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Effect of Laser Beam on Microorganisms Resistive to Carcinoma

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MICROBIAL THERAPY is being used to control the tumor growth. However the control of the activity of the microbe faces several problems which limited its applicability. Therefore, the aim of the present study was to assess the possibility of controlling the activity of *Pseudomonas aeruginosa* by 650 nm diode laser beam; power density $_{150}$ mW/cm^2, the spot area of the laser beam was 1 cm^2, incident doses of 30 joules/cm^2 was applied for 30 min per day. The tested mice were divided into 7 groups A, B, C, D, E, F and G. Group A was used as control, group B injected in the right thigh by 0.2 ml from a suspension containing 10^6 cell /ml of the Ehrlich tumor, group C injected with $4x10^9$ CFU of *Pseudomonas aeruginosa*. group D injected in the right thigh by 0.2 ml from a suspension containing 10^6 cells/ml of the Ehrlich tumor and exposed to Laser radiation for 30 min /day along four successive days. The group E injected with $4x10^9$ CFU of *Pseudomonas aeruginosa*, and exposed to Laser radiation for 30 min /day along four successive days. The group F mice injected in the right thigh by both 0.2 ml from a suspension containing 10^6 cell/ ml of the Ehrlich tumor and $4x10^9$ CFU of *Pseudomonas aeruginosa*. The group G injected in the right thigh by both 0.2 ml from a suspension containing 10^6 cell/ml of the Ehrlich tumor and $4x10^9$ CFU of *Pseudomonas aeruginosa* and exposed to Laser radiation for 30 mins/day along four successive days. The tumor growth characteristics were followed for all animals. Cellular changes were evaluated using cells viability (ATP production) and cytokines expression (IFN- γ and IL-6) the treated and untreated tumors were studied and survival rate were demonstrated. The results showed that injection with the microorganism alone caused the death of all animals till fourth day. While, the mice with post tumor implantation (PI) which exposed to laser irradiation decreased the volume of the tumor. On the other hand, tumors infected with microorganisms then irradiated for 1 day post infection showed a sudden decrease in tumor volume during next three days of infection then the tumor began to grow again with higher rate. More than 70% of the animals survive till day 35 PI. The groups A, B, C and F in the 1st, 2nd, 3rd and 4th days produced a non significant increase in ATP luminescence. While, the groups D, E and G irradiated once at the 1st, 2nd, 3rd and 4th day produced a significant increase in ATP. In addition, the groups A, B, C and F cells showed a significant increase in the level production of

IFN-γ and IL-6 in serum. Moreover, Irradiated groups D, E and G produced a significant change of both cytokines, but, the group G exhibited a highly significant increase in the production of IFN-γ and IL-6 in serum more than the groups D and E.

Keywords: Diode laser, Microbial therapy, *Pseudomonas aeruginosa*, Ehrlich tumor .

Therapeutic low level laser beam

Studies using therapeutic lasers have reported an effect on inflammation, mainly by shortening the inflammatory process–which in itself is essential for healing[1,2]. Sawasaki *et al.*[3] and Silveira *et al.*[4] reported that mast cell degranulation significantly increase after 670 nm laser irradiation of human mucosa and gingiva, respectively. The degranulation leads to a release of histamine and should theoretically stimulate an increased inflammatory response. It was speculated that the increased mast cell degranulation accelerates the inflammatory process, which eventually leads to wound healing via fibroblast proliferation and collagen synthesis. Chronic periodontal inflammation leads to the destruction of the periodontal ligament and subsequently to loss of alveolar bone. The latter is mediated primarily by osteoclasts and triggered by the pro-inflammatory molecule prostaglandin E2 (PGE2)[1]. An important aspect of laser-tissue interaction was the coherence of the laser light. Many studies have compared the biological effect of coherent and incoherent light and to date, all studies indicated that a superior effect by lasers producing a long length of coherence. These light sources have a spectral width of 30-100 nm, while, the spectral widths of the lasers are in the range 0.01 – 1 nm. A study by Rosner *et al.*[5] investigated the effect of diode laser on regeneration of crushed optical nerves. While diode laser delayed the degenerative process, non-coherent infrared light was ineffective or affected the injured nerves adversely. Coherence seems to be an important parameter in light stimulation of biological scattering in bulk tissue. Karu *et al.*[6,7] studied the importance of different light characteristics in cell stimulation, such as wavelength, coherence, dose and time regimens and concluded that coherence had no effect. Laser phototherapies (LPT), its mechanisms are achieved an effect, while, the photon must be absorbed by photoreceptors. There are many photoreceptors in the human body, *e.g.* the porphyrins. However, the most important receptor has been identified as cytochrome c-oxidase, the terminal enzyme of the Kreb's cycle. Cytochrome c-oxidase is an ATP producer[8,9]. A cell in a reduced condition can be revitalized by stimulating production of ATP. The laser light in the red spectrum serves the bond between NO and cytochrome c-oxidase, allowing the enzyme to initiate production of ATP[10] . This production in itself leads to a cascade of events, such as increased permeability of the cell wall and the Ca^{++} circulation. It has been speculated that infrared laser light by passes this process and acts directly on the cell membrane permeability and the calcium ion channels. Cells in a normal redox situation are not particularly responsive to LPT: the best effect is seen in cells in a reduced redox situation[11].

History of using bacteria for the treatment of solid tumors

The normal microflora in humans and animals may restrain malignant tumor regeneration.

