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The Effect of 650 nm Diode Laser on Growth Curve of Gram-negative Bacteria and Their Phagocytic killing Assay by PMN Cells.

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Abstract
Nowadays laser in medicine is a rapidly growing field in both researches and applications and many studies have been done in bacteriology against different types of lasers. The effect of the laser depends on many factors, one of the laser factors was the wavelength. The red wavelength band has been considered as a stimulated wavelength for prokaryotic and eukaryotic cells. In this study, three clinical species of Gram-negative bacteria (*E. coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*) were exposed to 650 nm band of red region wavelength by a diode laser. The effect of that wavelength appeared on the growth curves for each species along 3 days after laser treatment. The energy density (E.D.) 2.8 J/cm^2 gave a positive effect on growth curve for these three species, while the 5.6 J/ cm^2 have a stimulation effect on both *P. aeruginosa* and *E. coli.* The Phagocytic killing Assay (PKA) by PMN cells was tested against irradiated bacteria, and the 2.8 J/cm^2 appeared to have a significant ($P<0.05$) effect on PKA of all that three types of bacteria, on the other hand the 5.6 J/cm^2 affected significantly ($P<0.05$) on the PKA of *P. mirabilis*, and *P. aeruginosa*. These findings suggested that the E.D. 2.8 J/cm^2 was much effective than the 5.6 J/cm^2 on the growth curves and PKA values of the three bacteria species.

Keywords: 650 nm diode laser, Gram-negative bacteria, Phagocytic killing Assay, growth curve, aerobic and facultative aerobic bacteria, LPS.

تأثير ليزر الدايود ذي الطول الموجي 650 نانو متر على منحني النمو للبكتريا السالبة لملون غرام وفحص القتل بالبلعمه للخلايا العدلة

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الخلاصة
في هذه الايام يعتبر استخدام الليزر في الطب من المجالات النامية على الصعيدين البحثي و التطبيقي و الكثير من الدراسات اجريت على البكتريا باستخدام انواع مختلفة من اجهزة الليزر. تاثير الليزر اعتمدت على العديد من العوامل، احد عوامل الليزر هو الطول الموجي المستخدم. تعتبر حزمة الطول الموجي الأحمر من الاطوال الموجية المحفزة للخلايا الأولية والحقيقية النواة. في هذه الدراسة تم تعريض ثلاث انواع من البكتريا السريرية السالبة لملون الغرام (الاشريكية القولونية *E. coli* ، *Proteus mirabilis*، والزائفة الزنجارية *Pseudomonas aeruginosa*) إلى الطول الموجي 650 نانومتر و هو من الاطوال الموجية الحمراء باستخدام ليزر الدايود. تأثير ذلك الطول الموجي ظهر على منحنيات النمو للأنواع الثلاث على مدى 3 أيام

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بعد التشعيع بالليزر. اعطت كثافة الطاقة 2.8 cm^2/J تأثيرا إيجابيا على منحنى النمو لهذه الأنواع الثلاثة، في حين كان لل 5.6 cm^2/J تأثيرا محفزا على كلا الزوائف الزنجارية *P. aeruginosa* والاشريكية القولونية *E. coli*. تم فحص القتل بالبلعمة (PKA) من قبل خلايا PMN ضد البكتيريا المشعة و اظهرال 2.8 cm^2/J تأثيرا نوعيا كبيرا ($P < 0.05$) على PKA للانواع الثلاثة من البكتيريا، ومن ناحية أخرى كان لل 5.6 cm^2/J اثرا نوعيا ($P < 0.05$)على PKA لبكتريا الزوائف الزنجارية *P. aeruginosa* و *Proteus mirabilis*. من هذه النتائج اقترح أن كثافة الطاقة 2.8 cm^2/J كانت اكثر تأثيرا من 5.6 cm^2/J على منحنيات النمو، وقيم PKA للأنواع الثلاث من البكتيريا.

Introduction

Irradiation of cells at certain wavelength can activate some of the native components within the cell. In this way specific biochemical reactions as well as whole cellular metabolism can be altered. This type of reaction is believed to form the basis for low-power laser effects. [1]

A photobiological reaction entails the absorption of a specific wavelength of light by the functioning photoreceptor molecule (a specialized photoreceptor like rhodopsin, phytochrome, chlorophyll, etc). On the other hand, the monochromic light can be absorbed by nonspecialized chromophores (photoacceptor), like cytochromes. This molecule often takes part in some metabolic reaction in the cell not connected with light responses at all. After absorbing a specific wavelength of light a measurable biological effect will occur in certain circumstances [1, 2]. These effects can conventionally be divided into short-term (or direct, seconds or minutes after irradiation) and long-term (or indirect, hours and even days after the end of the irradiation). [3]

When cells are irradiated with various visible-light bands, light is absorbed by cytochromes; those are electron transfer proteins that carry heme as a prosthetic group and can absorb light at specific wavelengths *via* that heme cofactor [4,5]. Most bacterial cytochromes function either in photosynthetic electron transport or in aerobic and anaerobic respiration. Since respiration in prokaryotic cells takes place in the cytoplasmic membrane, cytochromes are often localized in this compartment and in the periplasmic space. After absorbing the visible light by the exited cytochromes a short-term activation of the respiratory chain will occur and leads to oxidation of the cytoplasm. [4]

The red region of visible light, (about 630 nm), is absorbed by the terminal cytochrome oxidases d (cyt. d) presence in bacteria, cyt. d is a protein that belongs to the family of cytochromes; its also known as cytochrome a_2. It is found in plenty of aerobic bacteria, especially when it has grown with a limited oxygen supply like *E. coli*. [1, 2, 3, 6], and *P. mirabilis* [7]. While in *P. aeruginosa* which is strict aerobic bacteria they highly depend on cyt. c in their aerobic respiration but they may have cyt. d as cyt.cd [8]. The cyt. d is a dimeric protein (composed of two subunits cyt. a and cyt. b) gives an absorption band of approximately 636 nm if it is oxidized and 638 nm if it is reduced. [5, 9]

Many experiments confirm the hypothesis about the critical role of photoinduced electrochemical potential on the bacterial envelope surface *via* photoactivation of the respiratory chain [2]. The activated electron-transport chain must result in an increase in many metabolic process like proton motive force (pmf, $\Delta\mu_{H+}$), proton electrochemical potential difference ($\Delta\mu_{H+}$) across the membrane [2, 4], and the value of Δ pH on the cytoplasmic membrane [2, 6].

