Al-Azhar Bull. Sci. Vol. 17, No. 1 (June.): pp. 57-67, 2006.

EFFECT OF HE:NE LASER ON NEUTROPHILS PHAGOCYTIC ACTIVITY

HUSSEIN M. EL KABBANI AND SOHEIR S. KORRAA

*** Health Radiation Research Department, National Center for Radiation Research and Technology, Atomic Energy Authority.*

Abstract

The aim of this study is to investigate. The effects exerted by using helium: neon **He:Ne** laser (632.8 nm) low level laser irradiation **(LLLI)** on neutrophil activity in response to opsonized zymosan. Human buffy coat leukocytes primed with opsonized zymosan were exposed to 10 mw **He:Ne** laser at energy densities of 1-5 J/cm^2. Phagocytic activity was assessed by measuring percent of phagocytosis, (hydrogen peroxide) **H2O2** and nitric oxide **(NO)** generation together with neutrophil-associated nitric oxide synthetase activity. Irradiation with low intensity red light stimulated neutrophil, an effect which was intensified, when neutrophils were dually stimulated by **LLLI** and opsonized zymosan within the dose range of 3-5 with maximum activity at 5 J/cm^2.

Introduction

He:Ne laser irradiation at a wavelength 632.8 nm is an interesting region of the electromagnetic spectrum with respect to its photobiological effects. It is a beneficial clinical modality in enhancing the process of wound healing, pain relief, and inflammatory suppression in cases of rheumatic arthritis and achilles tendonitis ***Kitchen et al., (1991) and Huang et al., (2000***). A thermic and non-destructive photobiological effects of He:Ne laser vary between biostimulation and bioinhibition of physiological, biochemical and proliferative phenomena in various cells, tissues, organs and organisms ***Schindl et al., (2000) and Basford, (1995).*** Most of the related current studies suggest that biostimulation is enhanced at energy density between 10^3-10^4 J/m^2, while inhibition is exhibited at energy density range 10^5 - 10^6 J/m^2 ***Karu, (1989).***

Studies concerning the elucidation of the mechanism encountered suggested that oxygen derived free radical as possible mediators for light activation in biological systems ***Karu et al., (1989) and Shchepetkin et al., (1993)***. It was suggested that the quantity of generated free radical depends on the power and efficacy of the laser being used ***Nakagawa (1990).*** Neutrophils are one component of blood known to mediate its phagocytic biological action via generating substantial amounts of nitric

oxide **(NO)** and reactive oxygen species **(ROS)** upon exposure to external stimuli ***Miller et al., (1995)*** including **He:Ne** laser ***Karu et al., (1989)***.

Polymorphoneutrophils **(PMN**) antimicrobial activity represents an important element in innate host defense against microorganisms. The contribution of **ROS** to **PMN** antimicrobial action is illustrated by the clinical complications in chronic granulomatous disease, a genetic disorder that results in lack of **NADPH** oxidase activity ***Klebanoff, (1982)***. Although **PMN** from chronic granulomatous disease patients exhibit normal migration, phagocytosis, and degranulation, they fail to produce oxidants and thus exhibit ineffective inhibiting to a variety of microorganisms. **PMN** also possess non-oxidative mechanisms that include an array of antimicrobial enzymes contained inhibiting within a variety of cytoplasmic granules that are released into the phagosome upon ingestion. The combination of both oxidative and non-oxidative mechanisms allows the **PMN** to effectively inhibit broad range of microorganisms ***Miller et al., (1995).***

The aim of the present study is to investigate the effect of **He:Ne** laser irradiation on the various functional parameters of neutrophils and look for possible correlations in the effects to understand the mechanisms involved.

