He:Ne laser irradiation induced survival and cell cycle progression effects on human circulating mononuclear cells *in vitro*

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ABSTRACT

The biostimulation and therapeutic effects of low-power laser radiation of different wavelengths and light doses are well known, but the exact mechanism of action of the laser radiation with living cells is not yet understood. The aim of the present study was to investigate whether He:Ne laser irradiation induced mitogenic stimulation on human blood circulating mononuclear cells in vitro displays peculiar features of cell cycle regulation. Buffy-coat human leukocytes were irradiated with He-Ne (10 mW) at energy densities of 1, 2.5 and 5 J/cm^2 and cultured in medium 199 without any supplements in the absence and presence of phytohaemagglutinin for120 hours. Detailed analysis of viability percentage, telomerase activity and gene p53 mRNA expression was performed and compared with a similar analysis of phytohaemagglutinin stimulated and non-stimulated mononuclear cells. Results showed that laser induced telomerase activity in blood mononuclear cells throughout the five consecutive days post laser irradiation, reaching its highest level at 72 hours post laser irradiation and was significantly higher in PHA stimulated cells compared to laser irradiated cells, where 5 J/cm^2 displayed the highest activity. There were no changes in gene p53 mRNA expression at zero compared to 72 hours post laser irradiation. However, it was non-significantly higher in PHA stimulated cells compared to laser stimulated cells. Results threw some light over the mechanism encountered by lymphocytes in the process of He-Ne laser induced biostimulation.

Key words: gene p53, Helium neon (He:Ne) laser, human mononuclear cells, low level laser irradiation (LLLI), telomerase.

INTRODUCTION

Mounting evidence from *in vivo* and *in vitro* data showed the significant influence of laser irradiation on cell functional state. The biostimulation and therapeutic effects of low-power laser radiation of different wavelengths and light doses are well known, but the exact mechanism of action of the laser radiation with living cells is not yet understood (Kujawa *et al.*, 2004). Low-level laser irradiation (LLLI) was found to promote the proliferation of various types of cells *in vitro* including stem cells. The results of previous studies supported the hypothesis that low level laser irradiation (LLLI) exerts a mitogenic effect on the peripheral blood mononuclear cells (Guslov *et*

al., 2006) without inducing any genotoxic effects (El Batanouny *et al*., 2002).

Cell cycle regulation is mediated by a large number of molecules that work in coordination to establish a balance among stimulatory and inhibitory signals (King and Cidlowski, 1995). Orderly progression of the cell cycle is positively controlled by periodic activation of cyclin-dependent kinase (CDK) complexes, and negatively controlled by the phosphorylation of CDKs and the expression of peptide CDK inhibitors that prevent uncontrolled proliferation (Sherr and Roberts, 1999). In addition, the replication machinery requires the activity of telomerase, an RNA-dependent DNA polymerase that directs the synthesis of telomeric repeats toward chromosome ends, by adding telomeric repeats to the 3' end of the telomeric strand, through copying of the template sequence present in its RNA moiety (Hodes, 1999; Buys, 2000). Substantial levels of hTERT/telomerase activity are seen in highly proliferating normal human cells and tissues, both *in vitro* and *in vivo* (Buchkovich and Greider, 1996; Belair *et al.*, 1997). Telomerase plays an essential role in regulating cellular replicative capacity (Bodnar *et al.*, 1998). Expression is absent in most normal human somatic cells (Kim *et al.*, 1994), but progenitor stem cells and cells of the immune system are unique among normal somatic cells in that they have the capacity to upregulate the telomere-extending enzyme, telomerase, albeit in a precisely controlled fashion upon stimulation (Weng *et al.*, 1998).