In prokaryotes, the envelope of Gram-negative bacterium is a complex structure. It consist of two membranes: the cytoplasmic or inner membrane (IM), and the asymmetrical outer membrane (OM), which holds phospholipids and lipopolysaccharides (LPS) in its inner and outer leaflet, respectively [10, 11, 12]. The components of the cell wall layers are synthesized at the level of the cytoplasmic membrane and released into the cell wall where they move outward until they "find" their appropriate layer or zone [11, 12].

LPS is a complex glycolipid that can be structurally divided in three parts: lipid A, the oligosaccharide region, and the O-antigen polysaccharide chain. LPS possessed all of the biological activity of endotoxin [12, 13, 14]. The size-distribution patterns of LPS are strain specific, but there are many reports documented a modification in O-side chain length and capping frequency of lipid A-core were affected by chemical and/or physical environment variations [15, 16, 17], as the length of LPS appears to effect on the sensitivity or resistance of Gram-negative bacteria to antibacterial system. [15, 16]

Bacterial defense is greatly dependent on innate immunity, particularly on PMN cells. PMN recognize bacteria by so-called “pattern-recognition receptors”. LPS is detected by some receptor of

PMN and moncyte cells. After binding to these receptors they will activate defense mechanisms, including phagocytosis, and oxidative burst. Many surface receptors are expressed on PMN like CD 16, CD 64 and CD14, but CD14 is a well-characterized receptor for LPS and other bacterial products. CD14 is a glycosylphosphatidylinositol-anchored 44-kD glycoprotein, and it is found on the PMN and monocyte cell surface .PMN, however, contain an intracellular pool of CD14 from which it is translocated to the surface in response to stimulation by LPS [18, 19, 20]. After the adhesion of PMN receptor to the target a phagocytosis process will start. This process is triggered upon the binding of opsonized microorganisms through PMN receptors that recognize certain lectine on target microorganisms and leads to phagocytosis and killing by creating a highly toxic micrenvironment in the phagolysosome.[21]

This study aims to evaluat the effect of 650 nm diode laser on the growth curves of three species of Gram-negative bacteria and if this wavelength effected on the LPS by measuring the PKA values against the PMN cells.

Materials and Methods

Bacterial isolates

Three types of clinical Gram-negative bacteria from laboratory department / Al-Yarmouk Teaching Hospital were used in this study, (*E.coli*, *P. mirabilis*, and *P. aeruginosa*), the identification of these isolates was done by ApI 20-E system (bioMerieux, Inc). Bacteria were grown over night in nutrient broth (Himedia, India) at 37˚C, and then washed three times by normal saline (N.S, 0.9% W/V sodium chloride) (El-Nasr pharmaceutical chemical company, Egypt) at 11000 r.p.m [22], finally bacteria were suspended in N.S. equivalently to 0.5 at optical density (OD_{600}) of spectrophotometer; and this was equal to 1×10^8 cell/ml (by a colony forming units (CFU)) [22], preparing for laser treatment.

Laser parameters

Photoirradiation was performed at 650 ± 10 nm by diode laser pointer (Shanghai Megicgoods Technology Co., China), the output beam power was 5 mW. The beam diameter was 0.4 cm, the power density was 9.32×10^{-3} W/cm^2. The laser exposure times were for 5 and 10 min, so the energy densities (E.D) were 2.8 J/cm^2 and 5.6 J/cm^2 for the 5 and 10 min exposure times respectively.

Isolation of PMN

30 ml of venous drained blood from healthy volunteers was added in heparinized silicon tubes (+BH, China) .The tubes were leaved in an incubator at 37˚C for ½ hr. the PMN cells were isolated from the hall blood cells by lymphoprep solution (Nyegaard&Co.As,Oslo, Norway) [21]. The PMN cells were washed and suspend in RPMI 1640 medium (Flow laboratories, England) to obtain a final concentration of $2\text{-}4\times10^6$ cell/ml viable PMN cells, cell viability was achieved by counting the blue cells that takes the (0.2 %) trypan blue stain (BDH, England) in heamocytometor chamber under 40X light microscope. [23]

Experimental procedure:

Bacteria irradiated by diode laser

0.1 ml of bacterial suspension (previously prepared) was put in eppendorf tube (triplicate each isolate) and irradiated at the 650 nm diode laser for 0 (L_0, control), 5 (L_5), and 10 min (L_{10}) for each bacteria species. The treated bacterial suspensions and control (0.1 ml) were transported to universal glass tubes which contained 10 ml nutrient borth (inoculums size 1:10 $^{(2)}$) and incubated for 24, 48, and 72 hr in aerobic condition. Bacterial growth was measured (at OD_{600}) for the three overnight incubation periods (a subculture was done after each day).