The Ehrlich ascites tumor cell is a spontaneous murine mammary adenocarcinoma[12] adapted to ascites form[13] and carried in out bred mice by serial intraperitoneal (i.p.) passage. All solid tumors undergo angiogenesis, as tumor cells which grow much faster than cells making up the blood vessel, it showed in biological changes and adaptive metabolisms, *i.e.* formation of defective vessels, appearance of apoptotic and necrotic cancer tissues, emergence of hypoxic areas and heterogeneous tumor cell populations [14]. Samuilov *et al*.[15] documented that cancer biotherapy including bacteriolytic therapy aimed to induce apoptosis in tumor cells, bacterial vector-based cancer vaccines, and inhibition of tumor neoangiogenesis by microorganisms.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an aerobic bacterium that switches over to the denitrification mode of metabolism under anaerobic conditions. It induces apoptosis in macrophages, epithelial cells, neutrophils, and tumor cells[15] . So, if *P. aeruginosa* injected at the core of the tumor, and began to grow toward the outside causing apoptosis to different areas of the tumor, it would help hopefully in getting rid of the tumor tissue. *P.aeruginosa* is also an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. In fact, *P. aeruginosa* is the epitome of an opportunistic pathogen of humans. The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect if the tissue defenses are compromised in some manner. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections**,** bacteremia, bone, joint infections, gastrointestinal infections, AIDS patients who are immunosuppressed. Moreover, it also causes a variety of systemic infections, particularly in patients hospitalized with cancer, cystic fibrosis, and burns. The case fatality rate in these patients is near 50 percent [16].

Azurin as P.aeruginosa anti-cancer agent

It has been recently reported that a bacterial redox protein, azurin, of *P. aeruginosa* acts as an anti-cancer agent both *in vitro* and *in vivo* in a nude mouse model. Azurin is a copper-containing oxide-reductase that is normally involved in the denitrification process in *P. aeruginosa*. Azurin is a member of a group of proteins collectively called cupredoxins, which are small, soluble redox proteins whose active site containes a type 1 copper protein. Azurin itself is involved in electron transfer during denitrification[17]. Azurin has several interesting characteristics. Not only is it released from *P. aeruginosa* in contact with cancer cells, but once released, it enters preferentially to cancer cells and only marginally to normal cells[18]. Moreover, on entry to cancer cells, it induces apoptosis in p53-positive cancer cells through complex formation and stabilization of the tumor suppressor protein p53 and significantly allows

regression of *in vivo* cancer growth [17,19]. Azurin also inhibits growth of various cancer cells by interfering in the signaling pathways and angiogenesis[20] demonstrating the multiple pathways through which it exerts its inhibitory action. Azurin is not only a potential anticancer drug candidate, but it forms complexes with many surface proteins present in parasites such as the malaria parasite *Plasmodium falciparum* or viruses such as the AIDS virus HIV-1, thereby interfering in their entry to the host cells and significantly suppressed their growth[21]. Samuilov[15] reviewed diverse approaches to cancer biotherapy including bacteriolytic therapy aimed at inducing apoptosis in tumor cells, bacterial vector-based cancer vaccines, and inhibition of tumor neoangiogenesis by microorganisms. The normal microflora in humans and animals may restrain malignant tumor regeneration. Punj *et al*.[17] documented various approaches for using bacteria in cancer therapy include the use of bacteria as sensitizing agents for chemotherapy, as delivery agents for cancer drugs and agents for gene therapy. The tumor regression stimulated by infecting microorganisms has been attributed to activation of the immune system of the host.

Materials and Methods

Pseudomonas aeruginosa samples

Pseudomonas aeruginosa was obtained from the faculty of medicine, Ain Shams University. The LB broth medium (pH7.1) is composed of NaCl (10 g), yeast extract (5 g), peptone from meat (10 g), and distilled H_2O (1000 ml) according to Kranendonk *et al.,* (2010)[36]. A calibration curve was done which relates the absorbance of the sample at 600 nm to the number of colony forming units (CFU). The LB broth medium was inoculated with the isolate and incubated with shaking (1400 rpm) at 37 ºC over night. 50 µl of the incubated culture was diluted with 2ml of the LB broth medium and observed while growing. The absorbance of the sample was measured at 600nm through the use of a spectrophotometer model (6405 UV/Vis) manufactured by Jenway in UK. Using the viable plate count technique 50 µl from the sample was diluted by distilled water to different concentrations of the bacteria and spread on agar plates then incubated at 37 ºC over night. The number of living colonies was then counted. The average number of CFU is calculated in the original concentration. The above steps are repeated for different concentrations of the microorganism. A relation is plotted between the absorbance of the sample and the number of CFU.

Injection of Ehrlich tumor

Animals were injected in the right thigh by 0.2 ml from a suspension containing 10^6 cells/ml isolated from Ehrlich ascites carcinoma in mice, prepared in the National Cancer Institute (NCI), Cairo University, Egypt.

Tumor volume estimation

It started from 10^{th} day post infection, wherever, the tumor volume of each mouse was measured twice a week using a vernier caliper, by measuring the

three mutually orthogonal tumor diameters. The volume of the tumor (V) was calculated through the relation suggested by Ning equation (1984):

$$V\ (cm^3) = (\pi / 6) * abc$$

where a, b and c, are the length, width and height of the tumor mass, respectively. After the bacterial injection the tumor volume was monitored daily. During the experiment a relation between tumor size and the number of days was made, to study the variation of tumor size of all groups with time.

