ISSN: 0067-2904

Photodynamic Inactivation (Effect of Laser with and Without Photosensitizer) on Viability of Streptococcus Pyogenes

Mawada M. Funjan
Department of Physiology, College of Medicine, University of Baghdad, Baghdad, Iraq

Received: 11/1/2021 Accepted: 30/7/2021

Abstract

This study focused on the bactericidal potency of toluidine blue" TBO"photosensitizer and red laser radiation of 635nmwith different doses against multi-drug resistant *streptococcus pyogenes* (*S. pyogenes*) isolated from infected burns wounds to see if it is susceptible to photodynamic inactivation .

Atotal of 45 isolates were collected from 38" patients" with infected burnwounds samples were collected from September to December 2019.Burns wounds swabs were employed using standard procedures of swab collection. Among these, eleven isolates were multidrug resistant"*S.pyogenes"*. More resistant isolates that has been proved to all antibiotics used. This multidrug resistant isolate used in all experiments. Bacterial suspension was diluted by using serial dilutions. The suspension of *S.pyogenes* in normal saline was treated with" red laser" radiation at a wavelength 635nm with and without "TBO", and investigated the effect of changing laser doses (3.6 ,7 and 10.8 J/cm^2) corresponding to laser exposure time (5,10 and 15 minutes and different photosensitizer concentrations on viability of "*S. pyogenes"*isolated from infected burnswounds. The results of this study suggest that multi- drug resistance"*S pyogenes"* isolated from infected burn wounds inhibited by using photodynamic inactivation mediated by "TBO" and red laser at a wavelength 635 nm. The effective technique for killing or inhibiting "*S. pyogenes* isolated from infected burns wounds is the combination of red laser at a wavelength 635nm and TBO. Thecombination of TBO and red laser light prohibit"*S. pyogenes"*, the optimum results of bacterial inhibition obtained at 50µg/ml, and laser dose 7 J/cm^2 corresponding to exposure time 10 minutes.

Keywords: Phototherapy, low level laser

التثبيط الضوئي (تأثير الليزر بوجود أو عدم وجود المتحسس الضوئي) على حيوية بكتريا المكورات العقدية المقيحة

مودة موسى فنجان

قسم الفسيولوجيا ، كلية الطب ، جامعة بغداد ، بغداد ، العراق

الخلاصة

تعد التهابات جروح الحروق من أكثر مضاعفات الحروق شيوعاً نتيجة تغلغل البكتيريا من الأنسجة المجاورة.وبسبب الاستخدام الواسع للمضادات الحيوية ، فإن انتشار البكتيريا المقاومة للأدوية المتعددة يؤدي الى ظهور مؤشر أنذار بالخطر على الصحة العامة.

*Email: mawadamousa4@gmail.com

أن العلاج الضوئي هو التقنية التي يمكن استخدامها لتثبيط وقتل هذه الكائنات الحية. ركزت هذه الدراسة على استخدام اشعاع الليزر الاحمر مع المتحسس الضوئي تولويدين الازرق (Toludine blue (TBO لقتل وتثبيط المكورات العقدية المقيحة *S.pyogenes المعزولة* من جروح الحروق الملتهبة الملوثة وبجرعات مختلفة من الليزر, ولاظهار فيما اذا كانت هذه البكتريا المقاومة للمضادات الحيوية ممكن تثبيطها وقتلها بأستخدام المتحسس الضوئي بالاشتراك مع اشعاع الليزر 635 نانومتر.تم جمع 45 عزلة من جروح الحروق الملوثة خلال 4 أشهر من سنة 2019 وتم عزل وتشخيص البكتريا واجراء اختبار الحساسية للعزلات البكترية الى 12 نوع من المضادات الحيوية حيث تم عزل وتشخيص 11 عزلة مقاومة للمضادات الحيوية ثم تم اختيار العزلة ذات المقاومة الاعلى لاكثر المضادات الحيوية المستخدمة حيث تم اجراء التجارب على عزلة المكورات العقدية المقيحة *S pyogenes* .الاكثر مقاومة للمضادات الحيوية.وتم اجراء عملية التخفيف لاختيار التخفيف الملائم .

حيث وضع 1ml من التخفيف في micro titter وتم خلط المحلول البكتيري مع TBO بتراكيز 50µg/ml 25µg/ml,وتشعع بالليزر بالازمان التالية 5,10,15 دقيقة الموافقه للجرع الليزرية التالية و(3.6 ,7 and 10.8 J/cm^2,) و تم قياس حيوية البكتريا cFu/ml .اظهرت النتائج ان استخدام المتحسس الضوئي TBO مع التشعيع بالليزر الاحمر يثبط حيوية ويقتل المكورات العقدية المقيحة وتم الحصول على القتل الامثل او التثبيط البكتيري الافضل عند تركيز المتحسس الضوئي 50µg/ml وجرعة الليزر 7 J/cm^2 الموافقة لزمن التعريض 10 دقائق.

