of animals

### 2.10 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 \[21\] to evaluate following histopathological parameters: polymorph-nuclear leucocytes (PMNL), re-epithelialization, fibroblasts, angiogenesis, granulation tissue formation and collagen fibers deposition. \[22\&23\]. The section were examined by two trained observers and assessed on a scale of 0-3. \[21\]. 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 [24].

2.11 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 student’s 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.

3. RESULTS:
During the post surgery period, the non infected animals remained healthy with no clinical evidence of infection. 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. Susbtability of all A. Baumannii isolates to 8 antibiotics, ceftazidime, amikacin, cefepime, gentamicin, tobramycin, impenem, cefotaxime and meropenem were investigated. Results show a high level of resistance of A. Baumannii clinical isolates to most of antibiotics under test. The present study revealed that all A. Baumannii clinical isolates had 90% resistance to ceftazidime, cefepime. This study also showed a highest resistance to gentamicin, (80%), amikacin(80%), cefotaxime(80%), ceftazime (80%), and impenem. Tobramycin and meropenem recorded 70% resistance.

3.1 Percentage of Wound closure
The results summarized in table (4, 5) and fig (1, 2).

Day 5
The wound closure in both non infected groups irradiated with 2.7J/cm², and non infected control groups expressed incomplete healing; while in the group exposed to 8.0J/cm² shows complete wound closure.
In the irradiated infected groups, both exposed to (2.7J/cm² and 8.0J/cm²) shows incomplete wound closure compared to control group which showed trivial wound healing.

Day 10
Both irradiated non infected and infected groups exposed to (2.7J/cm² and 8.0J/cm²) shows complete wound healing compared to control group which showed incomplete healing. Fig (1, 2).

3.2 Histological evaluation
The results summarized in (Fig 3- Fig 8).

Day 3
Enormous inflammatory cells infiltration and necrotic tissue could be recognized in all specimens. No evidence of re-epithelialization, mild granulation tissue formation (new vessels and fibroblasts) was recorded in all non infected and infected groups except for no evidence of granulation tissue was seen in infected group. No difference in new collagen fibers in all irradiated groups (infected and non infected) exposed to (2.7J/cm² and 8.0J/cm²) compared to all control groups. Fig (3, 4)

Day 5
In non infected group exposed to (8J/cm²) shows complete re-epithelialization, mature granulation tissue formation, extensive collagen deposition with decrease inflammation (P=0.001) compared to incomplete re-epithelialization in control non infected group.
In non infected group exposed to (2.7J/cm²) shows incomplete re-epithelialization, moderate granulation tissue formation and mild inflammation, present collagen fibers and absence of necrotic tissue. Significant difference between non infected irradiated groups and non infected control groups for re-epithelialization, collagen fibers (P≤0.001), and granulation tissue formation (P=0.005). In the infected irradiated group exposed to (2.7J/cm² and 8.0J/cm²) there was significant (P=0.001) incomplete re-epithelialization with mild to moderate granulation tissue formation and presence of inflammatory cells and necrotic tissue compared to no evidence of re-epithelialization, granulation tissue formation and absent collagen deposition with marked inflammation and necrotic tissue in infected control group. Fig (5, 6)

Day 10
Acute inflammatory phase of wound healing was entirely accomplished; there were few random polymorph-nuclear leucocytes in all infected and non infected groups left without statistically importance after 10 days of regimen.
Complete re-epithelialization of all groups have covered almost all wound, capillary gradually closed, granulation tissue gradually replaced with fibrous scar, increased collagen fibers. The fundamental factor in exploring the effect of red laser is re-epithelialization in both non infected and infected groups. Non infected groups exposed to red laser (2.7J/cm² and 8.0J/cm²) showed complete re-epithelialization, while non infected control group showed incomplete re-epithelialization.

