In vivo N₂ Laser Effect on Lymphocyte Transformation Capacity and Phagocytosis Activity in Mice

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Abstract: The objective of this in vivo study is to investigate the effects of 337.1 nm pulsed N₂ laser on cellular immune response represented by lymphocyte transformation capacity and phagocytosis activity in laboratory animals. The samples include 60 adult male BALB/c mice, were divided into control group and experimental groups. The experimental groups were divided into two main groups according to the time period after N₂ laser irradiation. Each group was divided into 9 subgroups which exposed to N₂ laser radiation at different values of pulse repetition rates and exposure times. The results of immunological tests demonstrated that the exposure to 180 J/cm^2 of N₂ laser radiation induce adverse effect to cellular immune response. The results of lymphocyte transformation assay showed that the capacity of lymphocyte to be transformed as response to mitogen PHA decreased only in subgroups that treated with 180 J/cm^2. While in the other subgroups the percent of transformed lymphocyte cells was unaffected. The findings of phagocytosis assay showed that the activity of phagocytic cells increased only in subgroups that treated with 80 J/cm^2, and decreased in subgroups that treated with 120 and 180 J/cm^2.

Introduction

There are many literatures that describe the effects of both coherent and non-coherent light radiations on the immune response. Some of these literatures showed that exposure to UV light will induce local and systemic immune suppression. Immune response is the ability of body to resist damage from foreign substances. A broad-based cellular immune response is the most important factor that contributes to the elimination of antigen from infected area (Roitt *et al*. and 1998; Dahl, 1996).

Assay of cellular immune response competence often requires (Hyde, 2000)
(1) Evaluation of phagocytic cells activities by using different assays such as phagocytosis assay.
(3) Evaluation of the role of T-lymphocytes by different assays such as;
a- Enumeration of T-cells subsets.

b- Evaluation of T-cells functions by lymphocyte transformation assay.

Lymphocyte transformation is a term used to describe the morphological changes that resulted when small, resting lymphocytes were transformed into lymphoblast as a result of stimulation by either non-specific mitogens or antigens. Lymphocytes stimulation is an in vitro technique commonly used to assess cellular immune response (Nowel, 1960 and Stites, 1994).

Phagocytosis is a highly efficient nonspecific host defense mechanism against the invasion of microorganisms. This process includes four steps: chemotaxis, opsonization, engulfment and killing. The most important phagocytic cells are polymorphnuclear neutrophils, monocytes and macrophages. (Hyde, 2000 and Stossel, 1974).

Both coherent and non-coherent light radiations have been shown to act on immune system cells in a number of ways. Infrared low

level laser therapy has been shown to increase both the phagocytic and chemotactic activity of human leukocytes in vitro (Tadukuma, 1993).

Luza *et al*., (2000) reported that, small dosage of He-Ne laser radiation increases the adherence and phagocytic activity of the leukocytes, but after higher irradiation dosage the leukocyte adherence and phagocytic activity is decreased.

Yu, W. *et al*. (1997) showed that, laser irradiation, at a wavelength 630 nm, significantly stimulate lymphocyte proliferation in the presence of mitogenic stimuli and enhanced lymphocyte ATP synthesis. Also previous investigations showed that the exposure to near UV radiation induce immune suppression.

Kripke and Daynes (1983) reported that chronic exposure of mice to high level of UVB turned out of skin tumor. This growing tumor, when transplanted into the same species inbred mice, which has non-UV irradiated, the tumor was rejected. So UV can alter the host immune system by producing generalized defects in antigen-presenting cells, and inducing the formation of suppressor T cells.

Some of literatures showed that a pulsed nitrogen laser with emission wavelength 337.1 nm was used for photosensitizer activation, in both photodynamic therapy and photodynamic detection of tumor tissues. Pascu *et al*., (1999) reported about the using of N_2 laser as a light source in the photodynamic therapy of brain tumors. Anidijar *et al*., (1996) assessed the ability of N_2 laser induced autofluorescence spectroscopy to distinguish neoplastic urothelial bladder lesions from normal or nonspecific inflammatory mucosa. Therefore, the present work was designed to evaluate cellular immune response represented by lymphocyte transformation capacity and phagocytosis activity in laboratory animals irradiated with N_2 laser radiation, at certain parameters.

Materials and Methods

Materials

The samples consisted of 60 healthy adult male BALB/c mice, 54 animals for experiments and (6) animals for control.