Subjects and Methods

PMN Isolation

Heparinized blood was drawn from healthy volunteers. Buffy coats were separated and concentrated in plasma at cell density 2 x 10^5 cells / ml. It was previously shown that neutrophils have a better response to He:Ne laser irradiation when irradiated in the presence of buffy coat leucocytes compared to isolated neutrophils ***El Batanouny and Korraa, (2002).***

Zymosan: Zymosan is a large carbohydrate fraction of the yeast-cell caused by fermentation or infection. Zymosan from ***Saccharomyces cerevisea*** was prepared ***Sorenson et al (1998)*** by being diluted in hanks balanced salt solution containing 1% autologous normal human serum prior to the experiment and incubated for 60 min. The description of ***Saccharomyces sp.***: white to cream, vegetative reproduction by budding, filaments, none or simple pseudohyphae; persistent asci containing up to 4 smooth, oval or round ascospores, on McClary acetate agar at 25°C found in sugar cane , baker, for brewer top fermenters and distiller strain . Mol % of G + C of DNA hybridization ***Sorenson et al (1998***)

Phagocytosis assay: According to standard methods ***Simons et al., (2005)*** approximately 2 x 10^6 cells were added to each well of the 24-well tissue culture plate, and allowed to adhere for 30 min at 37°C. Zymosan was added to PMN for 15 minutes to allow the yeast to bind to PMN but not be ingested. Cells received He:Ne laser irradiation with different power densities and supernatants were removed and replaced with HBSS + S warmed to 37°C to initiate phagocytosis. The latter experiment was repeated with and without laser irradiation where 100 neutrophils were counted for phagocytosis in every experiment. Other buffy coat leucocytes do not interfere with phagocytosis counting based on the fact that only neutrophils adhere to surfaces. A test for immunity to or infection by a pathogenic organism based on the assumption that the serum contains specific opsonins (a standard by which the power of resistance to disease is estimated) capable of facilitating phagocytosis of the organism.

Irradiation Experiments: **He: Ne** Hundred µl aliquots of cells with and without fungal opsonization **were** distributed in 24 well tissue culture plates. To minimize cross-irradiation between cells, at least two empty wells separated the experimental (irradiated) well. Plates were shaken immediately prior to irradiation to maintain homogenecity of cells in suspension. For every treatment duplicate experiments were carried out for each of 10 individuals. Irradiation was carried out with 10 mw He:Ne laser at energy densities of 1, 2, 3, and 5 J/cm^2. This required 2, 4, 6 and10 minutes for each well.

Generation of nitric oxide and Hydrogen Peroxide

Measurement of NO generation from opsonized stimulated neutrophils with and without laser irradiation was determined by the Griess reaction ***Green et al., (1998).*** Sixty minutes post stimulation with different doses of He:Ne laser. Briefly, the Griess reagent was prepared by mixing equal volumes of 5% orthophosphoric acid containing 1% sulfanilamide and 0.1% napthylethylenediamine and was kept at 4°C until used. Triplicates of 50 µl of culture supernatant or standards of serially diluted sodium nitrite were added to 96-well Immulon 4 microtiter plates, and 50 µl of the Griess reagent supplemented with nitrate reductase (final concentration 0.1 U/ml) was then added. Any colour generated was measured spectrophotometrically at 570 nm using a microtiter plate reader. Protein was determined by using protein sigma kit ***Lowry et al., (1951).***

Generation of Hyrogen Peroxide

The evolved hydrogen peroxide was measured by the assay method based on the horse raddish peroxidase mediated oxidation of phenol red by H2O2 which leads to the formation of a compound, which at alkaline pH exhibits increased absorbance at 660 nm ***Pick et al; (1980)*** Readings were recorded at 30, 60 and 90 minutes. Between readings the plates were incubated at 37^{o} C. 1N NaOH was added to each well 2 minutes before reading the absorbance. The absorbance value was converted to µg moles using standard curves of serial dilutions of H2O2 .