**TABLE 4**

<table>
<thead>
<tr>
<th>Day of examination</th>
<th>Duration of irradiation (min)</th>
<th>Closure % (healing) of Not infected wound</th>
<th>Calculated (P-value)</th>
<th>t-test*</th>
<th>ANOVA* F value (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not infected wound (m±SD)</td>
<td>Control (m±SD)</td>
<td>t-test*</td>
<td>ANOVA* F value (P-value)</td>
</tr>
<tr>
<td>Day 3 (n=10)</td>
<td>5 (n=5)</td>
<td>22.655±10.167</td>
<td>19.239±7.854</td>
<td>0.594 (0.57)</td>
<td>0.2853</td>
</tr>
<tr>
<td></td>
<td>15 (n=5)</td>
<td>23.239±8.962</td>
<td>0.750 (0.47)</td>
<td>(0.76)</td>
<td></td>
</tr>
<tr>
<td>Day 5 (n=10)</td>
<td>5 (n=5)</td>
<td>59.352±10.894</td>
<td>39.074±6.337</td>
<td>3.60 (0.007)**</td>
<td>11.82</td>
</tr>
<tr>
<td></td>
<td>15 (n=5)</td>
<td>76.544±16.962</td>
<td>4.63 (0.0017)**</td>
<td>(0.0015)***</td>
<td></td>
</tr>
<tr>
<td>Day 10 (n=10)</td>
<td>5 (n=5)</td>
<td>84.212±7.634</td>
<td>65.021±15.315</td>
<td>2.51 (0.037)**</td>
<td>5.558</td>
</tr>
<tr>
<td></td>
<td>15 (n=5)</td>
<td>85.608±8.0213</td>
<td>2.66 (0.029)**</td>
<td>(0.020)***</td>
<td></td>
</tr>
</tbody>
</table>

*Bootstrap ing 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. ***= one way ANOVA; statistically significant at level of significance of 0.05, 0.005.

**TABLE 5**

<table>
<thead>
<tr>
<th>Day of examination</th>
<th>Duration of irradiation (min)</th>
<th>Closure % (healing) of Infected wound</th>
<th>Calculated (P-value)</th>
<th>t-test*</th>
<th>ANOVA* F value (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Infected wound (m±SD)</td>
<td>Control (m±SD)</td>
<td>t-test*</td>
<td>ANOVA* F value (P-value)</td>
</tr>
<tr>
<td>Day 3 (n=10)</td>
<td>5 (n=5)</td>
<td>23.562±10.707</td>
<td>20.011±7.037</td>
<td>-0.620 (0.55)</td>
<td>0.4451</td>
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<tr>
<td></td>
<td>15 (n=5)</td>
<td>16.570±15.742</td>
<td>-0.504 (0.63)</td>
<td>(0.65)</td>
<td></td>
</tr>
<tr>
<td>Day 5 (n=10)</td>
<td>5 (n=5)</td>
<td>51.207±13.120</td>
<td>34.751±5.852</td>
<td>-2.56 (0.034)**</td>
<td>7.235</td>
</tr>
<tr>
<td></td>
<td>15 (n=5)</td>
<td>56.619±7.907</td>
<td>-4.97 (0.0011)**</td>
<td>(0.0087)***</td>
<td></td>
</tr>
<tr>
<td>Day 10 (n=10)</td>
<td>5 (n=5)</td>
<td>89.244±5.367</td>
<td>48.256±18.000</td>
<td>-4.88 (0.0012)**</td>
<td>18.06</td>
</tr>
<tr>
<td></td>
<td>15 (n=5)</td>
<td>85.330±8.488</td>
<td>4.17 (0.0031)**</td>
<td>(0.0002)***</td>
<td></td>
</tr>
</tbody>
</table>

*Bootstrap ing 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. ***= one way ANOVA; statistically significant at level of significance of 0.05, 0.005.
Figure: (1) Wound closure % during experiment in (non infected) groups

Figure: (2) Wound closure % during experiment in (infected groups)

Figure: (3) The semi-quantitative histopathological evaluation at day 3 after wounding (non infected groups).

