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Low Power He:Ne Laser Radiation in Killing Photosensitized *Helicobacter pylori* in Vitro

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Abstract: Four photosensitizers were used to test inhibitory effect of *Helicobacter pylori* bacteria using low power helium: neon red laser radiation. Biopsies were collected from 176 patients and *H. pylori* were isolated, identified and bacterial suspension was prepared. Samples of this suspension were mixed with various low concentrations of the test sensitizer. The mixture samples were exposed to different laser radiation doses. The samples were then inoculated and the inhibition zones were studied and compared with their analogues of control samples. The most effective sensitizer with optimum concentration and irradiation dose was determined. Statistical analysis of results was performed. The sensitizers' toluidine blue and the methylene blue with concentration of 100 μg/ml were able to produce the same effect of complete killing when irradiation energy density 13 J/cm^2. However, thionin and crystal violet sensitizers when used with the same concentration and exposed to the same laser dose, showed minor inhibitory effect. Irradiation of bacterial samples with absence of sensitizer or sensitized samples at the concentrations employed with out laser radiation has no effect on *H. pylori* viability in all of the experiments.

Introduction

Helicobacter pylori are known as pathogenic bacteria associated with gastric diseases, duodenal ulcers with possibility of causing carcinoma of stomach (Vaira *et al.*; 1990, Moss *et al.*, 1992 and Eurogast 1993). Conventionally, eradication of the bacteria is performed using a combination of antibiotics with achieved eradication rates over 80% (Rauws *et al.*, 1990). The risks involved include rising rates of antibiotic resistance with possibility of several side effects (Gorbach, 1990).

Lately, an alternative method of killing pathogenic bacteria is involved in using the red radiation of low power He: Ne laser in presence of sensitizing agent (Kandela, 1994; Kandela *et al.*, 2002; Malk *et al.*, 1990 and Milson *et al.*, 1996). The killing effect may be due to cytotoxic radicals that produced by the energy which can be absorbed by the sensitizer molecule and delivered to the surrounding constituents of the defected area; other reasons are possible (Kandela, 1994). This technique was successful in our laboratory, optimized and monitored in procedure, type of laser, photo-sensitizer and the irradiation dose for killing several kinds of bacteria both gram positive and gram negative, aerobic and microaerphilic, facultative also opportunistic types. In all cases, it was found that the bacteria can be sensitive to a type of sensitizer and to a low power laser irradiation dose, such that the killing process in all cases is of nonthermal type (Kandela, 1994; Bakkour, 2001 ; Al-Khafaje, 2002 and Kandela *et al.*, 2002).

Materials and Methods

Organism

Samples were collected from 176 patients resting in Baghdad local hospitals suffering from

peptic ulcers, gastritis and duodenities. Biopsies were taken by gastroduodenal endoscope. The *H. pylori* were isolated, identified from 140 patients using biochemical and histopathological methods and bacterial suspension was prepared (Bakkour, 2001).

Photosensitizers

Four kinds of photosensitisers were selected (Merck Company); these are: the toluidine blue O (TBO), the methylene blue (MB), the crystal violet (CV) and the thionin (Th).

Each photosensitizer was prepared with a concentration of 10 mg/ml then diluted using distilled water. Five low concentrations were then prepared for each sensitizer and these ranged between 10 μg/ml to 100 μg/ml. To study the effect of laser irradiation on sensitized *H. pylori*, bacterial suspension was mixed with the prepared concentrations of the test sensitizer, samples were irradiated for three different periods; the inhibition effect of bacteria in each case was studied with and without laser irradiation and the results were compared with those of control samples. The procedure was repeated for the other sensitizers.

Laser source and irradiation procedure

Helium:Neon laser (MWK industries, USA) of about 9 mW measured power and wavelength 632.8 nm was used. The laser unit was mounted on a stand base furnished with possibility of adjustment in three dimensions. The horizontal laser beam was reflected vertically on sample pit using a plane mirror. To determine the irradiation dose accurately in each experiment, the spot size of the laser beam was arranged to fit exactly the exposed area of the test sample and measured; the laser output power was measured by a digital power-meter. The exposure time was varied from 1-3 minutes; for each period the radiation dose was calculated in J/cm^2; the corresponding values range from $2.6 – 13\ J/cm^2$.

Bacterial samples, total viable cell count number 1.29×10^9 without sensitizer was irradiated first (L+,S-) using three different exposure times (1, 2 and 3) min; next sensitized samples with five different concentrations (10,30,50,75,100) μg/ml of the test sensitizer were irradiated successively (L+,S+) and also for the three exposure periods. This was repeated for other sensitizers under test. Samples after each test were inoculated and the inhibition zones were studied. Total viable cell count number was adopted to determine the inhibition effect. Results were compared with their analogue of control samples (L-, S+) and (L-, S-). An average of the results of twenty experiments for each sensitizer was obtained.