Laser treatment parameters

The laser used was diode laser (NEC, Japan), Laser has a given wavelength of light. Its energy density is the most important factor in determining the tissue reaction[22]. It is the output power density which determines the time required to deliver a particular energy density (Joules/cm^2) dosage that means, the output power determines the corresponding energy (Joules) which delivered during that time. Power density 150 mW/cm^2, wavelengths, 650 nm, the spot size of the laser beam was 1 cm^2 with exposure durations 30 min per day. Incident doses of (30 Joules/cm^2) and treatment schedule of once/day were used in the experiments and wave emission continuous. Laser therapy devices are generally specified in terms of the average output power of the laser diode, and the wavelength of light, where, they emit. The output power of the laser refers to the number of photons emitted at a particular wavelength and the power density measures the potential thermal effect of those photons at the treatment area [23].

Animals were anaesthetized prior to and during creation of the tumor. The dorsal fur of the animals was shaved with an electric clipper, and the anticipated area of the tumor to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision tumor of circular area 250 mm^2 and 2 mm depth was created along the markings using toothed forceps, a surgical blade and pointed scissors. The low energy diode laser with average power of 150 mW/cm^2, wave length (650 nm) was directed over the animal for an exposure time of 30 min /day according to their groups.

The optical penetration of the light varies with several parameters. The short wavelengths in the red spectrum have less penetration than those in the infrared spectrum. The type of tissue also influences the penetration.

Experimental animals and treatment schedule

Seventy female Balb/c mice of average weight 20 $\pm$ 2 g were used in this experiment. Ten animals were kept as control group and received no treatment. The 70 animals were divided into 7 equal groups namely; A, B, C, D, E, F and G.

Group	Experimental conditions/ treatments
A	Normal animals didn't receive any further treatment.
B	Animals injected in the right thigh by 0.2 ml from a suspension containing 10^6 cell /mL of the Ehrlich tumor only.
C	Animals injected with100µl PBS which contains $4x10^9$ CFU of *Pseudomonas aeruginosa* .
D	Animals injected in the right thigh by 0.2 ml from a suspension containing 10^6 cells/mL of the Ehrlich tumor and exposed to Laser radiation for 30 min/day along four successive days.
E	Animals injected with 100µl PBS which contains $4x10^9$ CFU of *Pseudomonas aeruginosa*, and exposed to Laser radiation for 30 min /day along four successive days.
F	Animals injected in the right thigh by both 0.2 ml from a suspension containing 10^6 cell /ml of the Ehrlich tumor and 100µl PBS which contains $4x10^9$ CFU of *Pseudomonas aeruginosa* .
G	Animals injected in the right thigh by both 0.2 ml from a suspension containing 10^6 cell /ml of the Ehrlich tumor and 100µl PBS which contains $4x10^9$ CFU of *Pseudomonas aeruginosa* and exposed to laser radiation for 30 min /day along four successive days.

Laser device and treatment parameters

The laser used was diode laser (NEC, Japan) with an output power density 150 mW/cm^2, wavelengths, 650 nm, the spot size of the laser beam was 1 cm^2 with exposure durations 30 min per day. Incident doses of 30 (joules/cm^2) and treatment schedule of once/day were used in the experiments and wave emission continuous. The output power of a laser, measured in mill watts, refers to the number of photons emitted at the particular wavelength of the laser diode[(23)]. Power density measured the potential thermal effect of those photons at the treatment area[(24)]. Despite the high output power this laser also has biostmulating effects[(25)] .The energy deposited was calculated through the relation.

Calculating laser treatment parameters

The laser used was He-Ne Laser (NEC,Japan) with an output power of 150 mW , wavelengths, 650 nm and beam diameter of 1 cm^2. The bacteria suspension was prepared and subjected to radiation dose by varying the exposure durations for 30, 60, 90 and 120 min . Non irradiated suspensions were used as control.

Laser Therapy devices are generally specified in terms of the average output power (mW) of the laser diode, and the wavelength (nanometers) of light they emit. This is necessary information, but not enough with which to accurately define the parameters of the laser system. To do this, one must also know the area of the laser beam (cm^2) at the treatment surface (usually the tip of the hand piece when in contact with the skin).

If the output power (mW) and beam area (cm^2) are known, it is a reasonably straight-forward exercise to calculate the remaining parameters which allow the precise dosage measurement and delivery.

The output power of a laser, measured in mW, refers to the number of photons emitted at the particular wavelength of the laser diode.

Power Density measures the potential thermal effect of those photons at the treatment area. It is a function of Laser Output Power and Beam area, and is calculated as:

$$1)\ \text{Power Density } (W/cm^2) = \frac{\text{Laser Output Power (W)}}{\text{Beam area } (cm^2)}$$

$$\text{Power Density } (W/cm^2) = 0.15(W)\ /3.14\ (cm^2) = 0.047\ w/cm^2$$

The total photonic energy delivered into the tissue by a laser diode operating at a particular output power over a certain period is measured in Joules, and is calculated as follows:

$$2)\ \text{Energy (Joules)} = \text{Laser Output Power (Watts) x Time s}$$

$$\text{Energy (Joules)} = 0.15\ (\text{Watts}) \times 1800\ s = 270\ (W/s)$$

It is important to know the distribution of the total energy over the treatment area, in order to accurately measure dosage. This distribution is measured as Energy Density ($Joules/cm^2$). "For a given wavelength of light, energy density is the most important factor in determining the tissue reaction" [12] . Energy Density is a function of Power Density and Time in seconds, and is calculated as:

$$3)\ \text{Energy Density } (Joule/cm^2) = \frac{\text{Laser Output Power (Watts) x Time (s)}}{\text{Beam Area } (cm^2)}$$

$$\text{Energy Density } (Joule/cm^2) = \frac{0.15\ (\text{Watts}) \times 1800\ (s)}{3.14\ (cm^2)} = 85.98\ (J/cm^2)$$