Figure: (4) The semi-quantitative Histopathological evaluation at day 3 wounding (infected wound)

* Significant (Chi-square=15, df=2, P=0.001).
Figure :( 5) the semi-quantitative Histopathological evaluation at day 5 after wounding (noninfected groups)
* 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 at day 5 after wounding (non infected groups)
* Significant (Chi-square=15, df=2, P=0.001)

Figure :( 7) the semi-quantitative Histopathological evaluation at day 10 after wounding (non infected groups)
* Significant (Chi-square=15, df=2, P=0.001)

Figure :( 8) the semi-quantitative Histopathological evaluation at day 10 after wounding (infected groups)
* Significant (Chi-square=15, df=2, P=0.001)
** Significant (Chi-square=30, df=4, P=0.005)
4. DISCUSSION:
Many studies have been concentrated on the benefit of cold laser therapy on wound healing. So the usage of cold laser therapy as therapeutic tool stays questionable. There are many questions regarding the quality or the biological effects promoted by laser.

It can be hypothesized that the mechanism of LLLT at the cellular level is based on an increase in the oxidative metabolism in mitochondria [25] [26]. Laser treatment is associated by a number of physical factors such as wavelength, spot size, dose, irradiance (power density) and irradiation time (illumination time). So, the effects of these factors on healing action several injuries and skin conditions remain uncertain. [27]

Considerable discrepancy has been identified in the used wound model and in laser parameters used in investigated researches. For this reason the clear comparison between studies and the organization of optimal irradiation parameters for cold laser therapy, as recommended wavelengths, doses and irradiance is not yet accessible [27]. It has been recorded in many studies that cold laser therapy positively affected wound healing by reducing inflammation, enhancing fibroblast proliferation and neo-genesis and facilitate collagen synthesis [28][ 29]. Although many researchers have shown that CLT on wound healing or that there has been no positive effect of CLT on wound healing process [30, 31, and 32]. So, the aim of this study was to evaluate the effect of constant irradiance on healing of infected wound. Samples of noninfected groups revealed more wound contraction, on the fifth day, the pattern of wound healing increase rapidly until day ten, in this portion of time, both infected and non inflected groups showed a significant difference compared to control group which showed more wound contraction.

Wound infection can prolong inflammatory phase, healing and decrease reepithelialization, collagen deposition and wound closure [33]. Both non infected and infected groups showed trivial response of healing after irradiation at (2.7] / cm²) and (8.0) / cm²) on day 3 after wounding compared to same finding seen in the control. While, on day 5, the non infected group showed positive effect of complete healing of the wound at (8.0) / cm²) expressed by complete re-epithelialization, granulation tissue formation, increase collagen deposition and decreased inflammation which goes with the wound closure percentage, this finding suggest that the beneficial effect may be due to the direct effect of laser on host tissue and the effect is immediate and lasting [34]. The results showed that both doses has the same noticeable effect on wound healing but the effect is more clear at (8.0) / cm²) and this in accordance with the results of Castano.etal study 2007 [35] who noticed that when irradiance is constant, the biological effect of laser needs enough time and this effect depend on the total dose (absorbed photon) more than on intensity of laser (irradiance) and phenomenon called (Importance of Irradiated or illuminated time).

Incomplete healing seen in the infected group on day 5 at (2.7) / cm²) and (8.0) / cm²) compared to no evidence of healing seen in the infected control group. Better results achieved on day 10, where the infected and non infected groups showed complete healing at both (2.7) / cm²) and (8.0) / cm²) compared to incomplete healing seen in both controls, and the finding correlated with the histological findings of increase re-epithelialization, granulation tissue formation and collagen deposition and absent of inflammation, this result is in agreement with the result of study published by (Do Nascimentoetal 2004.) (Yu, J. O. Naimetal1997). [36, 37]. Finally, we concluded that the most important parameter in determine the optimal light delivering regimen is irradiated or illuminated time.

5. CONCLUSION
Cold Laser therapy provides a protective mechanism against inflammatory tissue response. Cold Laser therapy with 635nm Laser is highly effective in treating non infected and infected wounds with A. Baumannii. Longer irradiation time were efficacious than short time irradiation without considering total dose or irradiance.

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