Standard microtiter plates were used for irradiation procedure of the samples, which carried out in sterilized condition, nearly constant conditions of temperature and illumination for all exposure trials.

Results and Discussion

No detectable effect on the viability of *H. pylori* was observed when the organism is irradiated with the red light of He:Ne laser and up to 3 minutes in absence of a photosensitizer in any of performed experiments (although for some tests irradiation were carried up to 10 minutes). Similar results were obtained when bacteria suspension was mixed with any of the four tested sensitizers or any of their five employed concentrations without the presence of laser radiation.

Table 1 illustrates the effect of laser radiation on sensitized bacteria samples for laser exposure time of 3 min and for the sensitizer different concentrations, also shows the effect of the four tested sensitizers. Results shown in this table are also displayed in Figs. 1 and 2, represent the average of twenty experiments for each sensitizer. As seen from this table and Fig. 1, the inhibition zones were first noticed when the *H. pylori* was irradiated in the presence of TBO or MB at concentration of 10 μg/ml. Complete killing was observed when the concentration of TBO or MB was 100 μg/ml and sample were exposure to laser radiation for 3 min. However, Table 1 and Fig. 2 indicate that zones of minor inhibition of bacterial growth were achieved when *H. pylori* were irradiated for the same time in presence of CV or Th with maximum concentration used of 100 μg/ml.

Figures 3 and 4 show detailed results of the effect of irradiation on time, also the effect of sensitizer concentration on the viability of the bacteria.

The results obtained with sensitizers employed are in agreement with those of Milson *et al.* (1996), but our overall results seen to be more optimized in laser dose and sensitizer concentration. Using the same sensitizer, TBO or MB and similar concentration, we obtained

Table 1: Effect of He:Ne laser on the average of total number of *H. Pylori*. Exposure time is 3 min.

Photo-Sensit.	Concen. (µg/ml)	Average of total no. of viable cells ($x10^7$ cfu/ml) L+S+	L-S+	L+S-	L-S-
TBO	10	100	126	125	129
	30	72	123		
	50	38	120		
	75	1.1	117		
	100	0	115		
MB	10	102	126	125	129
	30	75	123		
	50	41	120		
	75	1.8	118		
	100	0	116		
CV	10	128	129	125	129
	30	128	128		
	50	126	128		
	75	125	127		
	100	123	125		
Th	10	129	129	125	129
	30	129	129		
	50	127	128		
	75	125	127		
	100	123	126		

- L+S+ Sample irradiation with laser light in presence of sensitizer.
- L+S- Sample irradiation with laser light, no sensitizer.
- L-S+ Sample with sensitizer, no laser irradiation.
- L-S- Control sample, no sensitizer and no laser irradiation.
- Results are the average of twenty experiments.

complete killing at radiation dose of 13 J/cm^2 using He:Ne laser but they obtained the same result for TBO at 50 µg/ml using 160 J/cm^2 dose from He:Ne laser and 21 J/cm^2 for MB at 100 µg/ml but using gallium aluminum arsenide (GaAlAr) red laser light of wavelength 660 nm. Also we have noticed a partial inhibition when using CV or Th sensitizers, whereas they reported that both CV and Th were ineffective as sensitizers.

To judge the goodness of the hypothesis presented in this work about the ability of photosensitizing technique by low power laser in inhibition and complete killing effects of *H. pylori* viability, the role of sensitizer used and its optimum concentration, also the radiation dose and its minimum value that produce complete killing, statistical analysis was performed for results obtained from 80 trails on four sensitizers with 20 experiments for each including three

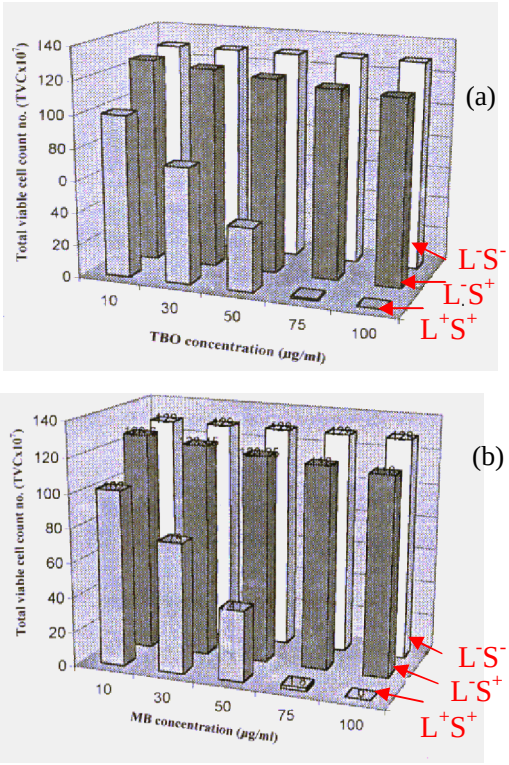


Fig. 1: Effect of He:Ne laser radiation on *H. pylori* for exposure time of 3 min. in presence of (a) TBO sensitizer (b) MB sensitizer.