To calculate the treatment time for a particular dosage, you will need to know either the Energy Density (J/cm^2), as well as the Output Power (mW), and Beam Area (cm^2).

$$4)\ \text{Treatment Time (Seconds)} = \frac{\text{Energy Density } (Joules/cm^2)}{\text{Output Power Density } (W/cm^2)}$$

$$\text{Treatment Time (s)} = \frac{85.98\ (J/cm^2)}{0.047\ (W/cm^2)} = 1829.5\ (s)$$

Cytokines expression (IFN-γ and IL-6)

Interferon gamma and Interlukine-6 are apheliotropic cytokine that acts on wide range of tissue and cells exerting growth including, growth, inhibitory and differentiation induction effects depend on the nature of the target cells, [26]. IFN-*γ* and IL-6 regulates immune activity the acute phase response to injury and infection, inflammation, ontogenesis and hematopoiesis, [26,27]. The photometric (ELISA) assay were used for the quantitative determination of IFN-*γ* and IL-6 in the culture medium of laser irradiated and un irradiated control cells .The assay is based on the quantitative sandwich Elisa principle using the IFN-*γ* and IL-6. ELISA kit II (Scientific Group. S. A, BD550788).

Cell viability (ATP)

Proliferating cells continuously pass through the cell cycle, which divided into four phases, namely G1 (GAP1), S (synthesis), G2 (GAP2) and M (mitosis). The cells are arrested in G1 phase until they receive signals that instruction them to divide. The most important parameters used for studying cell viability in cell populations are metabolic activity and DNA synthesis. Viability of cell was determined by the ATP luminescence. The effects laser light is controversial as it has been found that visible light including laser light has both stimulatory and inhibitory or no effect[28, 29]. Cellular ATP was measured by using the cell Titer-Glo^{TM} Luminescent cell viability assay (Promega S.A, G7573). This is homogeneous method of determining the number of viability cells in culture based on the quantization of ATP present, which signals the presence of metabolic activation of cells (Promega Technical Bulletin,TB 288). The production of luminescent signal is proportional to the number of cells present, An ATP standard curve performed by using a 10 fold serial dilution of 1µM ATP in culture media (1µM to 10 pM containing 10^{-10} to 10^{-15} moles ATP). An equal amount of cell Titer-Glo^{TM} reagent was added to 50µl of suspended cells, mixed for 2 min and the luminescent signal was allowed to stabilize by incubation at room temperature for 10 min.

Survival percentage of the animals of each group

$$\text{Survival percentage} = \frac{\text{pre exposure survived animals} - \text{after exposure survived animals}}{\text{Pre-exposure survived animals}} \times 100$$

Statistical analysis

All laser exposures or non exposures were performed four times on different cell populations. Each test performed in duplicate statistical analysis was done by using the one tailed student t-test and one-way ANOVA with the Newman-Keul's Multiple Comparison Test. Results were considered statistically when P value of < 0.05 . The results are expressed as mean ± S.E .

Results

Mice were un -irradiated and irradiated to output power density 150 mW/cm^2, wavelengths, 650 nm, the spot size of the laser beam was 1 cm^2 with exposure durations 30 min per day. Incident doses of (30 Joules/cm^2) and according to treatment schedule of once /day were used in the experiments and wave emission continuous. Cellular activity was determined by evaluating various cellular and cytokines expression.

Effects of continuous exposure to 650 nm Laser on Pseudomonas aeruginosa with or without infected Ehrlich tumor

Figure 1 showed the variation of the tumor volume as a function of incubation time post tumor implantation (P.I) (days) for animals of group B which left without any further treatment. The results indicate progressive growth of tumor volume in both animals' thighs. Animals of group F were injected in the right thigh by 0.2 ml from a suspension containing 10^6 cells/ ml of the Ehrlich tumor (50µLbacteria /100µL PBS which contains $4x10^9$ microbial cells). As a function of incubation period in group F, the results indicated the decrease of tumor volume and stop of its further growth. However, most of the animals began to die and no one survived at day 25 PI. But in group C, the animals which injected by dose of *P. aeruginosa* injection (50µl bacteria /100µl PBS which contains $4x10^9$ microbial cells), remained more than 4 days**.** Figure 1 also represented the thermal analysis of the tumor volume as a function of the incubation period for animal exposed laser output power 500 mW, λ=650 nm and beam diameter of 1cm^2 for 30 min/day. The results exhibited that the part body exposure of the animal to laser beam resulted in the deterioration of the tumor growth activity as can be noticed in the animals of group D. However, the animals of group E which injected by dose of *P.aeruginosa* injection (50µLbacteria/100µL PBS which contains $4x10^9$ microbial cells) and exposed to laser output power 500 mW, λ=650 nm and beam diameter of 1cm^2, remained more than 8 days**.** But, in group (G), the tumor volume was found to be inhibited more than other groups, where, the tumor treated with bacteria.