Phagocytic killing assay (PKA)

Phagocytic killing assay in this study was determined as following [26]: 1.0 ml of PMN suspension, 2.0 ml of laser treated bacterial suspension (suspended equivalently to 0.5 at OD_{600} in RPMI 1640 medium); 0.4 ml of 22% Bovine serum albumin (Plasmatec,Germany), and 1.6 ml of RPMI 1640 medium, all were mixed well in glass test tube. The tubes were incubated for 1 (T_1) and 2 hr (T_2) at 37˚C. After that period the PMNs had been lysed in 4.5 ml distilled water to release the bacteria, a CFU for each tube was made before the time (T_{zero}) and after the periods T_1 and T_2 incubation on nutrient agar. The PKA (% survival of bacteria) was calculated as follows:

$$\frac{\mathrm{T1,2}}{\mathrm{Tzero}} \times 100 = \%\ survival\ of\ bacteria \ ^{(26)}$$

Results

Laser effect on bacterial growth

The effect of laser irradiation on the growth of the three bacteria species showed different results, arranged between activation and inhibition. *P. aeruginosa*, *E.coli*, and *P.mirabilis* growth was obtained by measured the absorption at OD_{600} for bacteria suspension after 24, 48, and 72 hr of laser treatment. A growth curved for each species is presented in figures 1, 2 and 3 respectively.

After 24 hr (first day) of laser irradiation the growth curved of *P. aeruginosa* appeared that there was no significant ($P>0.05$) increasing in growth curves for L_5, L_{10} and L_0 (control). On the second day (48 hr) the sub culture showed a significant ($p<0.05$) increasing in growth curve for L_5 compared with L_0, and more than that for L_{10}. The growth curve for L_5 continued in rising significantly ($p<0.05$) without big difference with control for the third day (72 hr), while a significant ($p<0.05$) decreasing in the growth curved of L_{10} was observed at that period (72 hr). Figure- 1

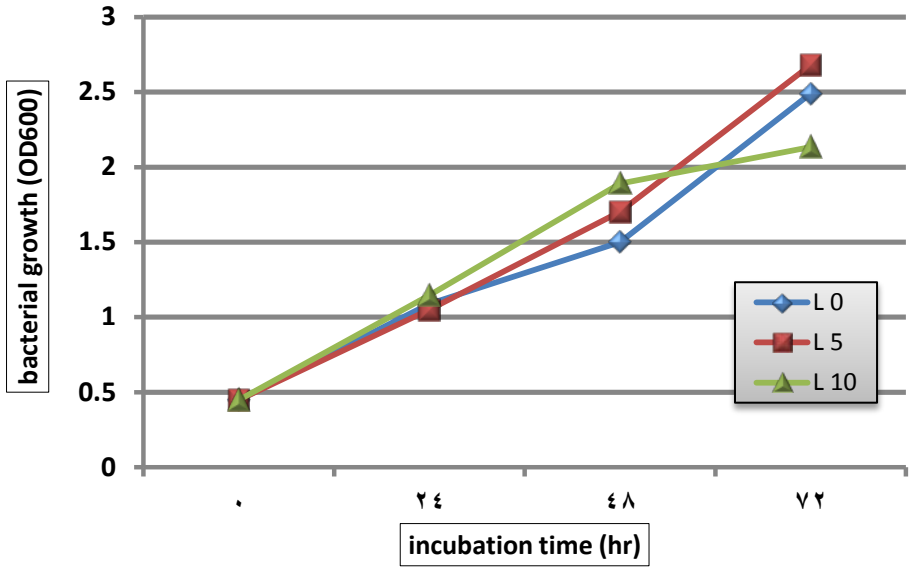

Figure 1- The effect of 650 nm diode laser treatment on the growth cured of *P. aeruginosa* (L_5, L_{10} and L_0 represented laser exposure times). The growth curve was obtained by reading bacterial suspension turbidity at OD_{600} for each three days (24, 48, and 72 hr) of sub culturing after laser treatment.

For *E.coli*, the growth curves showed a no significant ($P>0.05$) decreasing for L_0 and L_5 after 24 hr incubation time, and leas than that for L_{10}. On the next day; after 48 hr, a non-significant ($P>0.05$) increasing in the curve was observed for the two laser exposure times and control with fronting of L_5. This increasing continued non significantly ($P>0.05$) after 72 hr of sub culture where the L_{10} raised more than L_5 this time. Figure-2

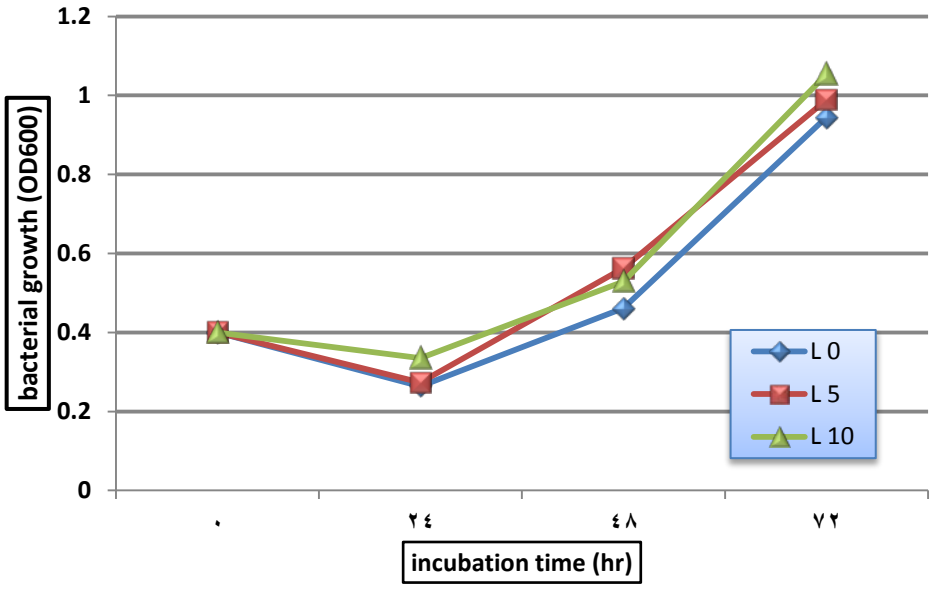

Figure 2- The effect of 650 nm diode laser treatment on the growth cured of *E. coli* (L_5, L_{10} and L_0 represented laser exposure times). The growth curve was obtained by reading bacterial suspension turbidity at OD_{600} for each three days (24, 48, and 72 hr) of sub culturing after laser treatment.