All mice were 6-8 weeks old and weighed between 30 and 35 g at the time of experiments. They were housed in subdued light, three or four per plastic cage, and were given standard laboratory diet and water.

Control group consists of six healthy animals without any treatment. The experimental groups were divided into two main groups, I and II, according to the time scale after the treatment Group I represents 12 hrs after the irradiation, while group II represents 24 hrs after the irradiation. Each one of the main groups I and II were subdivided into nine subgroups (3 animals in each one) according to which laser parameters were applied. Table (1). Pulsed N_2 laser (Molectron UV 24-model) was used in this study. The emission wavelength is 337.1 nm. It can be operated with a pulse repetition rate that ranging from 1 to 50 pulses per second. The pulse energy is 1 mJ with pulse duration of 10 ns.

Irradiation Procedure

Irradiation procedure was performed as the following:
1) The mice were anesthetized with pentobarbitone sodium, which was supplied as a solution for injection (Sanofi, France). Each 0.1 ml of that solution was diluted with 1 ml of normal saline. Intraperitoneal injection of 0.4 ml of diluted solution was performed; this produced a full state of anesthesia after 5 min and lasted for about one hour. No abnormal activity was seen in anesthetized animal after the anesthesia was vanished.
2) The dorsal hair of anaesthetized mice was removed with clippers.
3) Laser irradiations were performed with 1mJ pulsed N_2 laser. Samples were exposed to laser irradiation for exposure times of 10, 20 and 30 min with 10, 20, 30 Hz repetition rates for each exposure time.

The laser beam was focused on 0.3 cm^2 of the skin surface of the animals via a lens with a focal length of 5 cm. The laser pulse energy was detected using joule meter (Rj-7610, Laser precision crop, England) before each experiment. The fluence was constant in all irradiation experiments. At the end of each experimental work about 0.8-1 ml of blood was taken directly from the heart of the animal and kept in heparinzed tubes in order to be used in immunological tests.

Blood samples were used to evaluate the cellular immune response by two assays lymphocyte transformation and phagocytosis.

Lymphocyte transformation assay had been done according to Shubber, *et al.* (1984)**.** It was carried out as follows:

• A 250 μl of heparinized blood cultured in 2.5ml of complete RPMI-1640 medium in sterile silicon coated tubes (Terumo, Japan), using duplicate to each test.

• A 250 μl of mitogen (PHA) was added to one of the tubes (test), and other without mitogen (control).

• The tubes had been incubated at 37ºC for 72 hrs humidified condition containing 5% CO_2, the tubes must be shacked every day.

• At the end of incubation time the cells sedimented by centrifugation at 2000 rpm for 10 minutes.

• 5 ml of hypotonic solution (KCl) was added to the sediment cells, incubated at 37ºC for 50 minutes, then centrifugation at 2000 rpm for 10 minutes.

• 5 ml of fixation solution (Three volumes of absolute methanol were mixed with one volume of acetic acid) was added to the sediment cells and kept in refrigerator at 4ºC for 15 min.

• Washing up by fixation solution 3-4 times with repeating centrifugation until colorless suspension of sediment cells formed.

• Then using Pasteur pipette (Biomerieux, France), one drop of sediment cells had been put on two clean slides, left to dry at room temperature, stained by giemsa stain for 10 minutes, washed by distilled water and examined microscopically under oil immersion lens by counting 200 cells.

• The percentage of transformed cells had been calculated from the ratio between the numbers of transformed cells to the total number of cells counted.

Phagocytosis assay had been done according to Furth *et al*., (1985) as follows:

• Equal volumes (250 μl) of heparinized blood and bacterial suspension of *S. aureus* (10^6/ml) (1:1) were mixed into sterile test tube.

• The mixture was incubated in water bath at 37ºC for 30 min with continuously shacking.

• Smear had been prepared by taking a drop of the mixture on the slide; duplicate slides were made for each tube.

• Slides had been air dried, fixed by absolute methanol, stained by giemsa stain for 10 minutes and then washed by D.W.

• The slides had been examined by oil immersion lens to calculate the number of neutrophils engulfed microorganism.

• The percentage of phagocytic cells had been calculated. It's the ratio between the number of phagocytic cells and the number of 100 cells agocytic and non phagocytic.

Statistical Analysis

The statistical analysis had been performed by analysis of variance (ANOVA test). The comparison between the groups was performed using one-way analysis of the variance. In order to find the source of this difference, the least significant of difference (LSD) was used.