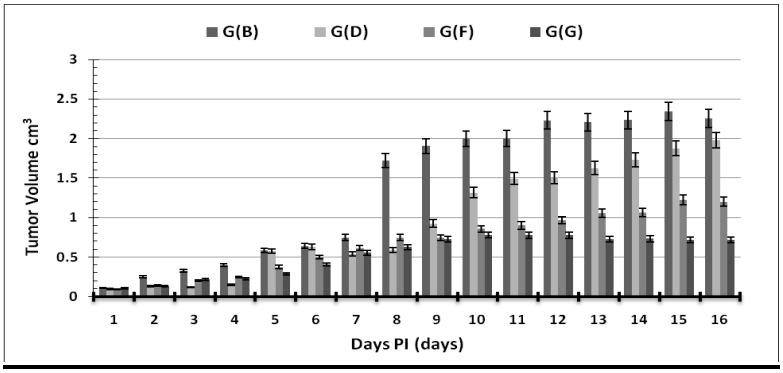

Fig. 1. The variation of the tumor volume as a function of the incubation period for animal exposed or non exposed to laser (laser output power 500 mW, λ=650 nm for 30 min/day). The data are expressed as mean ± S.E.

Effects of continuous exposure to 650 nm laser on level production of IFN-γ and IL-6 profile with or without Pseudomonas aeruginosa infected Ehrlich tumor

Sera of mice groups pre and post irradiation with laser were tested to evaluate the cellular immune response concerning the Th1 (IFN-γ) and Th2 (IL-6) cytokines using ELISA. When compared the un -irradiated group A control and the groups B, C (0 J/cm^2) cells and F, the results showed a significant increase in the level production of IFN-γ and IL-6 ($P< 0.01$ and $P < 0.05$, respectively) (Fig. 2 and 3). Irradiation groups D, E and G with (30 J/cm^2) cells produced a significant change but, group G induced a highly significant increase in level of IFN-γ and IL-6 ($P<0.001$ and $P<0.005$, respectively) when compared to groups D and E.

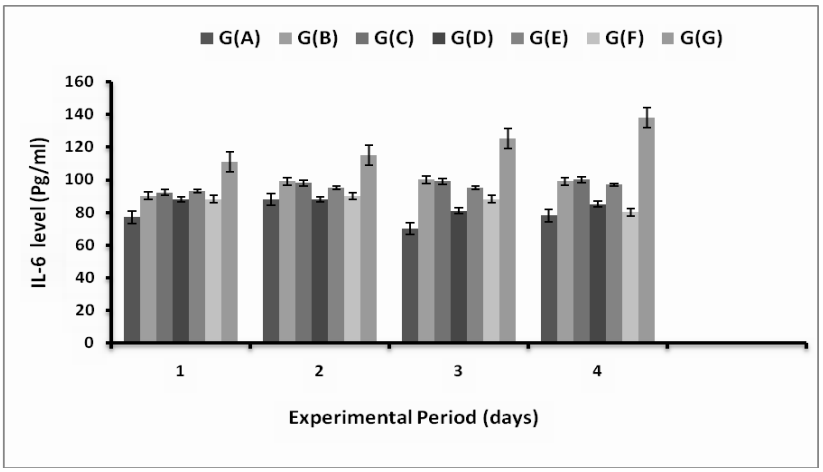

Fig. 2. Level of IL-6 in serum of mice in un- irradiated cells and irradiated cells at 650 nm of laser. The data are expressed as mean ± S.E.

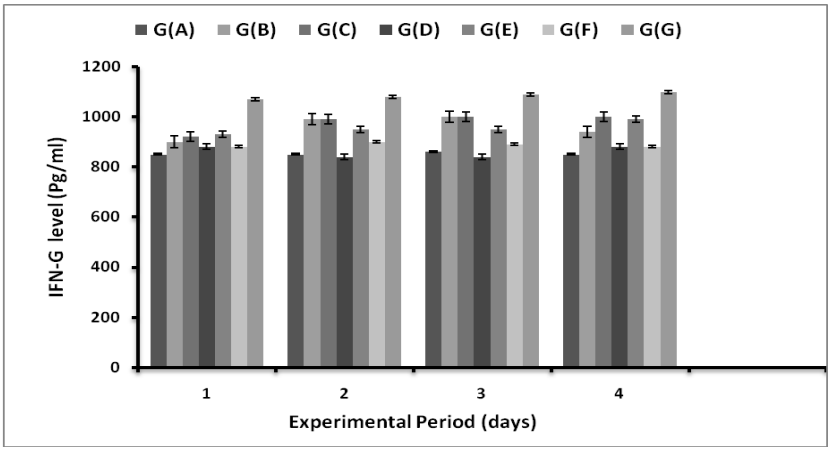

Fig. 3. Level production of IFN- γ in serum of mice in unirradiated cells and irradiated cells at 650 nm of laser beam. The results are expressed as mean ± S.E.

Effects of continuous exposure to 650 nm laser on cell viability (ATP)

As shown in Fig. 4, when compared the results of un irradiated group A (control) and groups B, C and F in the 1^{st}, 2^{nd} 3^{rd} and 4^{th} days with (0 J/cm^2), it exhibited a non significant increase in ATP luminescence's ($P< 0.02$ and $P < 0.04$, respectively). The irradiation groups D, E and G which irradiation once in the 1^{st}, 2^{nd} 3^{rd} and 4^{th} days with (30 J/cm^2), induced a highly significant increase in ATP ($P<0.002$ and $P<0.005$, respectively).