The three growth curves for *P. mirabilis* decreased similarly to *E. coli* after 24 hr of incubation time for L_5, L_{10} and control. The first sub culture (48 hr after laser treatment) showed no significant ($P>0.05$) increasing in the growth curves of L_5, L_{10} and control, but the L_{10} was slower than the two others. The latter two curves continued in their non significant ($P>0.05$) rising to reach a close point after 72 hr, while the curve of L_{10} remained slower than the others. Figure- 3.

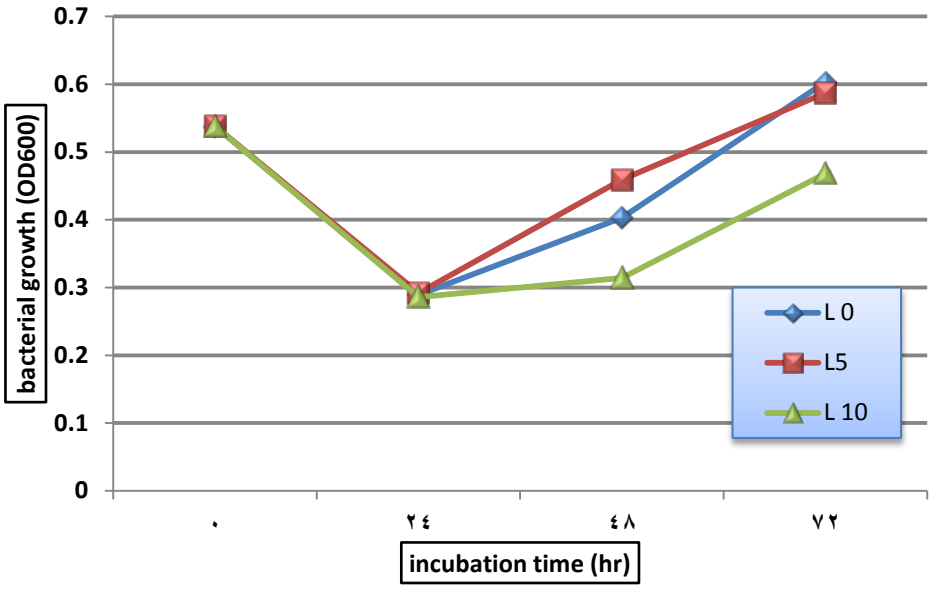

Figure 3- The effect of 650 nm diode laser treatment on the growth cured of *P. mirabilis* (L_5, L_{10} and L_0 represented laser exposure times). The growth curve was obtained by reading bacterial suspension turbidity at OD_{600} for each three days (24, 48, and 72 hr) of sub culturing after laser treatment.

Effect of laser treated bacteria on PMNs phagocytic killing assay (PKA):

The effect of laser irradiation on phagocytic killing of PMNs was tested for L_0, L_5, and L_{10} treated bacteria and for the three periods (24, 48, and 72 hr) after laser treatment. The PKA in the next figures (4-9) is explained by bacteria survival in per cent after incubation for T_1 and T_2 hr at 37° C.

The PKA for L_5 and L_{10} of *P. aeruginosa* appeared in figures 4, and 5 respectively. In figure -4 for L_5 *P. aeruginosa*, the PKA for T_1 were 85%, 78%, 80% and 75% for the control, 1, 2, and 3 days after laser treatment respectively, a significant ($P<0.05$) decreasing in the PKA observed as compared with control. At T_2 the PKA per cent remained non significantly ($P>0.05$) lower than control, except for the second day where bacteria reached non significantly ($P>0.05$) the same point of control.

The L_{10} bacteria showed different results, the per cent of survived bacteria for 1, 2, and 3 periods were much lower than in L_5 bacteria for T_1, and the differences remained clear for the T_2 as appeared in figure -5. A significant differences ($P<0.05$) were clear in T_1 and T_2 for the four curves.