**Table (1):** laser parameters for Group I and II

| Subgroups No.	Pulse Repetition rate (Hz)	Exposure time (min.)	Total energy deposit/ area (J/cm^2)
1L$_1$,2L$_1$	10	10	20
1L$_2$,2L$_2$	10	20	40
1L$_3$,2L$_3$	10	30	60
1L$_4$,2L$_4$	20	10	40
1L$_5$,2L$_5$	20	20	80
1L$_6$,2L$_6$	20	30	120
1L$_7$,2L$_7$	30	10	60
1L$_8$,2L$_8$	30	20	120
1L$_9$,2L$_9$	30	30	180

Results

Lymphocyte Transformation Assay

The results were expressed as lymphoblast percentage from the total lymphocyte had been calculated. See Figure (1). For the first experimental group, the results of the lymphocyte transformation assay are illustrated in Figure (2) Statistical analysis of the results showed that the transformed lymphocyte cells (Mean) were decreased significantly ($P < 0.05$) only in the subgroup 1L$_9$ (180 J/cm^2) that was treated with 30 Hz repetition rate and 30 min. exposure time of N$_2$ laser radiation, while there

was no significant difference (P > 0.05) in the other subgroups, when compared with the control group.
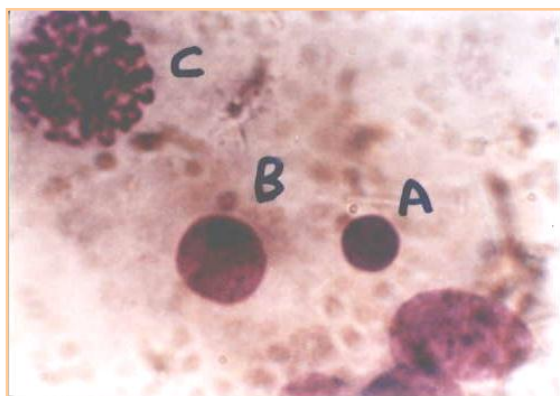

Fig. (1): Lymphocyte transformation assay. A- Large lymphocyte B- lymphoblast. C- Lymphocyte in metaphase.
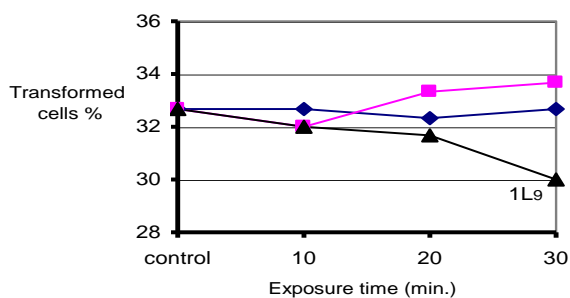
**Fig. (2):** Transformed lymphocyte cells (Mean) for Group I. Fluence is 3.3 mJ/cm^2.

For the second experimental group, the results of the lymphocyte transformation assay are illustrated in Figure (3). Also the percentage of lymphocyte-transformed cells showed a highly significant decrease (P< 0.01) in the subgroup 2L$_9$, while there was no significant difference in the other subgroups.
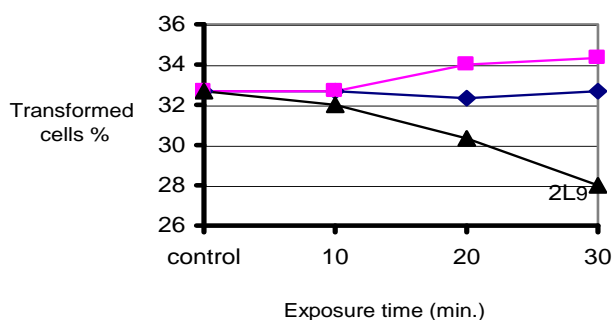
**Fig. (3):** Transformed lymphocyte cells (Mean) Group II. Fluence is 3.3 mJ/cm^2.