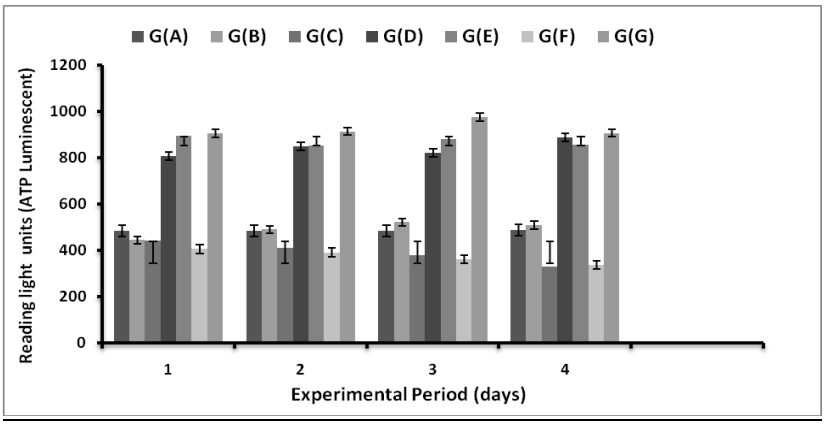

Fig. 4. Cellular viability as determined by the ATP Luminescent in unirradiated cells and irradiated cells at 650 nm of laser beam . The results are expressed as mean ± S.E.

Effect of single or fourth exposure

As shown in Fig. 4, and based on the results obtained in the present study, it exhibited the effects of a single radiation in the 1^{st}, 2^{nd} 3^{rd} and 4^{th} days on cells responded as far as the best exposure regime. But, the best one when enough time was given between irradiation to allow them to responded to the 650 nm laser stimuli. While, in group G, cells irradiated with (30 J/cm^2), it showed no differences in cellular viability and cytokine expression between 1^{st} and 2^{nd} day duration. In groups D and E, with (30 J/cm^2) cells irradiation in the 1^{st}, 2^{nd} 3^{rd} and 4^{th} days produced no significant change in viability ATP in all irradiated cells compared to control group A. On the other hand, when irradiated once or fourth in group G with (30 J/cm^2), it induced a highly significant change ($P<0.003$, $P<0.004$, $P<0.004$, $P<0.005$, respectively). But when compared to unirradiated cells there was no significant change in ATP luminescence in all cells irradiated with (30 J/cm^2). IFN-γ and IL-6 irradiation with (30 J/cm^2) in 1^{st} day to 4^{th} day with 650 nm laser showed significant change in IFN-γ and IL-6 ($P<0.05$) (Fig. 3). There was difference in cells irradiated only once day compared to the same cells that were irradiated once to 4 day with (30 J/cm^2) ($P < 0.002$, $P <0.003$ and $P <0.002$, $P <0.004$ and $P <0.001$, $P <0.005$ and $P <0.001$ and $P <0.005$, respectively).

Survival rate

Figure 5 illustrated the variation of the survival rate percentage as a function of the incubation period following tumor implantation for the animals from all groups were investigated. The variations of the survival rate percentage for the animals from all groups investigated were observed (Fig. 5). The results showed that animals of group A exhibited no animals lived longer than 40 days. In group D animals which injected with the exposed Ehrlich tumor began to die after 20 days post injection while animals of group B which injected with Ehrlich tumor began to die after 16 days. No animals from group (E) that injected with bacteria and exposed to laser output power 500 mW, λ=650 nm remained more than 8 days but, those of group C that injected by bacteria the animals died in the 2 days, which is considered as a marker to the high toxicity of this group. In group (F) animals, which injected with bacteria and Ehrlich tumor, it was observed that changes were in body weight, and ascites tumor volume began to die 25 days post injection. It seems interesting to find out that more than 60% of the animals of group G injected with bacteria and Ehrlich tumor and exposed to laser output power 500 mW, λ=650 nm were alive at day 35 PI which indicate that there is positive effects of the laser beam on both tumor growth and survival period of the animals. At the same time one may notice that for group A no animal lived longer than 40 days.

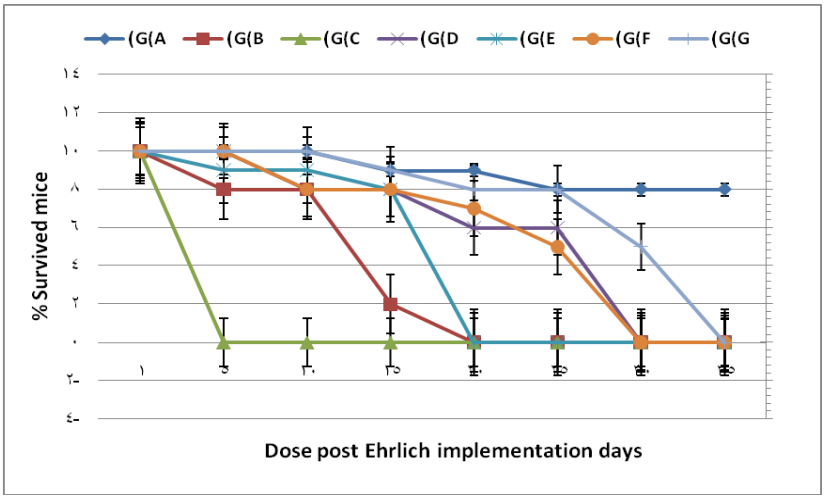

Fig. 5. Variation of the survival rate percentage as a function of the incubation period for animals from groups A, B, C, D, E, F and G. The data are expressed as mean ± S.E .