Concurrently, cell proliferation is linked to apoptosis by stress-integrating proteins, such as p53, a transcription factor that have the dual capacity of ensuring the proper execution of the cell cycle program but also promoting cell death when genomic damage occurs (Amundson *et al.*, 1998; Green and Schuler, 2000). The tumor-suppressor gene product, p53, is a crucial component of the cell's defense machinery against damage, and it is often activated by many stress signals (Zhan *et al.*, 1993). In addition to its function as a transcriptional activator, p53 was also shown to be involved in the suppression of several genes, including *hTERT* (Shats *et al*., 2004), where telomerase activity was reported to be activated concomitantly with the loss of the remaining functional wild-type *p53* allele in Li-Fraumeni skin fibroblasts, suggesting the existence of a p53-dependent regulatory pathway for hTERT control in human cells (Gollahon *et al*., 1998).

The aim of the present study was to investigate the effect of He:Ne laser on the activity of telomerase and gene p53 mRNA expression that represent both stimulatory and inhibitory components in cell cycle progression.

MATERIALS AND METHODS

Preparation of human peripheral blood mononuclear cells

Heparinized blood was obtained at different times from 10 healthy, non-smoker volunteers who had no recent diagnostic or occupational exposure to ionising radiation, laser, or chemicals and had not had any experience to recent allergic responses or drug administration.

Buffy coats were separated and concentrated in plasma at a cell density of 2 x 10^5 cells / ml. Also, whole blood from the same individuals was subjected to Histopaque 1077 (Sigma, Saint Loius MO, USA) separation in order to separate lymphocytes according to standard methods. Lymphocytes were then washed with Hank's balanced salt solution and suspended in its native plasma at the concentration of 2 x 10^5 cells/ml. Aliquots (100 µl) of cells were distributed in 96 well tissue culture plates (Nunc, California, U.S.A.). To minimize cross-irradiation

between cells, at least two empty wells separated each experimentally irradiated well. Plates were shaken immediately prior to irradiation to maintain homogeneity of cells in suspension. Every treatment was investigated in duplicate.

Laser Irradiation

Irradiation was carried out with a 10 mW He-Ne laser (wavelength 632.8 nm) at energy densities of 1, 2.5 and 5 J/ cm^2. This required 2, 5 and 10 minutes irradiation for each well, respectively.

Cell Culture

Two hours after laser irradiation, mononuclear cells were isolated from the irradiated buffy coats by Histopaque-1077 (Sigma Chemicals, St Louis, MO). Cells were transferred into 15-ml sterile plastic round bottom tubes containing only medium 199 (Sigma, Sigma, Saint Loius MO, USA). Cells were incubated for 24, 48, 72, 96 and 120 hours post laser irradiation. Cell growth was assessed at counting cell numbers on a hemacytometer. The viability of cells was determined by trypan blue dye exclusion assay. In addition to the main experiment of laser exposed cells, mononuclear cells from each individual were exposed to the following irradiation and cell culture exposure conditions: Cells treated with the chemical mitogen phytohaemagglutinin (PHA) and not exposed to laser, **c**ells neither exposed to laser nor PHA and Histopaque separated lymphocytes exposed to laser without PHA. No foetal calf serum or antibiotics were added in order to eliminate the suspicion of growth factors or antigen stimulation of lymphocytes; instead medium 199 was sterilized just before use with a 0.22 μm Millipore filter ((Nunc California, U.S.A.).

Telomerase assay

Telomerase activity was determined using telomerase repeats amplification protocol (TRAP) kit (Kim and Wu, 1940) that utilizes a PCR - ELISA assay according to the manufacturer's protocol (Boehringer Mann-heim Biochemicals, Mannheim, Germany).