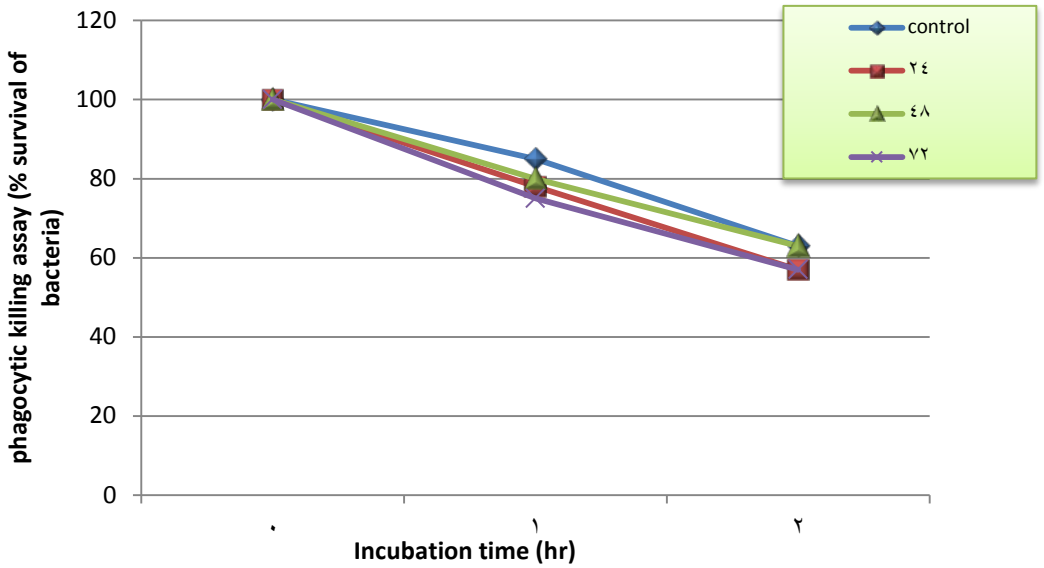

Figure 4- The PKA of 5 min laser treated (L_5) *P. aeruginosa*. The PKA was expressed by bacteria survival per cent against PMNs for T_1 and T_2 hr incubation periods. The four curves referred to the bacteria after 0, 1, 2, and 3 days from laser treatment.

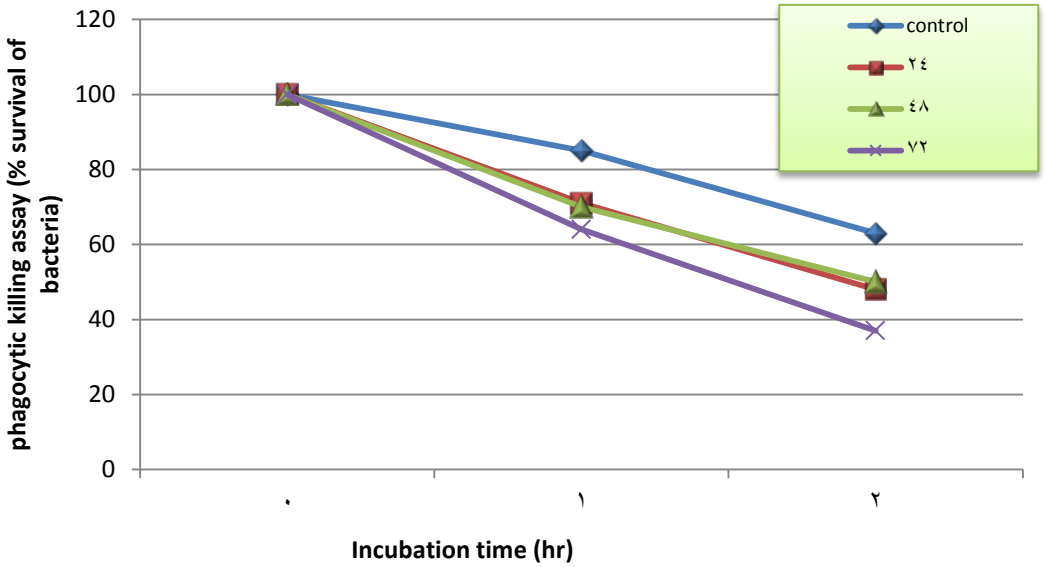

Figure 5- The PKA of 10 min laser treated (L_{10}) *P. aeruginosa*. The PKA was expressed by bacteria survival per cent against PMNs for T_1 and T_2 hr incubation periods. The four curves referred to the bacteria after 0, 1, 2, and 3 days from laser treatment.

The PKA of *E. coli* appeared in figures -6 and -7. After T_1 incubation period, the per cent of survived L_5 bacteria were significantly ($P<0.05$) closed to the control point (at T_1) and continued significantly ($P<0.05$) to the next hour (T_2) with slight increasing for the third day carve; figure 6,

while the PKA four curves of L_{10} bacteria were clearly non significant (P>0.05) closed to each other for T_1 and T_2, as appeared in figure -7.

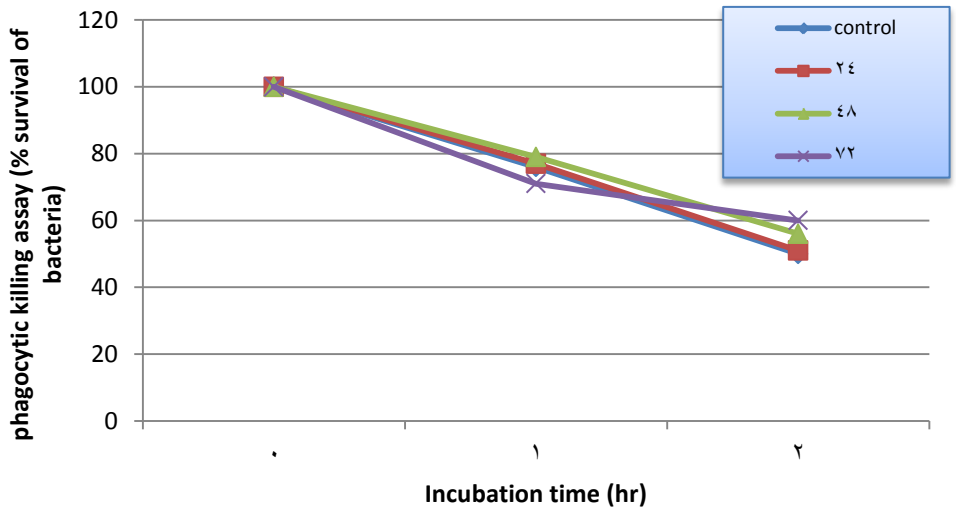

Figure 6- The PKA of 5 min laser treated (L_5) *E. coli*. The PKA was expressed by bacteria survival per cent against PMNs for T_1 and T_2 hr incubation periods. The four curves referred to the bacteria after 0, 1, 2, and 3 days from laser treatment