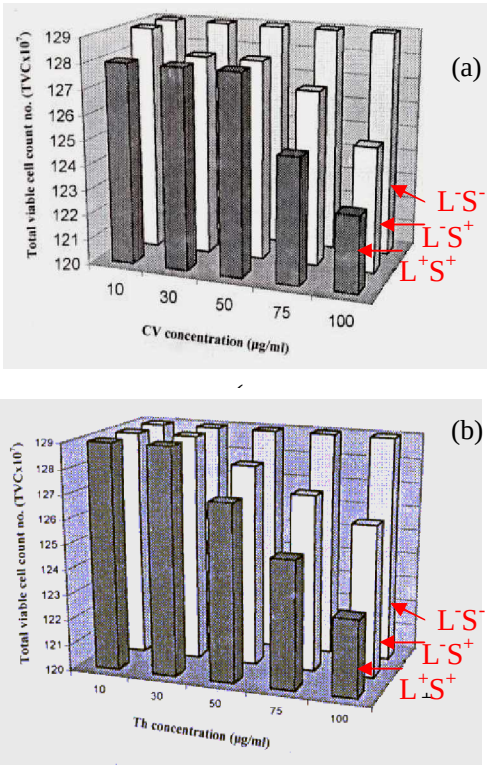
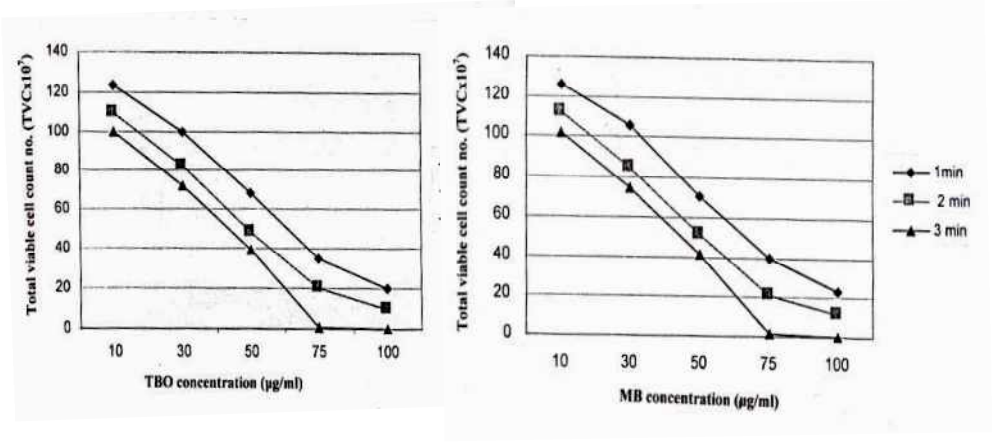
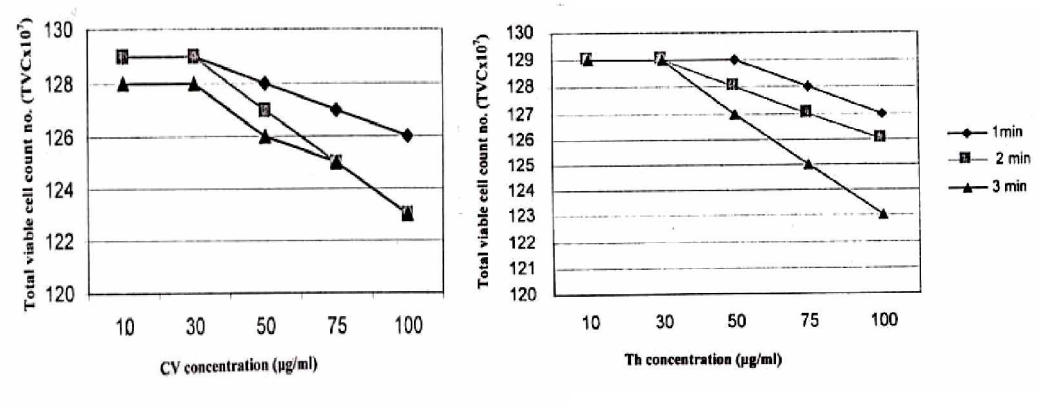


Fig. 2: Effect of He:Ne laser radiation on *H. pylori* for exposure time of 3 min. in presence of (a) CV sensitizer ; (b) Th sensitizer



(a) (b)

Fig. 3: Effect of He:Ne laser exposure dose on the viability of *H. pylori* in presence of (a) TBO sensitizer, (b) MB sensitizer.