RT-PCR for Gene p53

RNA was extracted from 2 x 10^5 cells, using QIAGEN RNeasy extraction Kit (Takara Inc, Japan). The RNA samples were reverse transcribed QIAGEN OneStep RT-PCR kit (QIAGEN Inc USA, Clini Lab). The thermal cycler was programmed as follows: 30 min 50 ^{O} C, 15 min 95 ^{O} C, 35 cycles of 3-step cycling 1 min at 94 ^{O} C, 1min at 55 ^{O} C and 1 min at 72 ^{O} C with final extension of 72 ^{O} C for 10 minutes. The resulting cDNA was amplified by PCR. The p53 primers sequences were: (forward) 5′-TTGGATCCATGTTTTGCCAACTGGCC-3′; and (reverse) 5′-GATGGAGT TGAAGGTAGTTTCGTG-3′. Samples were incubated at 95°C for 45 sec, 60°C for 1 min, and 72°C for 2 min for 35 cycles. Reaction samples were then incubated for an additional 7 min at 72°C and cooled to 4°C. The expected size of the product is 357 bp (Zapata et al., 2005). β-actin (540 bp) was used as a positive control; the oligonucleotides were (forward: 5'-GTG GGG CGC CCC AGG CAC CAT-3'; reverse: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'). Conditiond for β-actin were run was at 95°C for 15 min, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, with final extension at 72°C for 10 min.PCR. Ten μL from each PCR reaction product were separated on a 1.2 % agarose gel then stained with ethidium bromide and was evaluated under ultraviolet light and photographed. Relative expression for gene p53 was calculated with reference to β-actin for each individual.

RESULTS

Telomerae activity

Table (1) displays Telomerase activity at different times post He-Ne laser irradiation and under different conditions of cell culture. Peripheral blood mononuclear cells that neither received laser irradiation nor PHA stimulation displayed no telomerase activity.

Results showed that PHA stimulated cells displayed the highest telomerase activity at 72 hours (mean = 41 ± 6 %) post stimulation followed by cells irradiated with 5 J/cm^2 (mean = 18.9 ± 2 %) then 2.5 J/cm^2 (mean = 19.5 ± 3). Irradiation with 1 J/cm^2 displayed the lowest percentage (mean = 14.5 ± 5.5). The activity percentage was significantly higher in PHA stimulated cells ($p < 0.001$) when compared to laser stimulated cells at all energy densities investigated using the student t-test. Histopaque separated lymphocytes without other blood cells exposed to laser at 1, 2.5 and 5 J/cm^2 exhibited null telomerase activity, when they were cultured in the absence of PHA. Similarly were cultures, which received no mitogen stimulation.

Table (1): Telomerase activity in Peripheral mononuclear cells during five days of He:Ne laser irradiation

Treatment Groups	Telomerase activity				
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
PHA & 0 J/cm^2	5 ± 2.5	12 ± 1.2	41 ± 6	35.3 ± 6	34 ± 3
0 J/cm^2	null	null	null	null	null
1 J/cm^2	3.9 ± 1.3	3.9 ± 4.5	14.5 ± 5.5	15 ± 3	17.9 ± 2
2.5 J/cm^2	7.2 ± 3.8	12 ± 3	18.9 ± 2	15.5 ± 4	17.2 ± 3.8
5 J/cm^2	6.4 ± 2.5	14 ± 2	19.5 ± 3	18.9 ± 2	16.4 ± 2.5
Histopaque separated cells + 1, 2.5, or 5 J/cm^2	null	null	null	null	null

