Human Telomerase Reverse Transcriptase (hTERT) Gene Expression in Rheumatoid Arthritis (RA) Patients after Usage of Low Level Laser Therapy (LLLT)

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Abstract: Purpose: This study aimed to study the effect of cold laser as a complementary drug to the regular anti-inflammatory drug protocols used in rheumatoid arthritis treatment. Design: Experiment was conducted on seventy five subjects with age range 50± 5 years. They were subdivided into three groups. The first group represented twenty normal adults (10 males, 10 females) within the same age range with no rheumatoid arthritis history. The second group represented 27 patients (13 males, 14 females) treated with non steroid anti inflammatory drugs. The third groups represented 28 patients (13 males and 15 females) treated with non steroid anti inflammatory drugs and subjected to soft laser irradiation produced from mid laser infra red medical instrument. Patients received laser sessions along four weeks every other day. The expression of the catalytic subunit of telomerase, hTERT, was measured in PBMC of RA patients and controls by using of RT-PCR quantification kit package Cat. No BSB04M1, for the determination of gene expression. Results: Both rheumatoid arthritis patients groups showed lower telomerase gene expression either those exposed to laser or not as compared to control. Gene expression enhancement was found in patients irradiated with laser combined with the anti-inflammatory protocol as compared to those did not receive cold laser irradiation. Conclusion: Cold laser irradiation in rheumatoid arthritis patients enhanced the hTERT gene expression that in role guarantees balanced DNA ends repair level.

Key words: Rheumatoid arthritis – telomerase – cold laser – anti-inflammatory protocols – Gene expression.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes irreversible destructions of tendons, cartilage and bone (Zvaifler, 1995). Despite many years of intensive investigation the etiology of this multifactorial disease has not been revealed yet. The cause of rheumatoid arthritis is unknown. Even though infectious agents such as viruses, bacteria, and fungi have long been suspected, none has been proven as the cause. The cause of rheumatoid arthritis is a very active area of worldwide research. It is believed that the tendency to develop rheumatoid arthritis may be genetically inherited (hereditary). It is also suspected that certain infections or factors in the environment might trigger the activation of the immune system in susceptible individuals. This misdirected immune system then attacks the body’s own tissues. This leads to inflammation in the joints and sometimes in various organs of the body, such as the lungs or eyes (Alvarez et al., 2005). However, accumulating evidence indicates that RA is an autoimmune pathology in which T cells play a major role (Fox, 1997). With increasing disease duration a number of phenotypic and functional T cell defects have been described in RA including hyporesponsiveness of T cells to stimulation, a decline in naive CD4+ T cells and a disturbance in the naive T cell repertoire (TCR) repertoire indicated by a loss of TCR diversity and clonal expansion of a proportion of T cells (Bakakos et al., 2002; Ponchet al., 2002; Wagner et al., 1998). The capacity of lymphocytes to clonally expand may be mediated, at least in part, through the upregulation of telomerase (Marielle et al., 2005).

Telomerase is a large ribonucleoprotein complex that synthesizes telomere repeats to maintain telomere length at a species-specific level. Telomeres shorten progressively with every cell division due to the inability of DNA-polymerase to fully replicate the extreme ends of chromosomes, the so-called end-replication problem (Allsopp et al., 1995; Watson, 1972). This shortening of telomeres has been proposed to act as a mitotic clock that monitors cell division and provides a measure of the residual replicative capacity of cells (Harley et al., 1990; Weng et al., 1998). Critically short telomeres may be the signal for replicative senescence and ultimately chromosomal instability in normal somatic cells (Allsopp et al., 1992; Vaziri et al., 1993). Telomere erosion

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can however be prevented by upregulation or reactivation of telomerase (Vaziri and Benchimol, 1998; Bodnar et al., 1998). The two most important subunits are an endogenous RNA subunit, human Telomerase-associated RNA (hTR), which contains a 11-base template sequence for the synthesis of telomere DNA, and the protein catalytic subunit, human Telomerase Reverse Transcriptase (hTERT) with reverse transcriptase activity (Weng, 2002). Telomerase is constitutively expressed in germ line cells and in the majority of malignant tumor cells, and is repressed in most human normal somatic cells (Shay and Wright, 1996). In some somatic cell populations, such as lymphocytes and hemopoietic stem cells, there is a highly regulated transient expression of telomerase (Broccoli et al., 1995). Telomerase activity has predominantly been studied in tumor cells, but it may also play a role in autoimmune diseases like RA (Marielle et al., 2005).