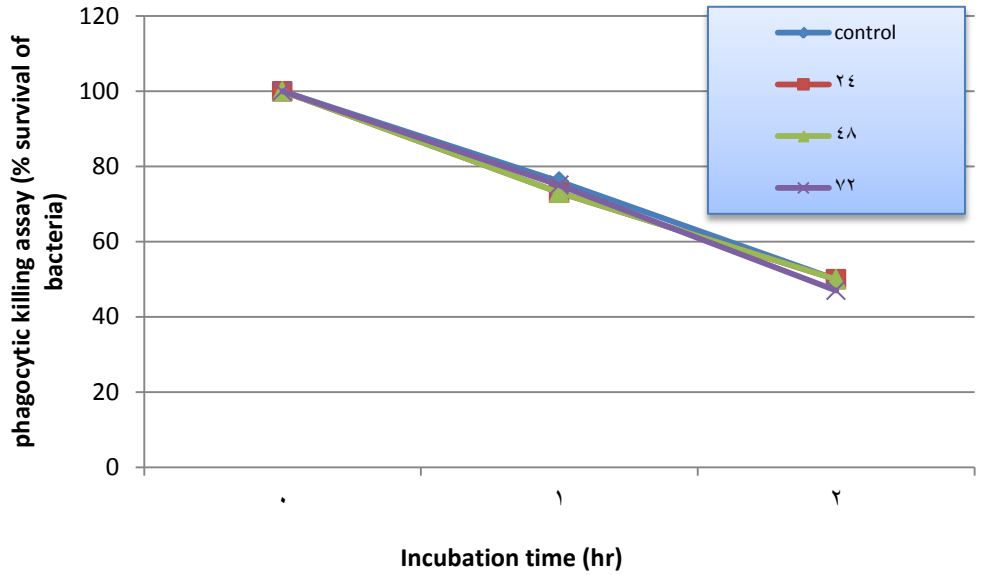

Figure 7- The PKA of 10 min laser treated (L_{10}) *E. coli*. The PKA was expressed by bacteria survival per cent against PMNs for T_1 and T_2 hr incubation periods. The four curves referred to the bacteria after 0, 1, 2, and 3 days from laser treatment.

For L_5 *P. mirabilis* the PKAs for the first, and second day after laser treatment at T_1 were significantly (P<0.05) closed to the control, for the third day bacteria, the curve was significantly (P<0.05) lower than the others. At the T_2 time the PKA for the second day curve raised significantly (P<0.05) upon the others, the third day curve reached the same point of control Figure-8.

The last figure Figure- 9 clarify a significantly (P<0.05) noticed differences in PKA at T_1 and T_2 in all four curves, the survived bacteria for 1, 2 and 3 days after laser treatment were significantly (P<0.05) lower than control in T_1 and also in T_2.

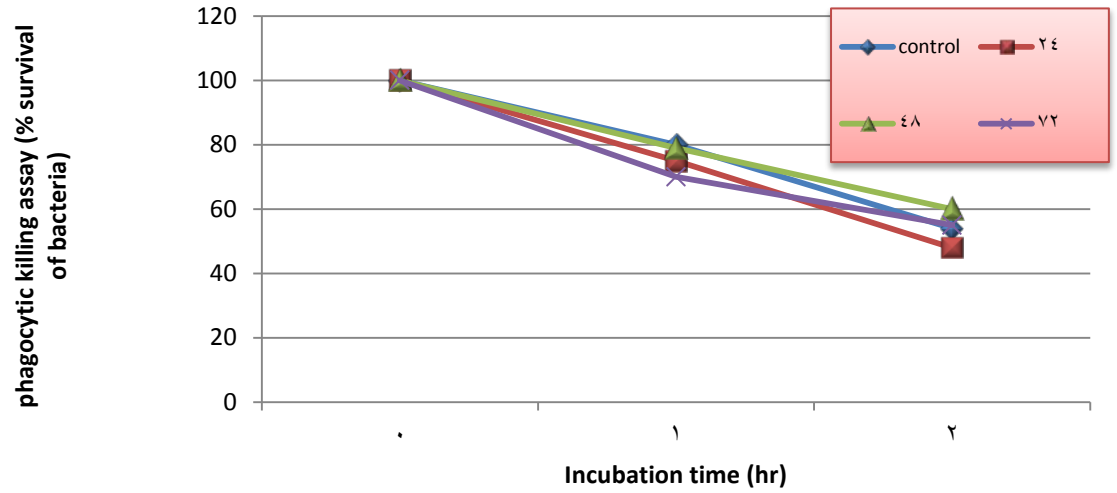

Figure 8- The PKA of 5 min laser treated (L_5) *P. merabilis*. The PKA was expressed by bacteria survival per cent against PMNs for T_1 and T_2 hr incubation periods. The four curves referred to the bacteria after 0, 1, 2, and 3 days from laser treatment