تشير نتائج هذه الدراسة الى ان حيوية المكورات العقدية المقيحة المقاومة للمضادات الحيوية والمعزولة من جروح الحروق الملوثة تم تثبيطها وقتلها باستخدام تقنية التثبيط الضوئي بواسطة استخدام المتحسس الضوئي TBO والليزر الاحمر بطول موجي 635 نانو متر

Introduction

"*Streptococcus pyogenes"* produce a lot of human diseases, it is "cocci""gram positive" bacteria organized in pairs or in chains .Some of the highest frequent microorganisms are related to wound infections and include"*pseudomonas aeruginosa","staphylococcus aureus" (S aureus),"streptococcus pyogenes " and " proteus species"*[1]. Inhumans, the infection associated with "*S. pyogenes"*may occur primarily in bloodstream, skin and respiratory tract infections. *"S. pyogenes"*is one of the commonest opportunistic pathogen in wound infection, which cause soft tissue and skin infection.[2] Because of frequent wrong use of antibiotics, there is a rise of bacteria with multi drug resistance which is a great danger to public health.The technique that can be used to reduce this defect and prohibit these organisms could be "photodynamic inactivation"[3] .Surface infection of wounds are fit to be treated byphotodynamic inactivationtechnique as these burns injuries are ready and convenient for local distribution of laser light and photosensitizer, The killingof bacteriain the infected wounds using"Photodynamic inactivation"has been expressed in previous studies[4], [5]" Toluidine blue (TBO)"is" phenothiazine dye" which is an effectual photosensitizing agent for inhibiting of yeasts, bacteria and viruses. [6]. Up to date, there are scattered and scanty researchers studying the lethal characteristics of "TBO" against few types of pathogenic bacteria. It is also ambitious to analyze the outcomes in various conditions." Photodynamic inactivation "treatment integrally manipulatedas photosensitizer accompanied with low dose visible light irradiation.[7] Photosensitizer react with oxygenandproduce reactive oxygen species" ROS" which cause cells to be killed.Two separate paths of reaction known as type one and type two will produce "ROS".Type one reactions required electron transfer from the triplet state of "photosensitizer" that contributes cytotoxic formation, like hydro peroxide, superoxide andhydro peroxide radicals. Type two reactions include the energy transfer producing single oxygen [8]."Photodynamic inactivation"wasstudied extensively, primarily in cancer therapy, recent experiments have shown that this mechanism can destroy microorganisms as well. After treatment with adequate "photosensitizer", 'yeasts", fungi,

viruses and bacteria may be killed in a"photo dynamical activation" process. [9] This approach has been proved to be efficient "*in vitro"* among resistant parasites, yeasts and bacteria.[10],Some of" Photodynamic inactivation"benefits are: a wide variety of actions influences both "gram negative" and "gram positive"bacteria, also the "photodynamic inactivation"does not allow mutagenic effects to evolve [11][12].The aim of this research is to study the effectiveness of "TBO" with various concentrations in combination with laser light and different laser exposure times (dose J/cm^2) on viability of"*S. pyogenes"*.

Methods

Isolation, identification and microbial sensitivity test.

This study focused on infection in burn wounds. Samples were collected during a period from September to December 2019.Patientin formation concerning baseline features and lethal infection of burn wounds were taken.

Study design:

Thirty-eight patients treated at burns unit in "AL-yarmook"teaching hospital/Iraq with acute infected burns wounds .Burns wounds swabs were taken using standard procedure of swab collection of microbiological specimens and were cultured under guidance of microbiologist.

The swabs plated aerobically on blood agar in 5% CO2.Samples were examined as in line with previous studies [13]. [14]. Azide "blood agar" possibly used for the generation of hemolytic reaction [15] Best incubation status for" streptococcal' isolates involve anaerobic conditions for existence of 5% CO2 at 37^{o}c [16]

"S.pyogenes" isolates were identified by gram stain, β- hemolysis bacitracin sensitivity test,catalase-negative and" API-20 strip "(identification of most "enterococci" and" streptococci") , from "(Biomerieux)" to identify "streptococci", as in line with the method reported by" Abraham" and "Sita"[16] the bacitracin sensitivity test was done by using 0.04 units bacitracin disc (bacitracin Discs," ThermoFsher scientific ,oxide") was achieved [17].

Antimicrobial sensitivity test

Antimicrobial sensitivity was detected by disc diffusion technique in alignment with "CLSI" 2019[18], as a consequence, various isolates were categorized as intermediate, resistant or sensitive.Various antibiotics were used in this project, specially "gentamycin" (30µg),"vancomycin"(30µg), levofloxacin (5µg),"tetracycline" (30µg)," amoxicillin" (10µg), "azithromycin"(15µg),"amoxicillin/clavulanic acid "(30µg),cefixime (5µg)," erythromycin(30µg)","cefepime" (30µg), "ceftriaxone"(30µg) and "clindamycin" (10µg)."Disc diffusion" technique for antimicrobial sensitivity test was used to determine the resistance trends of the isolated bacteria. Isolates considered as "a multi-drug resistant"when it is resistant to three or more various groups of antibiotics. [19][20]

Laser and photosensitizer

Laser used in all experiments was a red laser diode ("UK-Scientific") with wavelength 635nm to activate the "photosensitizer". Treatment parameters of laser used in this project as show inthe Table 1.