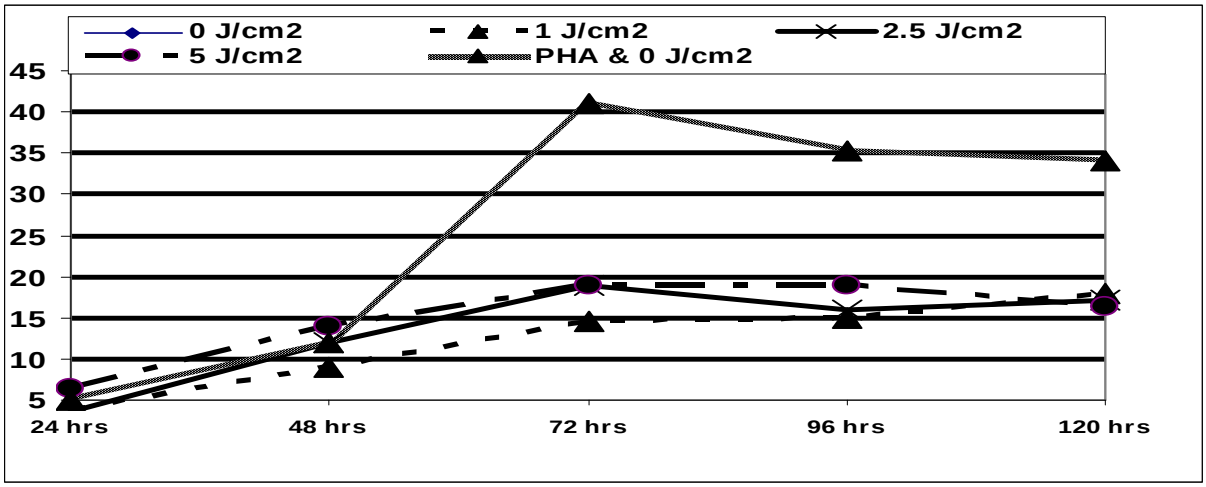

Fig. (1): Effect of 1, 2.5, 5 joules He-Ne laser irradiation or PHA induced telomerase activity percentages in mononuclear cells at 24, 48, 72, 96 and 120 hours post irradiation.

Percentage viability of mononuclear cells irradiated with a He-Ne laser

Mean percentage viability was 95 ± 1.8, 96 ± 2.5, 92 ± 5.6, 93.5 ± 5, and 47.5 ± 8.9 for 1, 2.5 and 5 J/cm^2 respectively, as well as for PHA without laser stimulation and for non-stimulated cells (all observed after 72 hours in culture). There was no significant difference in percentage viability between lymphocytes irradiated with 1, 2.5 or 5 J/cm^2, or cells stimulated with only PHA ($p > 0.05$), using the student t-test. Cells irradiated with 5 J/cm^2 displayed the lowest percentage viability among the energy density stimulated cells. Lymphocytes, which did not receive any mitogen stimulation, had significantly lower in percentage viability when compared to cells, which received laser or PHA stimulation for five consecutive days.

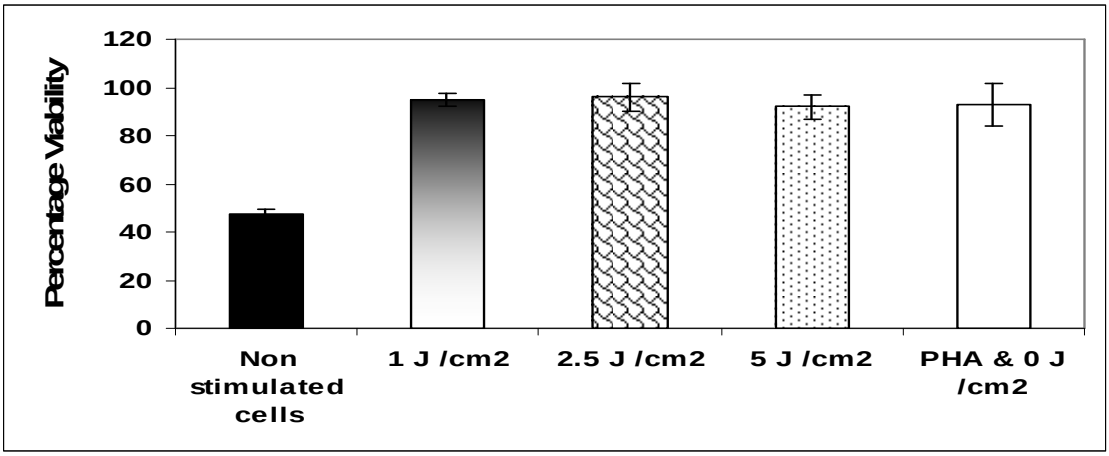

Fig. (2): Percentage viability of mononuclear cells irradiated with 1, 2.5, 5 joules He-Ne laser irradiation, PHA or unstimulated cells 72 hours post irradiation.