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis: Inducible NOS mRNA was detected using RT-PCR as previously described (18) Total RNA was extracted from cells using AB Analytica RNA extraction kit (AB Analytica, Padova, Italy). The RNA samples were reverse transcribed using superscript reverse transcriptase, oligo (dT) primers (Gamma Trade) and deoxynucleoside triphosphate as specified by the manufacturer. The synthesized cDNAs were amplified by PCR (30 and 25 cycles for iNOS and B-actin, respectively) with *Taq* DNA polymerase in the presence of deoxynucleostide triphosphate, and appropriate pair of primers. Aliquots (5 µl each) from the RT reaction were then used for PCR amplification with primer pairs for iNOS. INOS primer sequences were chosen for their ability to distinguish iNOS from the other NOS isoforms and were designed to flank a known intron-exon boundary of the genomic iNOS sequence. Therefore, only products corresponding to the iNOS mRNA was amplified in the RT-PCR reactions. This eliminates the concern that products might be generated from genomic DNA contaminants in the RNA samples. The iNOS primer pair used was as follows :
Forward : 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'
Reverse : 5'GGCTGTCAGAGCCTCGTGGCT-TTGG-3' .

INOS was amplified in the same reaction. The PCR was adjusted with the following cycle parameters: 95°C for 3 minutes; 94°C for 45 seconds; 59°C for 45 seconds, 72°C for 2 minutes for 35 cycles, then 72°C, for 7 minutes, and 4°C for 24 hours. Reaction products were then separated on a 1.2% agarose gel, ethidium stained, and photographed. The size of the PCR fragments representing iNOS was 557 bp.

Statistical Analysis

Data are the mean of at least three replicates and are presented as standard error of the mean values. Statistical analysis was performed using the Statistica software package.

Results

He:Ne laser Irradiation Augments Respiratory Burst in Adherent PMN

Irradiation with He:Ne laser increased the production of NO and H2O2 in opsonized irradiated PMN compared to non-irradiated ones**.** Also phagocytosis of opsonized zymosan was augmented by He:Ne laser irradiation.

Phagocytosis of opsonized zymosan induced the expression of NOS mRNA. The later effect was augmented on exposure to He:Ne laser irradiation.