(a) (b)

Fig. 4: Effect of He:Ne laser exposure on the viability of *H. pylori* (a) CV sensitizer, (b) Tb sensitizer.

irradiation doses for each sensitizer concentration. For this purpose, the students t-test was applied and the correlation coefficient in each case was determined.

Analysis of results confirms that the hypothesis of killing effect is true for significant level of 5% for TBO and MB at sensitizer concentration of 75µg/ml with 3 min irradiation. Also it is true for no killing effect of laser alone at any of the used irradiation periods or for each sensitizer alone at any of the employed concentrations. Analysis of results showed that a quasi-complete killing (>99%) or for a significant level of 1% at concentration of 100 µg/ml for both TBO and MB when the irradiation period was 3 min. Analysis showed also that for the above significant levels, no effect of photosensitizing action was noticed for both CV and Th sensitizers using the same irradiation period for all concentrations used.

To explain the effect of the sensitizer kind on inhibition of bacteria viability and in connection with the laser used, we return to our recorded spectra of the four tested sensitizers in the visible region (Kandela *et al*., 2002). The absorption peak for TBO and that for MB were located at 628nm and 664nm respectively which are near to the wavelength of the laser used, whereas the absorption peak of CV and Th sensitizers were located at 604.6 nm and 598.7 nm respectively. The maximum absorptivity at the laser wavelength was measured for TBO then MB, the minimum value was for Th. These results may explain the role of the sensitizer used and the laser source employed. Also it may describe why MB sensitizer was more effective for killing with GaAlAr laser used previously (Milson *et al*., 1996).

The laser interaction mechanism for killing process, as mentioned earlier, is of non-thermal type. It may be due to the cytotoxic radicals which involve in the production of singlet oxygen ($^1O^*_2$). This approach is well known as photodynamic action (PDA).

In conclusion, this study on the killing effect of sensitized *H. pylori* with TBO or MB exposed the red light of He:Ne laser, the first done in this country, offers a new technique to treat diseases affected by this colonized bacteria. Supporting this ability, it is expected that no side toxic effect would be observed because of using such low concentration of TBO or MB dye; further investigations about this point is needed when the technique is applied in vivo.

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تأثير اشعة ليزر الهيليوم - نيون واطئ القدرة في قتل جراثيم *Helicobacter pylori* المحسَسة ضوئياً

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الخلاصة

استخدمت اربع انواع من المتحسسات الضوئية في اختبار للقدرة التثبيطية لاشعة ليزر هليوم: نيون الواطئ القدرة على حيوية جراثيم *Helicobacter pylori* . جمعت 176 خزعة استئصلت من مرضى مصابين بامراض القرحة الهضمية والتهابات المعدة والعفج من خلال عملية التنظير. عزلت الجراثيم من 140 مريضا ثم شخصت وتم تحضير العالق الجرثومي . مزجت عينات من هذا العالق بتراكيز واطئة مختلفة من المتحسس الضوئي. عرضت نماذج من المزيج وعند كل تركيز بجرع مختلفة من اشعة الليزر. اخذت هذه النماذج وحضنت وتم دراسة مناطق التثبيط بالمقارنة مع نماذج للسيطرة ولكل متحسس . هدفت الدراسة الى تحديد المتحسس الضوئي ذو التركيز الامثل مع الجرعة اليزرية الادنى المسببة للقتل الكامل للجراثيم وبطريقة احصائية . بينت النتائج ان كل من المتحسسين التولدين الازرق والمثيلين الازرق بتركيز 100 مايكروغرام /مليلتر يكون قادرا على احداث قتل كلي عند التشعيع باشعة الليزر بجرعة 13 جول/ سم2 بينما لا يكون للمتحسسين الثايونين والبنفسج البلوري بالتركيز ذاته وباستخدام الجرعة الليزرية ذاتها تأثير يذكر على تثبيط فعالية الجراثيم . اثبتت الدراسة ايضا بان لا يكون لتشعيع الجراثيم بالليزر وبالجرع المستخدمة في البحث دون حضور للمتحسس الضوئي ، وايضا في حالة مزج العينات بأي من المتحسسات الضوئية وبالتراكيز المعتمدة في البحث دون استخدام اشعة الليزر اي تأثير على حيوية الجراثيم .