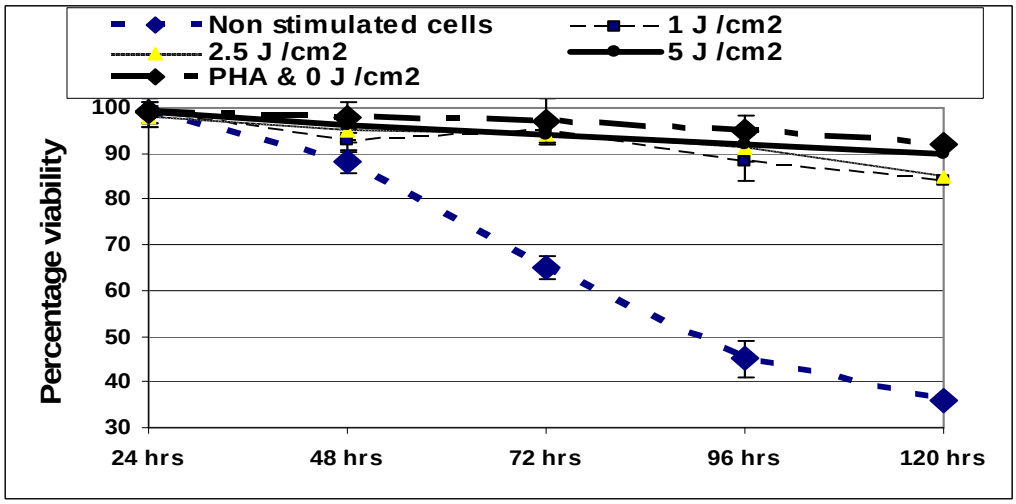

Fig. (3): Percentage Viability curve of mononuclear cells irradiated with 1, 2.5, 5 joules He-Ne laser irradiation, PHA or unstimulated cells after different hours of cell culture

Gene p 53 mRNA expression

There were no significant differences in gene p53 relative expression before laser irradiation compared to 72 hours post laser irradiation.

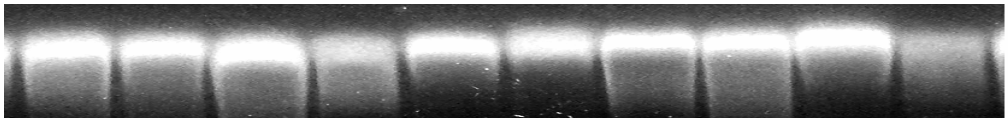

Fig. (4): gene p53 RT-PCR product gel micrograph, before laser irradiation compared to 72 hours post laser irradiation. Lanes 1, 3, 5, 7 and 9 represent PHA, 1 J /cm^2, 2.5 J /cm^2, 5 J /cm^2 and unstimulated cells before laser irradiation. Lanes 2, 4, 6, 8 and 10 represent PHA, 1 J /cm^2, 2.5 J /cm^2, 5 J /cm^2 and unstimulated cells post laser irradiation.

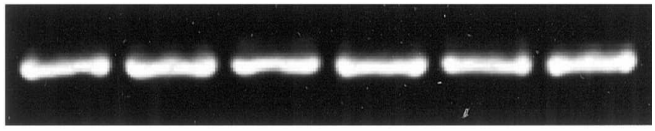

Fig. (5): gene β-actin RT-PCR product before laser irradiation compared to 72 hours post laser irradiation. Lanes 1, 3, 5 are before laser irradiation, while lanes 2, 4 and 6 are post laser irradiation.