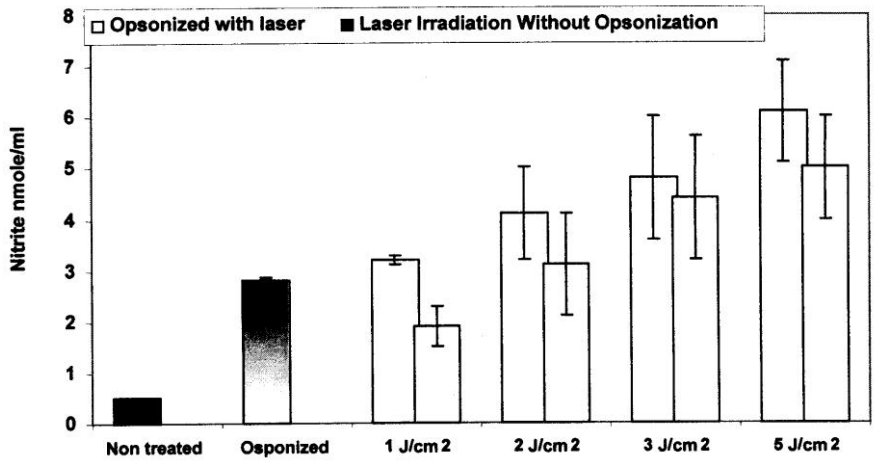

Fig 1. Effect of He: Ne laser irradiation on NO production

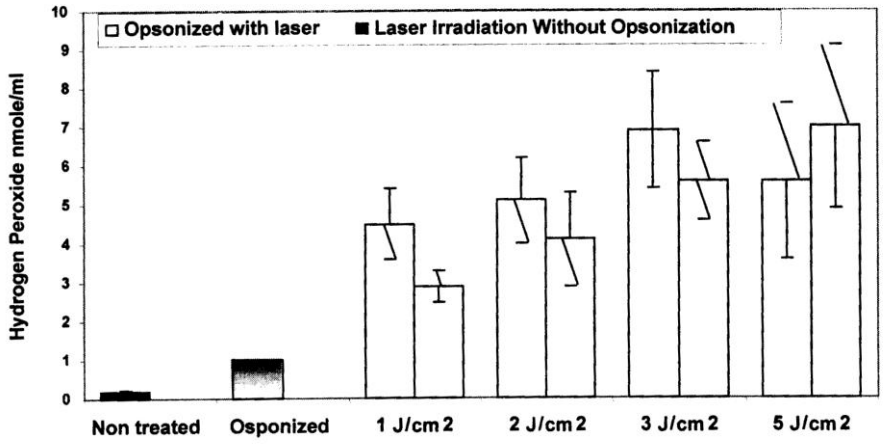

Fig 2. Effect of He:Ne laser irradiation on H2O2 production

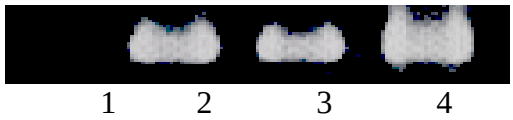

Fig 3. RT-PCR analysis of iNOS mRNA expression: lane 1 untreated, lane 2 opsonized, lane 3 He:Ne laser irradiated, lane 4 dual opsonized and laser irradiated.

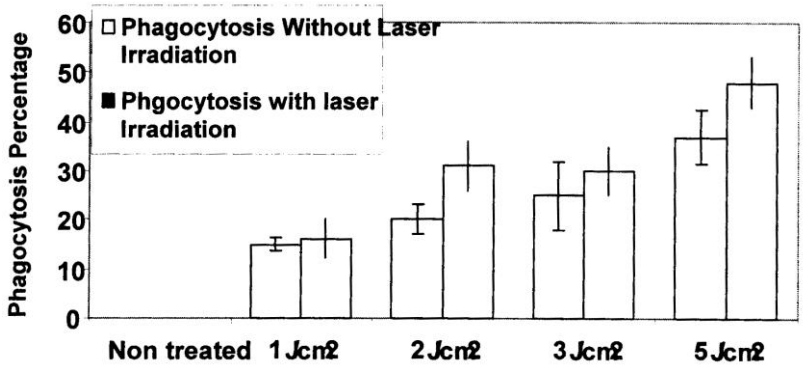

Fig 4. Effect of He: Ne laser irradiation on phagocytic percentage

Discussion

He:Ne laser radiation has been previously shown to produce chemiluminescence's on Candida ablicans-stimulated mouse spleen cells ***Karu et al., (1989)*** **and** calcium ionophore (A23187) and protein kinase activator (phorbol-12-myristate-13-acetate, PMA) stimulated human neutrophils ***Shchepetkin et al., (1993)***. Results of the present study, provides further proof to this phenomena, but the stimulant in this study was opsonized zymosan from ***Saccharomyces cerevisea***. Thus revealing the role of He:Ne laser irradiation on stimulating phagocytosis of individual serum opsonization.

A previous study carried out by ***Dima et al.,*** **(1996)** has shown that LLL-irradiation had no effects on serum opsonic activity at low doses used for the rapeutic purposes. An effect was observed only when they applied a power output of 60 mW. The power output in the present study is 10 mW and the reason for the discrepancy is that in their study they irradiated separated neutrophils, while in the

present study neutrophils were irradiated in the presence of other blood leucocytes. Several studies indicated that NO cannot be detected in purified neutrophils ***Yan et al., (1994) and Holm et al., (1999),***while other studies demonstrated that detectable amounts of NO in PMA stimulated neutrophils ***Wang et al., (2000) and Amin et al., (1995)***. Results of the present study provide support to the latter group as we have identified detectable amounts of NO in purified neutrophils stimulated with both PMN and laser. Increased generation of NO provides further proof to previous studies, which suggested that NO plays an important role in functional respiratory burst responses of human PMNs ***Larfars et al., (1998) and Powledge (1997).***