Discussion

The results revealed that the irradiation of visible laser light was better than the invisible laser light in the treatment of microbial infection of tumor on mice[30]. Several investigators documented that energy densities in the range (0.5

to 4 Joules/cm^2) are most effective in triggering a photo biological response in tissue, with 4 Joules/cm^2 having the greatest effect on wound healing[31,32]. The present work in this study showed a new method to control growth of Ehrlich tumor injected with (Ps.) by using output power density 150 mW/cm^2, wavelengths, 650 nm, the spot area of the laser beam was 1 cm^2 with exposure durations 30 min per day. Incident doses of 30 Joules/cm^2 and treatment schedule of once/day were used in the experiments and wave emission continuous on microbial infection of tumor on mice. The exposure level to the laser beam demonstrated was 650 nm which all can be found elsewhere in the close area to power lines. The investigation of the biophysical mechanisms of action is important because it examines the nature of the initial physicochemical interaction of 650 nm diod laser with biological systems, and the expression of these physicochemical changes as a biological response and that immune responses play an important role as antiviral and antimicrobial actions of IFN- γ and IL-6. Later on, the inflammatory process sets in with the presence of numerous chemical mediators and inflammatory cells (polymorphonuclear leukocytes, macrophages, and lymphocytes)[33,34].

Laser phototherpy mechanisms achieve an effect; the photon must be absorbed by photoreceptors,[34]. There are many photoreceptors in the mice body, *e.g.* the porphyrins. However, the most important receptor has been identified as cytochrome c-oxidase, the terminal enzyme of the Kreb's cycle. Cytochrome c-oxidase is an ATP producer[8,9,35]. A cell in a reduced condition can be revitalized by stimulating production of ATP. The laser light in the red spectrum severs the bond between NO and cytochrome c-oxidase, allowing the enzyme to initiate production of ATP[10].

The presented results also showed that there is non significant on cell viability (ATP) with exposure to 650 nm laser compared with the control in the 1st, 2nd 3rd and 4th days with (0 J/cm^2). Moreover, it produced non significant increase in ATP luminescence's. On the other hand, irradiation groups D, E and G irradiation once in the 1st, 2nd 3rd and 4th days with (30 J/cm^2) produced a significant increase in ATP. Treatment of injected organs by *Pseudomonas aeruginosa* with 650 nm laser for 30 min/day seems successful and applicable. Because of increasing mitochondrial ATP production, lymphocytes and mast cells activation, and proliferation of fibroblasts and other cells, besides promoting analgesia and anti-inflammatory effects[36,37]. At cellular level, the mechanism of low level laser therapy is based on an increase in mitochondrial oxidative metabolism caused by the excitation components of respiratory chain[28,38] (leading to an increase in ATP . The investigator, Karu,[38] provided evidence that wave length of 632 nm can increase respiratory activity, irradiation with (30 J/ cm^2) on any given days was above the bio stimulated threshold for cells and produced a decrease in cellular viability. Irradiation once a day had a negative impact on cytokines expression when irradiation once with (30 J/cm^2) on 1st day, However, there was no significant change. This result was in contrast with Hawkins & Abrahams,[39] who found an increase on IL-6 when irradiated twice with (5 J/cm^2) compared to cells

that were irradiated once. The increase in proliferation in these studies may be explained by the lower fluencies used such that deposited there being accumulative effect the total close given is still within the bio stimulated zone[40]. Kreisler and their colleagues[41] found an increase in proliferation of human larynx carcinoma cells irradiated with an 809 nm Gas laser power output 10 mW, fluencies 1.96, 3.92 and 7.84 J/cm^2, 2 or 3 consecutive days.

Gavish *et al.* [42] found that irradiation Hacat human keratin oocytes to a 780 nm titanium sapphire laser with a fluence of (2 J/cm^2) caused an up regulation of IL-6 genes. These results are in agreement with our results correspond to change in group (G) irradiated with (30 J/cm^2). There was no significant change when irradiated once on consecutive days possibly due to an inhibition of cytokines expression due to an accumulated effect. The results of unirradiated group A, B, C (0 J/cm^2) cells and F showed a significant increase in cytokines production IFN-γ and IL-6. While, Irradiation groups D, E and G with (30 J/cm^2) cells produced a significant change but, group G induced a highly significant increase in level of IFN-γ and IL-6 when compared to groups D, E.

Cells responded better when enough time was given between irradiations to allow cells to react and respond,[43]. On the other hand, higher fluencies (30 J/cm^2) clearly inflicted cellular damage and had on inhibitory effect on cellular migration. This corresponded with Hawkins & Abrahawse,[44] who reported that *Pseudomonas* is known as one of the most aggressive microorganisms because of its toxicity to biological tissues and its resistivity against almost all known antibiotics. To get the benefit of the high toxicity of this microorganism, it was used to attack the tumor cells, as microbial therapy. Several trials had been reported to use the microbial therapy for treatment of malignant tumors, [45-47]. However, this modality still needs a lot of investigations in order to be applicable .In the preset work the Ehrlich tumor was implanted in the thigh of mice and the tumor began to be noticed in the thigh by naked eye starting from day 10 Post Implantation (PI) and the mass of the tumor was large enough at day 16 PI. The results also indicated the progressive growth in tumor volume with incubation time, and no animal survived at day 40 PI. In most recent work by Fadel *et al.* [48] who reported that metastasis of the Ehrlich tumor can occur in all animals at day 25 PI. This finding may explain the reason for gradual death of animals till day 40 PI. Infection of the tumor with *Pseudomonas* at day 16 PI caused the sudden decrease in the volume of the tumor. The aggressive attack of the microorganism to the tumor caused no further growth in tumor volume. However, those animals which didn't receive any treatment after being infected with the microorganism began to die 3 days post infection and the last one died at the 4th day. On the other hand ,in group G, the animal which infected with microorganism at day 16 PI then exposed to 650 nm laser for 30min day, then, after 1 hr of infection, it showed sudden drop in tumor volume during the three following days. Moreover, the tumor began to grow again but, with lower values as compared with group. In addition, fifty percent of the animals from this group survived till day 40 PI.