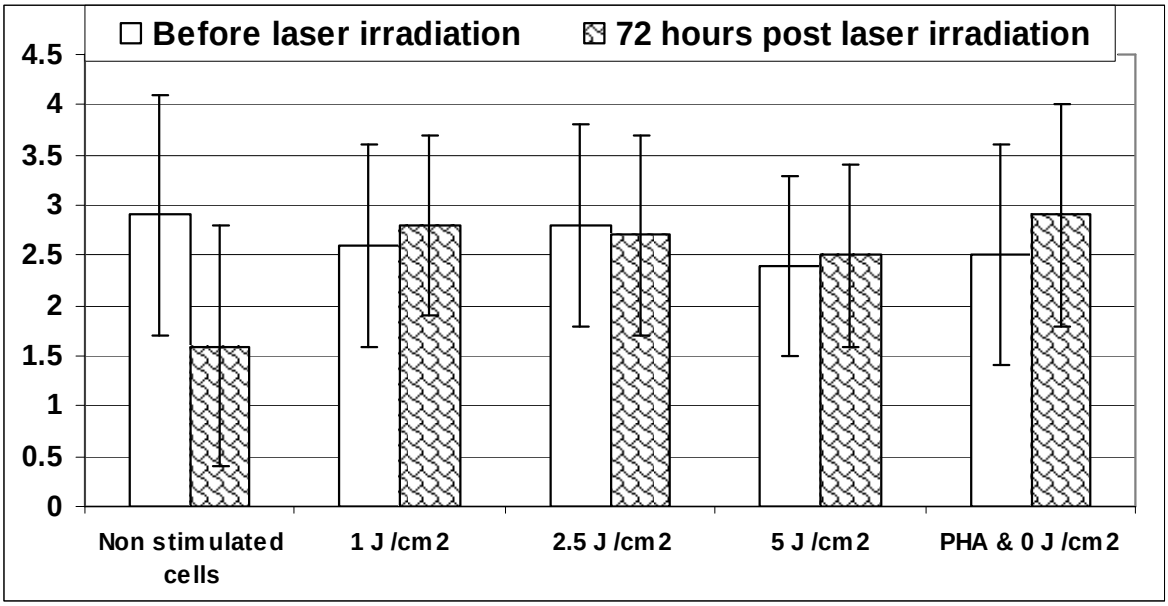

Fig. (6): gene p53 relative expression before laser irradiation compared to 72 hours post laser irradiation.

Gene p53 mRNA

Before laser irradiation mean relative expression of gene p53 mRNA was (2.8 ± 0.9) (Fig. 6). Twenty four hours post laser irradiation there was no significant differences between all groups of the study. Means were (2.9 ± 0.5), (2.6 ± 1), (2.4 ± 1.3), (2.8 ± 0.9) and (2.4 ± 1) for unstimulated, PHA, 1, 2.5, 5 and J/cm^2 respectively. However, results showed that PHA stimulated cells displayed the highest non-significantly level for gene p53 mRNA expression at 72 hours (mean = 2.9 ± .1.2). There were no significant differences between laser stimulated cells at 72 hr. post laser irradiation. Mean for cells irradiated with 1, 2.5 and 5 J/cm^2 was 2.5 ± 1.2 and 2.6 ± 0.7 and 2.7 ± 1 respectively. Non stimulated cells gene p53 relative expression was (mean = 1.6 ± 0.9) displayed the least p53 mRNA relative expression and was significantly lower compared to other laser treatments. Figures 4 and five represents expression of gene p53 and its reference.

DISCUSSION

Leukocytes are a heterogeneous mixture of monocytes, T and B lymphocytes, polymorp-honuclear granulocytes (Roitt *et al*., 1998) and endogenous progenitor cell (EPC) that can be detected in blood at a percentage ranging from 0.041% to 0.074% (Povsic et al., 2007 and Massberg *et al*., 2007). Activation of T and B cells through antigen receptor results in the expansion of a limited number of these cells that can be activated in response to specific antigen and enhance an immune response. In general, the clonal expansion of hemopoietic cell lines and lymphocytes is associated with transient activation of telomerase, an RNA-dependent DNA polymerase (Globerson, 1999). Kinetic analysis of telomerase activity in long-term T cell cultures has documented that the high level of telomerase induced in concert with activation reaches a peak at 3–5 days, then declines by 3 weeks (Weng *et al*., 1998). Also**,** telomerase has been found to be expressed at a basal level in all progenitor cells and is down-regulated upon further proliferation and differentiation (Yui *et al*., (1998); Imanishi *et al.,* (2006). Thus the telomerase activity observed in the present study through out the five consecutive days of culture can be suggested to be related to both lymphocytes and circulating progenitor stem cells.