In multicellular organisms, general biological principles postulate that the organization of cells (particularly immune cells) depends on regulated cell surface interactions both with molecules on the surface of other cells and with immune mediators in the extracellular fluid. Such interactions have been shown recently to generate and / or require reactive free radicals or derived species to successfully transmit their signals to the nucleus ***(Lowry, 1993)***. An example is that neutrophils undergo massive prolonged generation of ROI at the command of macrophages and lymphocytes, which react with microbial products and antigens ***(Nathan, 1997)***. Lymphocytes have been lately shown to respond to ROI released by neutrophils ***(Lander, (1993),*** suggesting that ROI act as competence signals involved in the regulation of cell cycle entry and in the control of early gene expression in T-lymphocytes ***Los et al., (1995) and Lander et al., (1993).*** NOI have also been shown to activate human lymphocytes leading to an increased secretion of lymphokines and cytokines that enhance the inflammatory process ***Lander et al., (1993)***. For the latter reason we irradiated buffy coat leucocyte in order to construct an *in vitro* model system that reflects the *in vivo* situation as close as possible.

In a previous study we revealed that 10 mw He: Ne laser (632.8 nm) at energy densities 1, 2, 3 and 5 J/cm2 stimulated the production of iNOS in human polymorphonuclear leucocytes in the presence and absence of zymosan. This effect was exhibited by purified neutophils and was more pronounced when neutrophils were irradiated in the presence of native plasma and lymphocytes ***(EL Batanouny and Korraa (2002)***. In the present study we also demonstrated the expression of nitric oxide mRNA as a consequence of exposure to the same fluencies but in the presence of an immune resonse stimulator, which was zymosan. There have been also other results regarding the expression of iNOS in human neutrophils. Several studies indicated that iNOS mRNA is not expressed in neutrophils ***Yan et al., (1994)***. This was further proofed by immunocytochemistry and RT-PCR transcripts,

which demonstrated iNOS human neutrophils ***Wallerath et al., (1997)***, it is suggested that exposure to electromagnetic fields alone induce iNOS mRNA expression and that secondary signal and are required for such expression ***Wallerath et al., (1997) and Yoshikawa et al., (2000)*** .Two similar studies conducted on ionizing radiation ***McKinney et al., (2000) and EL Batanouny and Korraa (2002)*** demonstrated that iNOS was not expressed in purified neutrophils but rather in buffy coat leucocytes. Accordingly, iNOS was measured only in buffy coat leucocytes and demonstrated that He: Ne laser induces iNOS mRNA in human neutrophils.

In conclusion He:Ne laser irradiation stimulates human neutrophils in buffy coat to produce NO by stimulating the activity and expression of iNOS enzyme. NO production was observed in zymosan stimulated and was augmented in buffy coat leucocytes. Also, this study demonstrates that laser induces NO and ROS when buffy coat neutrophils were dually stimulated by **LLLI** and opsonized zymosan within the dose range of 3-5 with maximum activity at 5 J/cm^2

References

1. AMIN AR, ATTUR M, VYAS P, LESZCZYNSKA-PIZIAK J, LEVARTOVSKY D, REDISKE J, CLANCY RM,. VORA KA, ABRAMSON SB. (1995). Expression of nitric oxide synthetase in human peripheral blood mononuclear cells and neutrophils. J Inflamm, 96; 47(4):190-205.
2. BASFORD J., (1995). Low intensity laser therapy: still not an established clinical tool, Lasers Surg. Med. 16 (4) 331-42 T.
3. DIMA VF, SUZUKI K, LIU Q, KOIE T, YAMADA M, SUZUKI KJ, NAKAJI S, SUGAWARA K. (1996). Laser and serum opsonic activity. Roum Arch Microbiol Immunol. ; 5 (4):277-83.
4. EL BATANOUNY M AND KORRAA S. (2002). Effect of low intensity laser on the activity and expression of nitric oxide synthesis in human polymorphonuclear leucocytes *in vitro.* Arab. J. Lab. Med: Vol. 28 (3): 289-297.
5. GREEN L, WAGNER J, GLOGGLOSKI P, SKIPPER J, WISHNOK H, AND TANNENBAUM R (1998). Analysis of nitrate, nitrite and [15Nl nitrate in biological fluids. Anal. Biochem, (9) 126: 131-138.
6. HOLM P, KANKAAURANTA H, OJA SS, KNOWLES RG, MOILANEN E (1999). No detectable NO synthesis from Larginine or N(G)hydroxy-L-arginine in fMLP-stimulated human blood neutrophils despite production of nitrite, nitrate, and citrulline from N(G)-hydroxy-L-arginine. J Leukoc BioI 66 (1): 127-34.