Table 1- characteristics of red laser and treatment parameters

Output power of the laser used (mw)	60mw		
Wavelength	635nm		
Irradiance measured at target area(watt/cm^2)	0.012watt/cm^2		
spot size of laser beam (cm^2)	5cm^2		
Mode of operation	Continues wave laser		
Dose= Irradiance (watt/cm^2) ×exposure time (sec) Dose= J/ cm^2[21].			
Exposure time(min)	5	10	15
Dose J/cm^2	3.6	**7**	10.8

At the beginning of each experiment, adjustment of the laser set up was done. Output power of laser measured by laser power meter ("genetic-Eo, com"). A convex lens was used as a beam expander of laser light and lighted a circular field (spot size) of 5cm^2. The intensity measured at spot size of 5cm^2 on "micro-titer" plate was 0.012 watt/cm^2.

Photosensitizer used in this project was TBO ("Sigma-Aldrich-merk") with peak absorption 630nm [22] which corresponding to a wavelength of laser diode so that it was chosen in this project. By dissolving 0.05g of TBO powder in 100ml of distilled water to get a concentration of 50µg/ml, also the same steps to get a concentration of 25µg/ml. The solution was sterilized by filtration through 0.22µm "Millipore filter paper" .The stock was kept in a dark place till use. Bacterial suspension was blended with various concentrations of "TBO" (50µg/ml and 25 µg/ml).

Experimental design

Using "*S .pyogenes"* suspension 10^{-5} CFU/ml ("colony forming unit/ml ")' experiments were accomplished to examine the effect of "photodynamic inactivation of" TBO". The essential parameters used in this study are CFU/ml to express the viability of "*S.pyogenes"*.

The experimental groups divided into four groups: First group (controls group), includes specimens without laser radiation and without adding" photosensitizer". Second group includes a bacterial suspension mixed with "TBO" alone without irradiation with laser. Third group consist of a bacterial suspension irradiated with laser at doses (3.6, **7**, 10.8) J/cm^2 corresponding to exposure time of (5, 10, 15) minutes without adding photosensitizer. Fourth group consist of both photosensitizer and laser light at doses (3.6, 7, and 10.8) J/cm^2.

Two steps novel in this procedure to reduce the influence of the heterogeneity of data assigned to bacterial suspension, a new preparation for every single experiment, and control plate for each experiment.

Irradiation procedure

One colony or more than one grow on selective media Azide blood agar, for 24 hours at 37^{o}c,then putting three coloniesfrom selective media with two milliliter of normal saline in sterilize tube then this bacterial suspension was put in a vortex to get homogenous suspension. The optimum optical density is 0.5 using spectrophotometer at wavelength 530nm, this bacterial suspension was diluted by using serial dilutions in normal saline to get 10^{-5}CFU /ml.

Laser irradiation with TBO

Aliquots of (0.1ml ,100µl) of "bacterial suspension" containing10^{-5}CFU/ml in normal saline solution were carried into "a micro titter" plate and equivalent amount of TBO in normal saline was added to every well to get last concentrations of 25and 50 µg/ml, then the wells were left for one minute in dark place ("pre-exposure time") and then irradiated with doses of laser radiation(3.6 ,7, 10.8) at constant intensity 0.012watt/cm^2 .To calculate the viability of bacteria, it was examined by finding CFU /ml, plates were processed for each experiment and incubated for 24hours at 37^{o}c. control groups including the suspension of bacteria and saline without dye solution were managed with the same steps to verify the influence of laser illumination only on viability of bacteria .

Viability of bacteria was examined by finding CFU/ml according to the following formula [23]

$$\frac{\text{CFU}}{\text{ml}} = (\text{Colony number}) \times \text{ dilution factor} \times \frac{1}{\text{volume plated}}$$

Volume plated =100µl=0.1 ml

Laser irradiation without'' TBO''

Two milliliter of bacterial suspension with 10^{-5} CFU/ml was distributed in microtiter plate over the area $5cm^2$ equivalently to $5cm^2$ of laser spot size and irradiated with laser doses (3.6,7,10.8 J/cm^2).

Statistical analysis

''The Statistical Analysis System- SAS'' (2012) [24] program was used to detect the effect of different factors in the studied parameters. The viability of ''*S.pyogenes''*CFU/ml was determined in comparison to control group." Data were provided as mean±standard deviation" (m±SD)", maximum and minimum values .comparison between irradiated groups and control group was done by student t-test. 'Least significant difference –LSD'' test "(Analysis of Variation-ANOVA)" was used to compare between means in this study. P value ≤0.05 indicating a statistically significant difference.

Resultsand Discussion

Over 4 months, a total of 45 isolates were collected from 38 patients with burn wounds,by using wound swabs. Among these, eleven isolates (24.4%) were multidrug resistant *S.pyogenes*. Under microscopic examination, all eleven isolates were in cooci shaped in chains or in couples,'' catalase production test was negative'','' β-hemolytic for all isolates and they were gray and small''.