Phagocytosis Assay

The findings of the phagocytic cells activities were evaluated by calculating the number of neutrophils engulfed microorganism from the total numbers of neutrophils. See Figure (4).
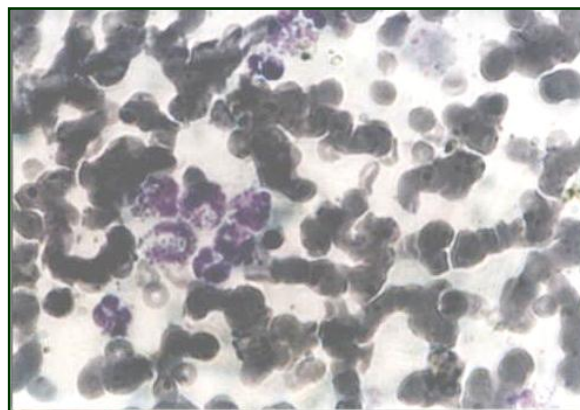
Fig. (4): Phagocytosis of S. aureus by phagocytic cell.

Figure (5) shows the phagocytic activity (Mean) of group (I). A significant (P < 0.05) increase can be seen in the subgroup 1L$_5$ (80 J/cm^2), when compared to the control group, while there was a significant decrease (P < 0.05) in the subgroup 1L$_9$ (180 J/cm^2) that treated with 30 Hz repetition rate and 30 minutes exposure time.
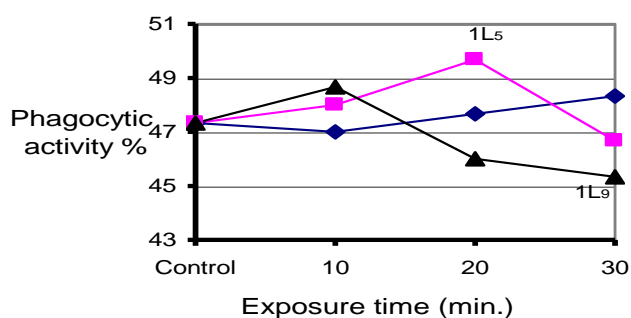
Fig. (5): Phagocytic index (Mean) for Group I. Fluence is 3.3 mJ/cm^2.

In the second experimental group (II), the results showed a significant increase (P < 0.05) in the phagocytic activity for subgroup 2L$_5$, as shown in Figure (6). Also there was a significant decrease (P< 0.05) and highly significant decrease (P< 0.01) in the subgroups 2L$_8$ and 2L$_9$.
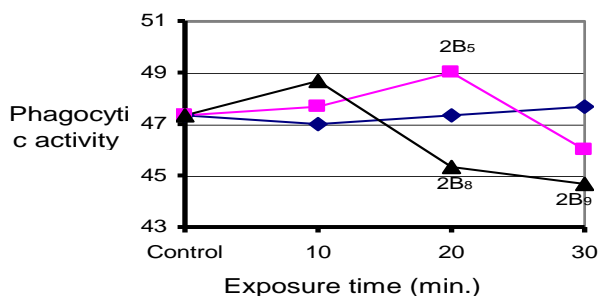
Fig. (6): Phagocytic index (Mean) for Group II. Fluenceis 3.3 mJ/cm^2.

Discussion

The results of lymphocyte transformation assay showed that the capacity of lymphocyte to be transformed as response to mitogen PHA is decreased only in subgroups $1L_9$ and $2L_9$ that treated with 180 J/cm^2 energy density, while in the other subgroups the percent of transformed lymphocyte cells was unaffected. These findings are in a good agreement with the findings reported by (Ullrich, 1985) and (Norbury *et al*., 1977).

The low response of lymphocyte cell to PHA may be attributed to the ability of UV light to defect the antigen presenting cells, to induce the formation of suppressor T cells or to induce the formation of immunosuppressive factors. Langerhans cells reside in the epidermis layer of the skin have the same function as macrophages; can present antigens to lymphocyte cells. UV radiation may cause either direct killing of Langerhans cells or release of soluble mediators, which alter the antigen presenting capability of Langerhans cells. So the result is failure to activate T helper cells and development of T suppressor cells (Vermeeret *et al.*, 1990; Ullrich *et al*., 1986 and Defabo *et al*., 1979).

The immunosuppressive effects of UV radiation is due to the production of some cytokines such as prostaglandin – E2, IL – 4 and IL – 10 from keratinocytes in the epidermal layer. (Rivas *et al*., 1992) UV radiation causes photoisomerization in the urocanic acids (UCA), which produced enzymatically in the stratum corneum of the epidermis. Its transferes UCA from trans to cis isoform. Cis isoform is an immunosuppressive factor. (Noonan *et al*., 1988).