7. HUANG H., A. QURESHI, J. BIUNDO (2000). Sports and other soft tissue injuries, tendinitis, bursitis, and occupation-related syndromes. Curr Opin Rheumatol. 12 (2) 150-4.

8. KARU T. (1989). Photobiology of low-power laser effects, Health Physics 56: 691-704.

9. KARU T, RYABYKH, T. FEDOSEYEVA G, AND PUCHLOVA N. (1989). Helium-neon laser induced respiratory burst of phagocytic cells, Lasers Surg. Med. 9: 585-588.

10. KITCHEN S.S., C.J. PARTRIDGE (1991). A review of low-level laser therapy, Physiotherapy 77 161-168.

11. KLEBANOFF, S. J. (1982). Oxygen-dependent mechanisms of phagocytes. J. I. Gallin, and A. S. Fauci, eds. In *Advances in Host Defense Mechanisms Vol. 1*:*111*. Raven Press, New York.

12. LANDER H.M., (1993). An essential role for free radicals and derived species in signal transduction, FASEB J. 11 (1997) 118-124.

13. LANDER H., P. SEHAJPAL, D. LEVINE, A. NOVOGRODSKY (1993). Activation of human peripheral blood mononuclear cells with nitric oxide-generating compounds, J. Immunol. 150; 509-1516.

14. LARFARS G, GYLLENHAMMAR H (1998). Stimulus-dependent transduction mechanisms for nitric oxide release in human polymorphonuclear neutrophil leukocytes. J Lab Clin Med 132(1):54-60.

15. LOS T., W. DRÖGE, K. STRICKER, P. BAEUERLE, K. SCHULZE-OSTHOFF (1995). Hydrogen peroxide as a potent activator of T-lymphocyte functions, Eur. J. Immunol. 25 (1995) 159-169

16. LOWRY O, ROSENDROUGH N, FARR A AND RANDALL R (1951). Protein measurement with Follin phenol reagent. J. BioI. Chern, 193:265-275.

17. LOWRY S.F. (1993). Cytokine mediators of immunity and inflammation, Arch. Surg. 8 1235-1241.

18. MCKINNEY L., AQUILLA E., COFFIN D., WINK D., VODOVOTZ Y. (2000). Ionizing radiation potentiates the induction of nitric oxide synthesis by interferon-y and/or lipopolysaccharide in murine macrophage cell lines: role of tumor necrosis factor. Annals of the New York Academy of Sciences, 899: 61-68.

19. MILLER, R. A., B. E. BRITIGAN. (1995). The formation and biologic significance of phagocyte-derived oxidants. *J. Invest. Med. 43*:*39*- 46.

20. NAKAGAWA K. (1990). Direct observation of laser generated free radicals from a myocardium target site. Free Radic. BioI. Med.; 12: 241 – 242.

21. NATHAN C.F. (1997). Neutrophil activation on Biological Surfaces, massive secretions of hydrogen peroxide in response to products of macrophages and lymphocytes, J. Clin. Ivest. 80: 1550-1560.

22. PICK E, KEISARY Y (1980). A simple colourimetric method for the measurement of hydrogen peroxide produced by cells in culture. J.lmmunol Methods; 38:161-165.

23. POWLEDGE N. (1997). A Modulation of human neutrophil inflammatory responses by nitric oxide: studies in unprimed and LPS-primed cells. J Leukoc Bioi, 62(6).805-16.