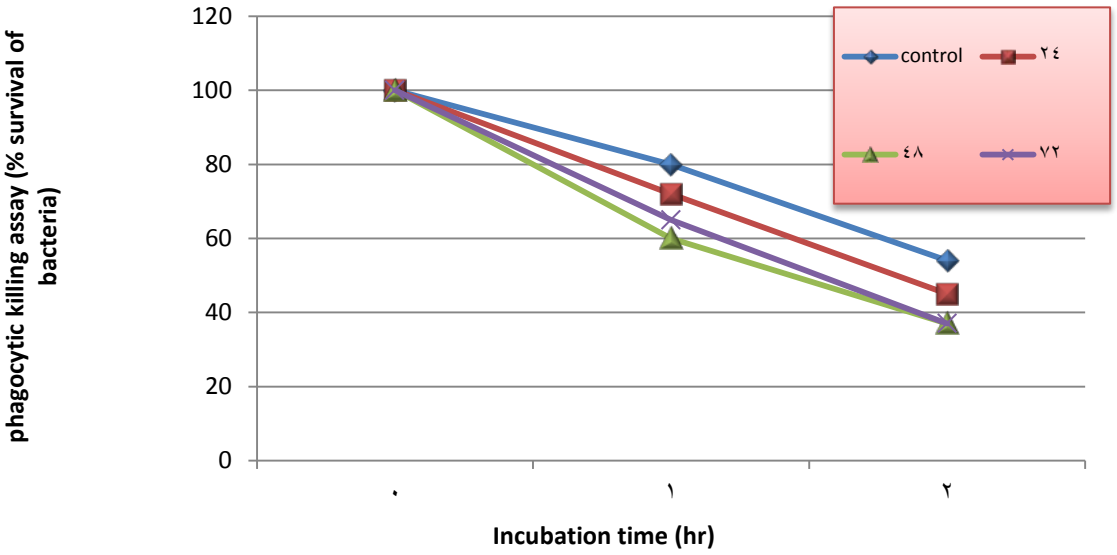

Figure 9- The PKA of 10 min laser treated (L_{10}) *P. merabilis*. The PKA was expressed by bacteria survival per cent against PMNs for T_1 and T_2 hr incubation periods. The four curves referred to the bacteria after 0, 1, 2, and 3 days from laser treatment.

Discussion

I- Laser effects on bacteria.

As many know how the light is one of the important and oldest effects on life on earth, and for many years an earlier experiments had been studded the effect of sun light, then monochromic light on biological bodies at the level of cells and organs. Some of these experiments described the physical or chemical or biological changes. The effects reflected form activation to inhibition. In this study it is related to the prokaryotic cell and its affects, for that some aspects must be taken in concentration:

First: laser irradiation experiment had been done in June month in Iraq, which is a summer months in this country, and that hot weather was reflected on the slight effects of laser (especially stimulation effect) on bacteria proliferation when compared with control to the three tested types of bacteria. When the cells are growing at their maximal specific growth rate; which affected by respiration process, the capacity of the electron transport chain for electron transfer to oxygen is fully utilized, as in summer, where no photostimulation growth occurs. This is exactly reversed in winter where the photostimulation is at its maximum rate. [2]

Second: the cyt. d; worked in limited oxygen condition, is found in *E. coli* and *P. mirablis* and these two species are facultative aerobic bacteria, while *P. aeroginosa* is strict aerobic bacteria and highly depends on cyt. c in their respiration process, for that the respiration bath ways are different between these two groups. Furthermore, prokaryotic cells have ability to used alternative bath ways in their respiratory chain as response to the changes in growth conditions. [2]

Third: because bacteria proliferation was tested after 1, 2, and 3 days in this study so the dark effect (indirect response) of laser on bacteria will be discussed. [1]

In the case of *P. aeruginosa* which are strict aerobic bacteria and have highly branched respiratory chain that ended by cyt. c and quinol oxidase for its aerobic respiration [8], so cyt. d is not involved in this respiration chain, and this fact supported the results which appeared significantly ($P<0.05$) on *P. aeruginosa* growth curve after 48 hr from laser irradiation for L_5 (where the laser E.D. is 2.8 J/cm^2, the laser effect time will be expressed by E.D.). However the slight increasing in *P. aeruginosa* growth curve may came from the activation of some components of respiratory chain other than cyt. d (the photoaccepter of 650 nm laser light) [1]. The activation of laser light stopped for the L_5 laser treated bacteria because the difference between it and control growth curves remains with the same range.

While that, the E.D. of L_{10} curve was 5.6 J/cm^2 appeared a significant ($P<0.05$) difference after 48 hr of incubation period, but after 72 hr the growth curve started a significant ($P<0.05$) clear decreasing when compared with control, this effect may related to the rapid cell division after activation of some respiratory component and changes in the redox state of cytoplasm (like, increase of pmf, ($\Delta\mu_{H+}$) and raising in ATP pool in the cell), which will result in the formation small daughter cells. A new division cycle will be latent until these cells have a size enough to start a new division cycle, because the mass and cell surface are highly related to the amount of synthesized ATP when cells decided to start a division, so when these two factors are not in equivalent, a new division cycle is impossible. [2]

For the facultative *E. coli* bacteria, the non significant ($P>0.05$) activation for L_{10} after 24 hr of laser treatment probably caused by the activation of the cyt. d in the respiratory chain after absorbing the quanta of 640 nm and led to changes in the redox state of cytoplasm, these changes result in an increasing in pmf, ($\Delta\mu_{H+}$) and raising in ATP pool in the cell, all these changes lead to the possibility of cell division immediately, resulting in two cells with reduced size [2], this is also conform to the non significant ($P>0.05$) rising in the growth curve of L_5 where the E.D. is (2.8 J/cm^2) enough to start this activation after 48 hr of laser treatment, so it fronted the L_{10} growth curve.

After the 72 hr the L_5 curve showed a non significant ($P>0.05$) decreasing in the cell proliferation than occurred in the L_{10} curve, and this may be related to the mass or surface of the activated cell and synthesized ATP. If the ATP-synthase were synthesized in double quantity above a critical level (and ΔpH decreases below the critical level) with small cell surface size, the daughter cells will wait until it achieve a size similar to the control cells size, then they will started a new division cycle, for that time the growth curves of irradiated cells raised non significantly ($P>0.05$) with no continues increasing difference with the control curve, as appeared in figure- 2 [1, 2]. These results agreed to what had been found by Karu and Tiphlova, they reported that the 632 nm light caused a sharp increase in *E. coli* cells number during the 45-60 min of incubation period after laser irradiation. They also found that the stimulated E.D.s of that wavelength were for 13 and 4×10^3 J/m^2. [2]