The results of sensitivity test of eleven "*S.pyogenes*"isolates were as following: Vancomycin showed a clear effectiveness with 60% of sensitive isolates, 9% with intermediate and 27% of isolates showed resistance. Seventy three percent (73%) of isolates that were sensitive to Amoxicillin/Clavulanic acid (AMC), while only 18% showed resistance to this antibiotic, 82% of isolates were resistance to "Amoxicillin (AML)",''Tetracycline (TE)'', and ''Erythromycin (E)''Figure1. All isolates were resistant to ''Levofloxacin (LEV)'', ''Azithromycin (AZM)'',''Cefixime (CFM)'', and ''Clindamycin (DA)''. 45% of isolates were sensitive to ''Gentamycin (CN)'' and 45% of isolates were resistance, While 60% of isolates were resistance to ''Ceftriaxone'' (CRO) with 18% showed intermediate "susceptibility" and 18% with good sensitivity .Figure1

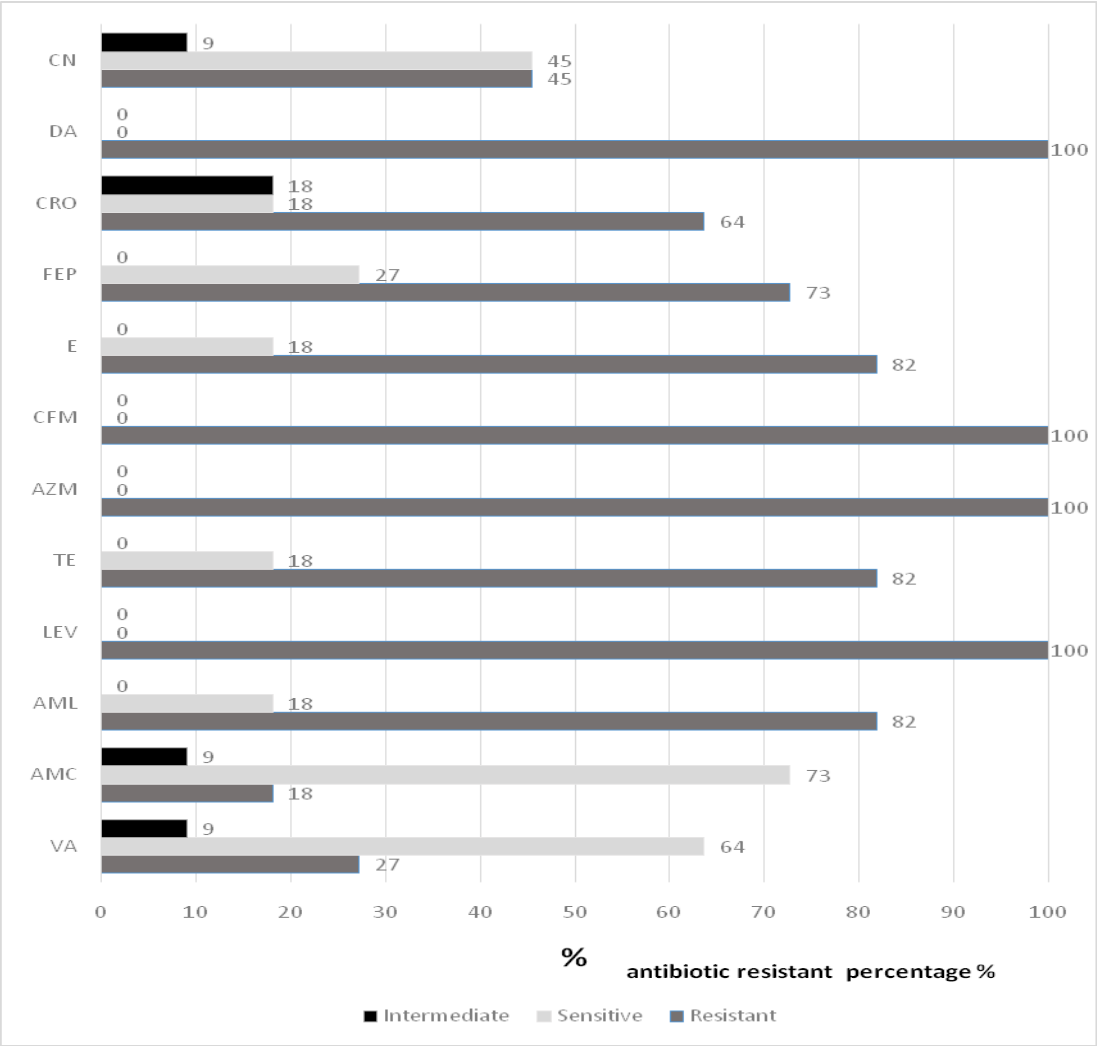

Figure 1- Antibiotic resistant percentage

The laser irradiation without" TBO" did not show an inhibition action of'' (CFU/ml) "Table 2 Figure 2, likewise, no statistical difference in" viability of*S.pyogenes"*between the control groups and groups treated with different concentrations of : TBO"only. Table 3.

Table 2- mean value of viability of *S.pyogenes* LOG CFU/ml treated with laser only.

Dose J/cm^2		**LOG CFU/ml**		
		Laser treatment	Control	T-test (P-value)
3.6	Mean ± SD	7.994 ± 0.200	7.999 ± 0.193	0.1018 NS (0.925)
	Range	7.67-8.39	7.69-8.38	
7	Mean ± SD	8.081 ± 0.205	8.041 ± 0.190	0.1204 NS (0.436)
	Range	7.71-8.37	7.69-8.35	
10.8	Mean ± SD	8.011 ± 0.157	8.013 ± 0.152	0.0799 NS (0.960)
	Range	7.68-8.35	7.74-8.36	
NS: Non-Significant.				