24. SCHINDL A., M. SCHINDL, H. PERNERSTORFER-SCHON, I. SCHINDL, (2000). Low-intensity laser therapy: a review, J Invest. Med. 48 (5) 312-26.

25. SHCHEPETKIN A, UDUT Y, AND KARPOV A. (1993). The effect of He-Ne laser radiation on the chemiluminescence's of human neutrophils, Radiobiologiia 33: 377-82 (Abstract).

26. SIMONS M., NAUSEEF, W., APICELLA A. (2005). Interactions of *Neisseria gonorrhoeae* with Adherent Polymorphonuclear Leukocytes. Infection and Immunity; 73 (4): 1971-1977.

27. SORENSON W., SHAHAN T., SIMPSON J. (1998). Cell wall preparations from environmental yeasts: effect on alveolar macrophage function in vitro. Ann Agric Environ Med. 5(1):65-71.

28. WALLERATH T., GATH I., AULITZKY W., POLLOCK J., KLEINERT H., FORSTERMANN U. (1997). Identification of the NO synthesis isoforms expressed in human neutrophil granulocytes, megakaryocytes and platelets. Thromb Haemost, 7(1): 163-7.

29. WANG MH, FUNG HL, CHEN YQ (2000). Regulation of the RON receptor tyrosine kinase expression in macrophages: blocking the RON gene transcription by endotoxin-induced nitric oxide. Immunol, 164(7):3815-21.

30. YAN L, VANDIVIER RW, SUFFREDINI AF, DANNER R. (1994). Human polymorphonuclear leukocytes lack detectable nitric oxide synthase activity.J Tmmunol; 153(4): 1825-34.

31. YOSHIKAWA T., TANIGAWA M., TANIGAWA T., IMAI A., HONGO H., KONDO. M (2000). Enhancement of nitric oxide generation by low frequency electromagnetic field. Pathophysiology, 7(2): 131-135.

m/z 301
(2.7%)

m/z 249

الملخص العربي

أثر أشعة ليزر الهليوم: نيون في تحفز وتنشيط من كرات الدم البيضاء متعددة الأشكال المهاجمة الأكولة

حسين محمد القباني و سهير سعد قراعة

أجريت تلك الدراسة للاستدلال على أثر الهليوم: نيون في تحفز وتنشيط إنتاج الأكسجين الحرة وأكسيد النيتريك من كرات الدم البيضاء متعددة الأشكال وأثر هذه الشوادر الحرة في تنشيط خلايا الدم الأكولة لمهاجمة الخلايا الميكروبية التي تم معاملتها بمصل الإنسان . تمت تلك الدراسة على تبرع أشخاص أصحاء وقد تم فصل كل كرات الدم البيضاء ثم تعريض كل منها مجتمعة وعلى حدة بجرعات 1, 2.5 , 5 جول/سم^2 في وجود وعدم وجود المنشط الميكروبي ثم قيس مستوي إنتاج شوارد الأكسجين الحرة وأكسيد النيتريك ومستوي أنتاج الحامض النووي المرسال لأنزيم أكسيد النيتريك المستحث. أظهرت النتائج أنه هناك ارتفاع جوهري في إنتاج شوادر الأكسجين الحرة وأكسيد النيتريك عند تعرضها لأشعة الهليوم في وجود الخلايا الميكروبية التي تم معاملتها بمصل الإنسان.

المستخلص من تلك الدراسة هو أن أشعة الهليوم:نيون تعمل على تنشيط خلايا الدم الأكولة لمهاجمة الخلايا الميكروبية التي تم معاملتها بمصل الإنسان عند إستخدام الجرعات جرعات 1, 2.5 , 5 جول/سم^2. و هي تعمل علي إنتاج شوادر الأكسجين الحرة وأكسيد النيتريك و زيادة مستوي أنتاج الحامض النووي المرسال لأنزيم أكسيد النيتريك المستحث ولتحقيق مأربها.