The findings of phagocytosis assay demonstrated that the activity of phagocytic cells increased significantly in subgroups $1L_5$ and $2L_5$ that treated with 80 J/cm^2 of total energy, and decreased in the subgroups $1L_9$, $2L_8$ and $2L_9$ that treated with 120 and 180 J/cm^2.

The stimulative and inhibitive effects of N_2 laser light on phagocytic activity may be attributed to morphological and functional changes. Photochemical reactions of UV radiation in the skin may be alters macromolecules morphologically in situ to become partial or complete antigens.

UV light induce release of chemotactic factors, such as, complement factors, IL – 1 and TNF – α, which increase the phagocytosis process by increasing the percentages and functions of circulating polymorphnuclear neutrophils (PMN's) in the peripheral blood (Rasanen *et al*., 1989).

N_2 laser light may cause a stimulative effect as a result of the excitation of NADH molecule, which leads to increase the Oxidation – Reduction reactions. High-energy electrons flow may occur through the respiratory chain leading to the high-energy production via ATP molecules, which as a result, increase the cell activity (Jeevan *et al*., 1990).

Cellular damage in the near UV region is strongly dependent upon the presence of oxygen, indicating a sensitized indirect photodynamic type of action involving reactive oxygen species.

The photons energy in the UV light is not transferred directly to ground state molecular oxygen but it's absorbed by an intermediate primary chromophore NADH molecule, which activates ground state molecular oxygen (Michael *et al*., 1985; Peak *et al*., 1983)

Through this reaction, one or more reactive oxygen species will be produced, such as, superoxide anion O_2^- (as well as the product of its dismutation hydrogen peroxide H_2O_2), singlet oxygen O_2^1 or hydroxyl radical OH·. These species are cytotoxic because they are strong oxidizing agents, they can oxidize sulphahydral groups and inactivate the active sites of enzymes; denature structural proteins and causes damage to DNA (Michael *et al*., 1985).

Because of 10% of body blood is in the superficial vessels of the skin, the prolonged UV exposure may make it possible to radiate and damage a large portion of blood cells. Therefore, the inhibitory effect of N_2 laser light at high repetition rates and long exposure times may

belong to the decrease of the numbers of PMN's in the peripheral blood (Vermeer *et al*., 1991).

Conclusion

The conclusion drown from this in vivo study demonstrated that N_2 laser radiation causes suppressive effects in lymphocyte transformation capacity and phagocytosis activity at high levels of energy density (120 – 180 J/cm^2).

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دراسة تأثير ليزر النتروجين على قدرة التحويل اللمفاوي ونشاط البلعمة في الفئران

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الخلاصة   الغرض من هذه الدراسة داخل الكائن الحي هو البحث في تأثيرات ليزر النتروجين (337,1) نانومتر على الاستجابة المناعية الخلوية المتمثلة بقدرة التحول اللمفاوي ونشاط البلعمة في الحيوانات المختبرية . عينات الدراسة تضمنت 60 فأرا بالغا من طراز (BALB/C) والتي قسمت الى مجموعة السيطرة ومجاميع مختبرية. المجاميع التجريبية قسمت الى مجموعتين رئيسيتين أعتمادا على الفترة الزمنية بعد التشعيع بليزر النتروجين . كلا المجموعتين قسمتا الى تسعة مجاميع ثانوية والتي عوملت باشعة ليزر النتروجين عند قيم مختلفة من معدلات التكرار النبضية وأوقات التعرض للتشعيع. أثبتت نتائج الفحوصات المناعية أن التعرض لكثافة طاقة (180 جول /سم^2) من أشعة ليزر النتروجين يؤدي الى تأثير عكسي على الأستجابة المناعية الخلوية . نتائج اختبار التحول اللمفاوي أظهرت ان قدرة الخلايا اللمفاوية للتحول نتيجة الأستجابة الى المايتوجين PHA انخفضت فقط في المجاميع الثانوية التي عوملت بكثافة طاقة (180 جول /سم^2) . بينما نسبة تحول الخلايا اللمفاوية في المجاميع الثانوية الأخرى لم تتأثر . نتائج اختبار البلعمة أظهرت أن نشاط الخلايا البلعمية أرتفع فقط في المجاميع الثانوية التي عوملت بكثافة طاقة 80 جول /سم^2 ، وأنخفض في المجاميع الثانوية التي عوملت بكثافة طاقة 120 و 180   جول / سم^2 .