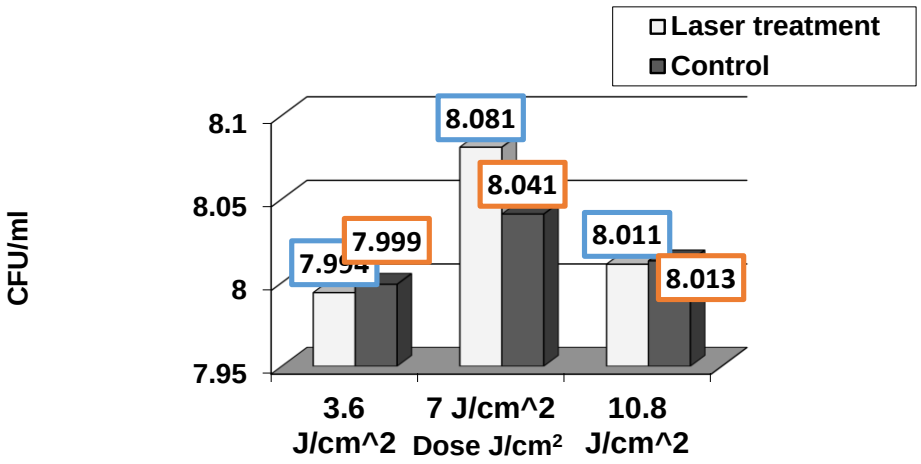

Figure 2-Viability of *S. pyogenes* (LOG CFU/ml) treated with laser only

Table 3-Mean value of viability LOG CFU/ml for *S. pyogenes* with photosensitizer only

Photosensitizer concentration		LOG CFU/ml		
		With PHS TBO	Control	T-test (P-value)
25 µg/ml	Mean ± SD	8.06 ±0.16	8.07 ±0.15	0.0809 NS (0.739)
	Range	7.77-8.30	7.80-8.32	
50 µg/ml	Mean ± SD	8.01 ±0.15	8.04 ±0.23	0.101 NS (0.490)
	Range	7.76-8.33	7.77-8.94	
NS: Non-Significant.				

Table 4 showed the influence of laser radiation on "*S. pyogenes"* at doses 3.6, 7 and 10.8J/cm^2 corresponding to exposure times 5, 10, 15 minutes and two different "TBO" concentration (25 µg/ml and 50 µg/ml). Significant reduction in viability of"*S.pyogenes"*between photo inactivation groups and control group < 0.01.The optimum inhibition in ''LOG CFU/ml" with light dose **7** J/cm^2 and TBO concentration 50 µg/ml.Table 4

Table 4-Mean value of viability" LOG CFU/ml for *S. pyogenes"*under photodynamic inactivation treatment

LogCFU/ml				
Laser doses + TBO photosensitizer Dose J/cm^2+Conc µg/ml			Control Groups	T-test (P-value)
3.6 J/cm^2+25 µg/ml	Range	7.47-7.95	7.96-8.34	0.064 ** (0.0001)
	Mean ±SD	7.68 ±0.12	8.14 ±0.12	
3.6 J/cm^2+50 µg/ml	Range	7.39-7.87	7.96-8.35	0.061 ** (0.0001)
	Mean ± SD	7.63 ±0.13	8.20 ±0.10	
7 J/cm^2+25 µg/ml	Range	6.60-7.23	7.88-9.31	0.066 ** (0.0001)
	Mean ± SD	6.98 ±0.17	8.09 ±0.13	
7 J/cm^2+50 µg/ml	Range	6.00-7.20	7.65-8.31	0.133 ** (0.0001)
	Mean ± SD	6.45 ±0.31	8.02 ±0.18	
10.8 J/cm^2+25 µg/ml	Range	6.60-7.49	7.81-8.32	0.0773 ** (0.0001)
	Mean ± SD	7.02 ±0.15	8.08 ±0.14	
10.8J/cm^2+50 µg/ml	Range	6.47-7.34	7.88-8.33	0.096 ** (0.0001)
	Mean ± SD	7.06 ±0.22	8.17 ±0.14	
** (P≤0.01), NS: Non-Significant.				

With constant "TBO concentration", rising at exposure time "(dose J/cm^2)" led to more inhibition in viability of "*S.pyogenes"*. With constant TBO concentration viability of"*S.pyogenes"* decrease with an increase in light dose (J/cm^2) P≤0.01. Table 5

Table 5-Mean value of viability LOG CFU/ml for *S. pyogenes* under photodynamic inactivation treatment, according to laser dose (exposure time).

Log CFU/ml			LSD (P-value)
Group irradiated by 5min (3.6 J/cm2) +25 µg/ml	Group irradiated by 10min (**7**J/cm2)+ 25 µg/ml	Group irradiated by 15min (10.8 Jcm2)+25 µg/ml	
Mean ± SD	Mean ± SD	Mean ± SD	
7.68 ±0.12 a	6.98 ±0.17 b	7.02 ±0.15 b	0.372 ** (0.0083)
range	range	range	
7.47-7.95	6.60-7.23	6.60-7.49	
Group irradiated by 5min (3.6 j/cm2) +50 µg/ml	Group irradiated by 10min (7 j/cm2)+ 50 µg/ml	Group irradiated by 15min (10.8 j/cm2)+50 µg/ml	
Mean ± SD	Mean ± SD	Mean ± SD	
7.63 ±0.13 a	6.45 ±0.31 c	7.06 ±0.22 b	0.287 ** (0.0001)
range	range	range	
7.39-7.87	6.00-7.20	6.47-7.34	
Means having with the different letters in same row differed significantly. ** (P≤0.01).			