To investigate the telomerase activity induced in human mononuclear cells when irradiated with He-Ne laser irradiation, it was necessary to construct an *in vitro* model system that reflects the *in vivo* situation as close as possible. Accordingly, in this present study mononuclear cells were irradiated with He-Ne laser in the presence of their native neutrophils and plasma in an attempt to approach the *in vivo* situation. Results indicated that He-Ne laser (632.8 nm) at an output of 10 mW and energy densities 1, 2.5 and 5 J/cm^2 stimulated a percentage of telomerase activity *in vitro* in the absence of any chemical mitogen like. Phytohaema-gglutinin. This phenomenon was observed from 24 up to 120 hours post laser irradiation and was maximal at 72 hours but only when lymphocytes were irradiated in the presence of neutrophils. Histopaque separated lymphocytes did not exhibit this phenomena.

The effect of He:Ne laser irradiation on telomerase activity has not been previously investigated. However, evidences based on measurements of cell proliferation shows that results of the present study are not different from results obtained by Karu *et al.,* (1991), who showed that irradiating Hypaque-Ficoll separated lymphocytes with He-Ne laser of

power output 6 mW / m^2 and energy densities 0.56 J / m^2 did not stimulate DNA synthesis in such cells measured in terms of radio-active thymidine uptake. Although there are differences in the magnitude of power output and energy densities used in this present study compared to their experiment, their experimental *in vitro* model system did not consider the role of other blood leucocytes as was done in the *in vitro* model of this present study. Results of this study also resemble those results that showed that lymphocyte proliferation was significantly higher in samples irradiated in presence of whole blood compared to lymphocytes irradiated after isolation from whole blood (Stadler *et al*., 2000). It has been shown that He:Ne by itself less effective in enhancing cell proliferation than PHA and dual laser and PHA stimulated lymphocytes (Gusloy, 2006).

In the present study it is observed that He:Ne laser irradiation had no action on gene p53 at 72 hours post laser irradiation. Gene p53 is a mechanism in the cells, which controls mechanisms to detect and respond to DNA damage, by the activation of the p53 tumor suppressor protein. High levels of activated and stabilized p53 protein accumulate in the nucleus in response to various forms of cell stress, including DNA damage (Vousden and Lu., 2002). The p53 tumor suppressor plays an essential role for preserving the integrity of the genome and for maintaining regulation of cell cycle progression (Wu *et al*., 2001). Several investigators have shown that the levels of p53 are acutely increased in both normal and malignant cells in response to DNA-damaging agents (Levine 1993). Although definitive mechanistic studies remain to be performed, the induced expression of p53 after DNA damage appears to be regulated at least in part, by translational and post-translational regulatory processes. The importance of translational regulatory mechanisms underlying the expression of p53 has been recently supported by studies that suggest that the expression of murine p53 is controlled by a negative autoregulatory feedback process (Fu *et al*., 1996).

He:Ne laser applied at the fluencies 1, 2.5 and 5 J / cm^2 did not increase the expression of gene p 53 indicating that at the applied fluencies He:Ne laser does not cause DNA damage. A previous study showd that He:Ne laser irradiation decreased stress induced skeletal muscle satellite cells due to nutrient deprivation showed reducing in the expression of p53 (Sheffer *et al.*, 2001). It was suggested that LELI overcomes p53-dependent apoptosis by inhibiting the increase in p53 induced by cellular stresses, such as growth-factor deprivation (Hasan *et al*., (1999), with subsequent induction of its downstream genes. In the present study there was no stress and thus there was no change in gene p 53. Previous studies showed that lower laser fluencies can enhance DNA repair (Karu *et al*., 1994). He:Ne laser has been shown to increase the induction of the frequency of sister chromatid exchanges, which is a measure of DNA repair (Tucker and Preston, 1996) in sheep peripheral blood mononuclear cells in a dose response dependent pattern (Quero *et al*., 1997).