Therefore, based on the above discussion, it may be concluded that microbial cells for treatment of malignant tumors are a promising technique after being well controlled as justified. The use of *Pseudomonas aeruginosa* in microbial therapy for the treatment of malignant tumors is promising and still some work is needed for its applicability in human. The low energy at wavelength of 632.8 nm can modulate the cell proliferation and the release of growth factors from fibroblasts[49] . The present study showed that the laser-treated group (G) were inhibition of microbial infection of tumor better and faster with $P < 0.0001$ as compared to the control group, and an increasing dose (30 J/ cm^2) was found to decelerating the reparative process and hence called a bio-inhibiting dose in microbial infection of tumor. The bactericidal effect of Nd:YAG laser has been tested *in vitro* by Kranendonk *et al.*[36].

Conclusion

At wave length 650nm decrease the volume of tumor was evident when irradiated (30J/cm^2) on 4^{th} day. Not only was there a stimulation of cytokines expression (IFN- γ and IL-6) cells which protected from the damaging effect of tumor. This study also showed that influence of (30 J /cm^2) was above the simulative threshold for group G at wavelength 650 nm. The influence of (30 J /cm^2) was no change in 1^{st} day, while, (30 J /cm^2) was inhibitive to tumor. A single exposure showed no change volume of tumor while fourth exposure is inhibitive and there is accumulative effect. In addition to the cumulative effect, irradiation on non consecutive days is better than irradiation on consecutive days which determined by the biochemical parameters depending on the cellular parameters being tested the duration effect should be determined for that specific test a wavelength of 650 nm is inhibitive tumor volume.

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تأثير شعاع الليزر على الكائنات الدقيقه المقاومه للورم

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في محاولة لاكتشاف علاج بديل فعال للسيطرة على نمو الأورام ، باستخدام شعاع الليزر ٦٥٠ نانوميتر حيث من المعروف أن الطرق التقليدية المستخدمة لعلاج السرطان هى الجراحة تليها العلاج الإشعاعي ثم العلاج الكيميائي. وتم مؤخرا استخدام العلاج الميكروبي للسيطرة على نمو الورم. ولكن السيطرة على نشاط الميكروب وجد عدة مشاكل و التي تقتصر على تطبيقه. ولذلك، كان الهدف من هذه الدراسة هو إمكانية السيطرة على نشاط الكائنات الحية الدقيقة المقاومه للورم، باستخدام شعاع الليزر الذي يتميز بالخصائص التالية :

(650 nm diode laser , power density 150 mW/cm^2, the spot size of the laser 1 cm^2, exposure duration 30 minute per day, Energy of incident dose 30 joules/cm^2)

تم تقسيم الفئران إلى ٧ مجموعات هي A، B، C، D، E، F وG . A المجموعة الحاكمة والمجموعةB تم حقنها في الفخذ الأيمن بنسبة ٠,٢ مل معلق يحتوي على $١٠^{٦}$ خلية / مل من الورم إيرليك .والمجموعة C تم حقنها بعدد $١٠x٤^{٩}$ CFU من الميكروب. وكذلك المجموعة D تم حقنها في الفخذ الأيمن بنسبة ٠,٢ مل من معلق يحتوي على $١٠^{٦}$ خلية / مل من الورم إيرليك وتتعرض لأشعة الليزر لمدة ٣٠ دقيقة / يوم لمدة أربعة أيام متتالية. المجموعة E تم حقنها بعدد $١٠x٤^{٩}$ CFU من الميكروب، وتم تعريضها لأشعة الليزر لمدة ٣٠ دقيقة / يوم لمدة أربعة أيام متتالية. والمجموعة F تم حقنها في نفس المكان وبنفس النسبه من معلق يحتوي على $١٠^{٦}$ خلية / مل من الورم إيرليك و $١٠x٤^{٩}$ CFU من الميكروب. و المجموعة G تم حقنها في الفخذ الأيمن أيضا من نفس المعلق ومن نفس تركيز الورم من الميكروب وتتعرض لأشعة الليزر خلال نفس الزمن المذكور سابقا . وتم دراسة خصائص نمو الورم لجميع الحيوانات. ثم تم تقييم التغيرات الخلوية باستخدام خلايا حيوية (ATP الإنتاج) و السيتوكينات الإنترفيرون (IL6-γ) كما تم دراسة معدل البقاء على قيد الحياة وأظهرت النتائج أن الحقن مع الكائنات الحية الدقيقة وحدها تسبب في وفاة جميع الحيوانات فى اليوم الرابع. فى حين أن الفئران التى زرع بها الورم مسبقا وتم تعريضها إلى شعاع الليزر أظهرت نقصان فى حجم هذا الورم. بينما الأورام التى تم حقنها بهذه الكائنات الدقيقه عندما تعرضت لشعاع الليزر حدث نقص مفاجئ فى حجم الورم بعد يوم من حقن الكائنات الدقيقه .