"Photodynamic inactivation" of bacteria is interesting antibacterial strategy against bacterial infection in burns wounds [25][26][27][28] [29].
Irradiation of "*S.pyogenes"* with light from red laser without photosensitizer had no influence on "CFU/ml", and "TBO" had no important effect on "CFU/ml" without laser radiation. These outcomes were identified to those achieved in prior studies [30][31][32][33].The application of ''photodynamic inactivation'' for infected wounds is greatly dependent on irradiation parameters, such as exposure time, type and concentration of the"photosensitizer''.[32] The essential point for ''photo inactivation'' of "*S.pyogenes"* is the exposure time whichis one of the most important factorsthat decisively affect bacterial inhibition.The selected range of energy in this project from (3.6, 7, and 10.8 J/cm^2) was calculated regarding the laser treatment parameters like intensity (watt/cm^2) and exposure time. [34][35]

Previous researches examining the antibacterial influence of" TBO" in photodynamic inactivation strategy against" pathogenic bacteria "operating at low intensity laser as it generate low output intensity which is not producing risk to Surrounding tissues.[34][35]

In this study, TBO photosensitizer is a member of' 'phenothiazinium, non-porphyrin family'' [36] [37]. It was elected because of its little poisonous effect to human cells,"*in vitro*"effectiveness, high proportion of reactive oxygen species formation, and remarkable variation regarding its broad band of absorption, which permitstimulation by different light sources.[38].This study designed to examine the influence of different concentrations of ''TBO'' on viability of bacteria" (CFU/ml)". The most commonly reported "TBO" concentration in theprevious literatures to express "photodynamic inactivation"effectiveness against" gram positive and gram negative bacteria" isolated from infected wounds were (25 and 50) µg/ml.[33][39][33][40][41]

Theexperiments in this research established more inhibition in the viability of"*S. pyogenes*" obtained with raising the dose''photoinactivation'' at (3.6J/cm^2) corresponding to 5 minutes exposure time with two concentrations of "TBO"(25,and 50µg/m) , the dose delivered cause a ''significant inhibition in viability of bacteria (CFU/ml) ''compared with control group. Table 4,5, the same effect noticed with light dose 7 J/cm^2 at 10 minutes exposure time with two concentrations of "TBO" (25,and 50µg/ml), but the concentration of" TBO" at 50µg/ml initiate effective influence on bacterial inhibition.

The results show that there is a great inhibition in viability of bacteria as the dose rise andthis is agreeable in comparison between these results and previous"*in vitro"*study [40]. Whichfocused on determining thebactericidal influence of "antimicrobial photodynamic inactivation"using TBO and "methylene blue" with various concentrations (12.5, 25, 6.25, 100 and 50µg/ml) correlated to red laser with various doses (4.8, 2.4, 6.9, 7.2 and 12 J/cm^2) on strain of"*S. aureus".* The results demonstrated that the correlation 100and 50 µg/ml with 12 J/cm^2showed the complete killing. Therefore it can be concluded that "photodynamic inactivation "is able to improve the antibacterial influence of" photosensitizers" and both, doses and concentrations of photosensitizers are an essential factors for highest influence of ''photodynamic inactivation". [40]

The results of this project also showed that the inhibition in" viability of *S.pyogenes"* depends on doses of red light, this results is in agreement with another previous study of ''al kashif.etal.''[33] which is focused on the effect of methylene blue activated by diode laser 660nm with output power 35mw, on three types of pathogenic bacteria ''(*Echerichiacoli.coli, Staphylococcus epidermidis and S. aureus''* isolated from infected foot ulcer of diabetic patients. The results of ''Al-Kashif'' study etal. [33] demonstrated that all isolates were sensitive to be inhibited by" photodynamic inactivation". The inhibition effect was principally depending on the dose." Red laser dose" (109.2 J/cm^2)was adequate to obtain maximum inhibition in viability of "*s.aureus* and *Staphylococcus epidermidis".*

In this study the important parameters used were spot size of the laser (irradiated area 5cm^2) which is suitable for irradiation of skin wound, the output power was 60mw and the intensity equal to 0.012 watt/cm^2.These parameters of "photodynamic inactivation technique" may have an essential clinical application , it may be used to prohibit "*S. pyogenes*" in infected wounds .The photosensitizer could apply to the wound by using syringes and laser light that could be controlled by optical fiber[34][12].

Conclusion:

This study found that TBO was efficient in a photo inactivation of *S.pyogenes*.TBO in association with red laser could be an effective means of destroying multi drug resistant *S.pyogenes* isolated from infected burn wounds. The optimum inhibition of *S.pyogenes*was accomplished by 50µg/ml of TBO stimulated by laser dose (7 J/cm2) delivered over 10 minute's irradiation.

References:

[1] R. W Haley, D. H . Culver, J. W. White, W. M. Morgan, T. G .Emori,V., P. Munn, & T. M. Hooton" The efficacy of infection surveillance and control programs in preventing nosocomial infections in US hospitals". *American journal of epidemiology,*vol. 121, no. 2, pp: 182–205, 1985.