In figure- 3, the non significant ($P>0.05$) raiding in the growth curve of L_5 (2.8 J/cm^2 E.D.) for *P. mirabilis* that appeared after 48 hr is similar to the effect of cyt. d excitation in *E. coli* (activation in the redox respiratory state that ended to a cell division increasing). After 72 hr incubation period the curve showed a non significant ($P>0.05$) decreasing when compared with control, and it can be explained as what happened in *E. coli* L_5 curve after 72 hr. [1, 2]

The negative effect (inhibition) of laser on the L_{10} growth curved of *P. mirabilis* may relate to the amount of 5.6 J/cm^2 E.D. that absorbed by cyt. d and the dependency of this bacteria species on that cytochrome in their respiration process. This negative effect can be explained by the high acceleration of the respiratory chain and overproduction of ATP-synthase, which led to inhibition of cell division and growth, and in a formation of membrane cisterns and vesicles within the cells. It is also supposed that the excessive synthesis of wasted energy caused a slowing in growth [2], and this agreed with the fact that the same wavelength of light which absorbed by the same molecule has both positive and negative effect, depending on the light dose and intensity, and this will determine the direction of the macroeffect end. The exciting effect stranded by a narrow dose range, and this also explained the continues decreasing in L_5 growth curve after 72 hr incubation period. [1, 2]

For the facultative aerobic bacteria, we concluded from this study that the E.D. 2.8 J/cm^2 for 650 nm diode laser had a non significant (P>0.05) activation effect for *E. coli* and *P. mirabilis* bacteria where the cyt. d is the photoacceptor for that wavelength which involved in their respiratory chain, on the other hand the E.D. 5.6 J/cm^2 caused a non significant (P>0.05) excitation to *E. coli* only. Any lateness occurred between the L_5 and L_{10} curves (provided that they started higher than control curve at any times of incubation days) may be related to the time needed by the dividing cell to achieved an equivalent value between the cell surface and synthesized ATP to start a new division cycle.

The strict aerobic bacteria; *P. aeroginosa*, were also activated by 650 nm diode laser as it is highly depend on cyt. c other than cyt. d in its respiration process. So this significant (P<0.05) activation might come from excitation of other component in the respiratory chain. For that, the 2.8 and 5.6 J/cm^2 of 650 nm diode laser light are also considered an activated E.D. for *P. aeroginosa*.

II- Effect of laser treated bacteria on PMNs PKA:

The PKA of L_5 (2.8 J/cm^2 E.D.) laser treated *P. aeroginosa* in figure 4 showed a significant (P<0.05) differences after 1 hr incubation period time (T_1), while after 2 hr (T_2) all PKA value appeared non significantly (P>0.05) closed to one point, so from this figure we concluded that the 2.8 J/cm^2 E.D. of 650 nm laser light has no effect on the structure of *P. aeroginosa* cell wall component; especially the chain length of LPS (the target of CD14 receptor in the surface of neutrophils [18, 19, 20]),

On the other hand a noticeable different in the PKA values were found for the E.D. 5.6 J/cm^2 (L_{10} laser treated bacteria) in figure -5, where all PKA values had significant (P<0.05) lower value than control (more killed bacteria). These increased number of killed bacteria may related to the fact that after the chemical changes in the redox stat of the cell it will effect on the LPS chain length in the outer surface of newly divided cells [15, 16], and as a result to this, it will surly affect on how strongly such external components (LPS) can interact with PMN, and ended by phagocytosis. [10]

For *E coli* bacteria, the PKA values showed a significant difference (P<0.05) for L_5 when compared with control figure- 6, while a non significant difference (P>0.05) was observed for L_{10} PKA values, there for there was no effect of 5.6 J/cm^2 E.D. of 640 nm laser on the LPS length.Figure-7

In figure -8, from the PKA values of L_5 *P. mirabilis*, it's also appeared a slight significant (P<0.05) effect of 2.8 J/cm^2 650 nm diode laser on the LPS chain length. But for the E.D. 5.6 J/ cm^2 (L_{10}), it caused a significant (P<0.05) increasing in the killed bacteria (low PKA). Since this E.D. had an inhibition effect on *P. mirabilis*(figure- 3), so the high number of killed by PMN might came from a changes in the LPS chain length or the resistant mechanisms to phagocytosis for the weekend new daughter cells. [15, 16, 25]

It appeared that the intensity 2.8 J/ cm^2 E.D. of 650 nm diode laser caused a significant (P<0.05) effected on PKA values of laser treated *P. aeroginosa, E. coli* and *P. merabilis*. While the 5.6 J/cm^2 E.D. cause a significant (P<0.05) change in PKA values for *P. aeroginosa* and *P. merabilis*, which might came from its effect on LPS chain length or the resistance of bacteria to PMN phagocytosis. Based on the data from some literature, it supposed that the interaction (adhesion) between two membranes of a Gram-negative bacteria cell play an important role in cell division as this process is ΔpH dependent, so this chemical and physical changes may effect on the characteristics of LPS; especially the O-side chain length and capping frequency of lipid A-core in a given strain [15], and adhesion with CD 14 of polymorphonuclear neutrophils (PMN) cell. [18, 19]

As one of the physical environmental effects on the LPS, Kropinski and his colleagues found that thermal adaptation of *P. aeroginosa* caused changes in the LPS, phospholipids and outer membrane protein. [26]. Another experiment done by Weiss and his colleagues, reported that the bacterial sensitivity or resistance to purified bactericidal PMN proteins is determined by the binding properties of the outer cell membrane, which in turn depends upon the LPS chain length. They inoculated *E. coli* and *Sallmonella typhimurin* on media containing galactos, and this sugar will synthesis a complete length of LPS, and will became more resistance than the bacteria that grown in the absence of galactos (short chain of LPS will be formed). [16]

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