In conclusion He:Ne lasr is of great beneficial therapeutic application, where, B and T lymphocytes, the major antigen-specific lineages of the immune system, require extensive cell division and clonal expansion for their functions. Telomerase activity is expressed and regulated stringently during lymphocyte development and differentiation (Weng et al., 1997 and 1998). Also, telomerase activity is induced dramatically in mature resting lymphocytes on antigenic activation (Bodnar et al., 1998). It is suggested that telomerase play an important role in the

control of human lymphocyte replicative life span. Because telomerase activity is highly regulated in lymphocytes, defining the effect of laser irradiation on its activation is of particular interest and may suggest mechanisms for manipulation of the immune response for therapeutic benefit. Also, peripheral mononuclear cells contain progenitor stem cells that circulate in blood to help regenerate damaged tissue and laser irradiation stimulates such cells providing a valuable tool to stimulate tissue repair.

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الملخص العربي

الأثر المستحث من أشعة الهيليوم نيون ليزر علي حيوية خلايا الدم وحيدة النواة و دفعها الي الدورة الخلوية

سهير سعد قراعة

قسم البحوث الطبية الأشعاعية, المركز القومي لبحوث و تكنولوجيا الأشعاع هيئة الطاقة الذرية – القاهرة – مصر

التنشيط الحيوي الذي يصاحب الأستخدمات العلاجية لأشعة الليزر منخفضة القوي بأختلاف طولها الموجي و جرعاتها المختلفة أثبت فاعليتته في كثير من الأمراض. و لكن تبقي ميكانيكة هذا النوع من الأشعاع علي الخلية الحية في حاجة الي ايجاد مبررات علمية. الهدف من هذه الدراسة هو التعرف علي كيفية دخول خلايا الدم البيضاء الي الدورة الخلوية. تم الحصول عل دم من أشخاص أصحاء و تعريضة لأشعة الهليوم نيون الليزرية (١٠ مليوات) عند جرعات ١, ٢,٥ و ٥ جول/ سم٢ و كذا تم تعريض خلايا الدم البيضاء وحيدة النواة بعد فصلها من الدم ثم تنميتها بأنبوبة الأختبار لمدة ١٢٠ ساعة باضافة و بدون أضافة مادة الفيتوهيمأجلوتنين المحفزة للأنقسام الخلوي. وقد تم قياس النسبة المؤية لحيوية تلك الخلايا و كذلك نشاط أنزيم التلومراز و مستوي التعبير للحامض النووي الرسول لجين البي ٥٣. أوضحت النتائج أن التعرض لأشعة الهليوم نيون الليزرية أدت الي زيادة في نشاط أنزيم التلومراز بلغ قمة المستوي بعد ٧٢ ساعة .كان أعلي مستوي للتلومراز بالخلايا المحفزة بالفيتوهيمأجلوتنين تلاها الخلايا المحفزه ب ٥ جول/ سم٢و كانت الخلايا التي لم تتعرض الي تنشيط حيوي منعدمة النشاط بالنسبة لنشاط أنزيم التلومراز. لم يكن هناك أي أختلاف بمستوي تعبير الحامض النووي الرسول لجين البي ٥٣ علي مدي ال ١٢٠ ساعة بالخلايا المعرضة لمادة الفيتوهيمأجلوتنين و المعرضة لأشعة الهليوم نيون الليزرية عند جرعات ١, ٢,٥ و ٥ جول/ سم٢. و لكن بعد ٧٢ ساعة كان هناك أرتفاع غير معنوي بالخلايا المعرضة لمادة بالفيتوهيمأجلوتنين. الدراسة أوضحت دور لنشاط أنزيم التلومراز لحث خلايا الدم وحيدة اللنواة الي التكاثر و تثبت أن أشعة الهليوم نيون الليزرية أشعة آمنة عند أستخدام جرعات ١, ٢,٥ و ٥ جول/ سم٢.