[2] W. Krzyściak, K. KPluskwa, A.Jurczak,& D. Kościelniak. "The pathogenicity of the Streptococcus genus". *European journal of clinical microbiology & infectious diseases: official publication of the European Society of Clinical Microbiology,*vol. 32, no. 11, pp. 1361–1376, 2013.

[3] L.S. Peloi, R. R. Soares, C.E.Biondo, V.R. Souza, N.Hioka, E. Kimura ."Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue" *J Biosci*. vol. 33, pp. 231-237,2008.

[4] S. K. Bisland, C. Chien, B.C .Wilson, S. Burch. "Preclinical in vitro and in vivo studies to examine the potential use of photodynamic therapy in the treatment of osteomyelitis."*PhotochemPhotobiolSci* ,vol. 5: pp. 31-38, 2006.

[5] C. K. Hope, &M.Wilson. "Induction of lethal photosensitization in biofilms using a confocal scanning laser as the excitation source." *The Journal of antimicrobial chemotherapy*", vol. 57, no. 6, pp. 1227–1230, 2006.

[6] M .Wainwright." Photodynamic therapy: the development of new photosensitizer. " *Anti-cancer agents in medicinal chemistry*, vol. 8, no. 3, 280–291, 2008.

[7] F. Gad, T. Z, T. Hasan , M. R. Hamblin ." Effects of growth phase and extracellular slime on photodynamic inactivation of Gram-positive pathogenic bacteria." *Antimicrobial Agents and Chemotherapy*, vol. 48, no. 6, pp. 2173-2178, 2004.

[8] N. A. Kuznetsova, D. A. Makarov, O. L. Kaliya, &G. N. Vorozhtsov ." Photosensitized oxidation by dioxygen as the base for drinking water disinfection." *Journal of hazardous materials*, vol. 146, no. 3, pp. 487–491. 2007.

[9] G. Jori, & S. B. Brown. "Photosensitized inactivation of microorganisms". *Photochemical &photo biologicalsciences Official journal of the European Photochemistry Association and the European Society for Photobiology*, vol. *3, no.*5, pp. 403–405. 2004

[10] E. N. Durantini, "Photodynamic inactivation of bacteria." *Current Bioactive Compounds*, vol. 2, pp. 127-142 , 2006.

[11] G. Jori, M. Magaraggia, C.Fabris, M. Soncin, M.Camerin, L.Tallandini, O. Coppellotti, & L. Guidolin. "Photodynamic inactivation of microbial pathogens: disinfection of water and prevention of water-borne diseases". *Journal of environmental pathology, toxicology and oncology : official organ of the International Society for Environmental Toxicology and Cancer*, vol. 30, no 3, pp. 261–271, 2011.

[12] M. R. Hamblin, & T. Hasan. " Photodynamic therapy: a new antimicrobial approach to infectious disease?" *Photochemical &photobiologicalsciences: Official journal of the European Photochemistry Association and the European Society for Photobiology*, vol. 3, no.5, pp.436–450, 2004.

[13] A. Gueye-Ndiaye, N.C.Sarr, K. Thiam, C.S Boye." In vitro activity of antimicrobial agents against Streptococcus pyogenes isolates from patients with acute tonsillopharyngitis in Dakar, Senegal*." Microbiol Insights*. vol. 2, pp. 25–29. 2009.

[14] M. A.CamaraDieng, & C. S. Boye." Antibiotic susceptibility of streptococcus pyogenes isolated from respiratory tract infections in dakar, senegal. "*Microbiology insights*, vol. 6, pp.71–75, 2013.

[15] R. M Patrick, J.B Ellen ." Manual of clinical microbiology "*Washington, D.C: ASM Press*, 2003.

[16] T. Abraham, & S. Sistla. "Identification of Streptococcus pyogenes - Phenotypic Tests vs Molecular Assay (spy1258PCR): A Comparative Study ". *Journal of clinical and diagnostic research*, vol .10, no. 7, pp. DC01–DC3, 2016.

[17] Ferretti , J .Joseph, et al., editors." Streptococcus pyogenes: Basic Biology to Clinical Manifestations". *University of Oklahoma Health Sciences Center*, 2016.

[18] National Committee for Clinical Laboratory. *Performance Standard for Antimicrobial Disk Susceptibility Test for Bacteria*. 29th Edition 2019. NCCLS.

[19] Ardanuy, Carmen et al. "Molecular characterization of macrolide- and multidrug-resistant Streptococcus pyogenes isolated from adult patients in Barcelona, Spain (1993-2008)." *The Journal of antimicrobial chemotherapy*. vol. 65, no .4, pp. 634-43. 2010

[20] A. P. Magiorakos, et al. "Multidrug-resistant, extensively drug-resistant and pan drug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance." *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases* vol. 18, pp. 3, 2012**.**

[21] M.H. Niemz ." Laser-tissue interactions: Fundamentals and applications" . *New York: Springer-Verlag*, 2007.

[22] J. Jebaramya , I . Malaichamy, S. Prabahar,, "Spectral Studies of Toluidine Blue O in the Presence of Sodium Dodecyl Sulfate."*Digest Journal of Nanomaterials and Biostructures"* .vol. 4, no. 4, pp. 789-797, 2009.

[23] J.G . Cappuccino, N. Sherman. "Microbiology a laboratory manual" .8th Edition. *Benjamin Cummings, New York*,pp: 335-337, 2002.

[24] SAS. Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA. 2012**.**

[25] V. G.Garcia, M. A de Lima, T. Okamoto, L. A.Milanezi, , Júnior, E. C., Fernandes, L. A., J. M.de Almeida, &, L. H. Theodoro." Effect of photodynamic therapy on the healing of cutaneous third-degree-burn: histological study in rats." *Lasers in medical science*. vol. 25, no.2, pp.221–228, 2010.

[26] S. ALambrechts, T. N.Demidova, , M. CAalders, T.Hasan, , & M. R.Hamblin,."Photodynamic therapy for Staphylococcus aureus infected burn wounds in mice". *Photochemical &photobiological sciences Official journal of the European Photochemistry Association and the European Society for Photobiology*, vol: 4, no. 7, pp. 503–509, 2005.

[27] M**.** Funjan, "Photodynamic Inactivation of Klebsiella Pneumonia Isolated from Burn Wounds." *Neuroquantology*, vol.18, no.6, pp.1-10, 2020.

[28] M . Ismail, S. Mohammed, F. Jabbar, K. Ibrahim. "Effect of Diode Laser (805) nm on alpha-toxin production and antibiotic sensitivity of Staphylococcus aureus." *Iraqi journal of science.* vol. 53.pp 755-759, 2012.

[29] A. Ali . The Effect of 650 nm Diode Laser on Growth Curve of Gram-negative Bacteria and Their Phagocytic killing Assay by PMN Cells .*Iraqi Journal of Science*.vol. 5, . pp. 2264-2274, 2015.

[30] A.A Balhaddad, M. S .AlQranei, M. S.Ibrahim, M. D.Weir, F. C. Martinho , H. Xu, , & M. Melo,. "Light Energy Dose and Photosensitizer Concentration Are Determinants of Effective Photo-Killing against Caries -Related Biofilms. " *International journal of molecular sciences*, vol. 21, no.20, pp. 7612, 2020.

[31] A., Hanakova, K.Bogdanova, K.Tomankova, K.Pizova, Malohlava, J., Binder, S., Bajgar, R., Langova, K., Kolar, M., Mosinger, J., &Kolarova, H." The application of antimicrobial photodynamic therapy on S. aureus and E. coli using porphyrin photosensitizers bound to cyclodextrin". *Microbiological research*, vol.169, no. 2-3, pp. 163–170, 2014.

[32] J. Shen, Q. Liang , Su, G., Y.Zhang, , Z. Wang. ,CBaudouin,, & A.Labbé .

[33] "In Vitro Effect of Toluidine Blue Antimicrobial Photodynamic Chemotherapy on Staphylococcus epidermidis and Staphylococcus aureus isolated from Ocular Surface Infection " . *Translational vision science & technology*, vol .8, no. 3, pp. 45, 2019.

[34] N. Kashef, G. Ravaei Sharif Abadi, & G. E. Djavid, . "Phototoxicity of phenothiazinium dyes against methicillin-resistant Staphylococcus aureus and multi-drug resistant Escherichia coli " . *Photodiagnosis and photodynamic therapy*, vol .9, no.1, pp. 11–15. 2012.

[35] F. Cieplik, D.Deng, , W. Crielaard, W. Buchalla, , E.Hellwig, , A. Al-Ahmad, , & T. Maisch. "Antimicrobial photodynamic therapy - what we know and what we don't " . *Critical reviews in microbiology*, vol. 44, no. 5, pp. 571–589. 2018.

[36] M. N. Usacheva, M. C. Teichert,., & M. A. Biel . "Comparison of the methylene blue and toluidine blue photobactericidal efficacy against gram-positive and gram-negative microorganisms" . *Lasers in surgery and medicine*, vol .29. no. 2, pp.165–173, 2001.

[37] A. E.O'Connor, W. M. Gallagher, &A. T. Byrne." Porphyrin and nonporphyrin photosensitizers in oncology: preclinical and clinical advances in photodynamic therapy." *Photochemistry and photobiology*, vol. 85, no.5. pp. 1053–1074, 2009.

[38] M. Tim. "Strategies to optimize photosensitizers for photodynamic inactivation of bacteria" . *Journal of photochemistry and photobiology." B, Biology*, vol. 150, pp. 2–10. 2015.

[39] I. C. Zanin, M. M. Lobo, L. K. Rodrigues, , L. APimenta, J. F. Höfling ,& R. Gonçalves. " Photosensitization of in vitro biofilms by toluidine blue O combined with a light-emitting diode" . *European journal of oral sciences*, vol .114, no. 1, pp. 64–69. 2006.

[40] N Kashef, et al. "Photodynamic inactivation of drug-resistant bacteria isolated from diabetic foot ulcers." *Iranian journal of microbiology*. vol. 3,no 1 , pp. 36-41. 2011

[41] Monteiro, S. C. Juliana et al. "Effectiveness of antimicrobial photodynamic therapy (AmPDT) on Staphylococcus aureus using phenothiazine compound with red laser." *Lasers in medical science* ,vol. 32,no.1,pp. 29-34, 2017 .

[42] C. E., Millson, M. Wilson, A. J J. Macrobert, & S. G. Bown,. The killing of Helicobacter pylori by low-power laser light in the presence of a phtosensitizer. "*Journal of medical microbiology*, vol.44, no.4, pp 245–252, 1996.