Low-level laser therapy (LLLT) (cold laser) is a medical and veterinary treatment that uses low-level lasers or light-emitting diodes to alter cellular function. LLLT is controversial in mainstream medicine with ongoing research to determine the ideal location of treatment (specifically whether LLLT is more appropriately used over nerves versus joints (Brosseau et al., 2005), dose, wavelength, timing, pulsing and duration (Huang et al., 2009). The effects of LLLT appear to be limited to a specified set of wavelengths of laser (Bjordal et al., 2008), and administering LLLT below the dose range does not appear to be effective (Bjordal et al., 2003). Despite a lack of consensus over its ideal use, specific test and protocols for LLLT suggest it is effective in relieving short-term pain for rheumatoid arthritis (Brosseau et al., 2005), osteoarthritis, (Jamtvedt et al., 2007), acute and chronic neck pain, (Chow et al., 2009) tendinopathy, (Bjordal et al., 2008; Tumilty et al., 2010) and possibly chronic joint disorders (Bjordal et al., 2003). The evidence for LLLT being useful in the treatment of low back pain, (Yousef-Nooraie et al., 2008; Middelkoop et al., 2010) dentistry (Cobb, 2006; Seulean et al., 2005) and wound healing is equivocal (Da Silva et al., 2010).

LLLT may reduce pain related to inflammation by lowering, in a dose-dependent manner, levels of prostaglandin E2, prostaglandin-endoperoxide synthase 2, interleukin 1-beta, and tumor necrosis factor-alpha, the cellular influx of neutrophil granulocytes, oxidative stress, edema, and bleeding. The appropriate dose appears to be between 0.3 and 19 joules per square centimeter (Bjordal et al., 2006). Another mechanism may be related to stimulation of mitochondrion to increase the production of adenosine triphosphate resulting in an increase in reactive oxygen species, which influences redox signalling, affecting intracellular homeostasis or the proliferation of cells (Tafur and Mills, 2008) The final enzyme in the production of ATP by the mitochondria, cytochrome c oxidase, does appear to accept energy from laser-level lights, making it a possible candidate for mediating the properties of laser therapy (Karu, 2008).

**MATERIALS AND METHODS**

This study was conducted on 75 subjects with range 50 ± 5 years old. These subjects were subdivided into three groups. The first group represented twenty normal adults (10 males and 10 females) within the same age range with no rheumatoid arthritis history. The second group represented 27 patients (13 males, 14 females) treated with non steroid anti inflammatory drugs. The third groups represented 28 patients (13 males and 15 females) treated with non steroid anti inflammatory drugs and subjected to cold laser produced from mid laser infra red medical instrument. Patients received laser sessions along four weeks every other day. Trigger points were irradiated, access points to the joint and striated muscles adjacent to relevant nerve roots. Irradiation sessions were carried out in the Air Force Hospital in Cairo after ethical approved from the National Research Center ethical committee.

All patients were subjected to detailed clinical history, past history and laying stress on compliant of patients, onset and course of diseases, the pattern of joint involvement and extra articular affection. Pregnant women and patients suffering from other inflammatory diseases were excluded. A pulsed diode laser, He-Ne mid laser with IR manufactured by space laser SRI was used. Turin with continuous emission visible light 632.8 nm wavelength (Output power 5 mw, output divergence after lens 60 mRad), in coaxial associated with 2 infra red diodes of wave length 904 nm, each with the following specification:

1-Infra- red laser emitters: Peak output power = 5 × 10 w. Average output power = 5 × (0.3 + 5) mw. Pulse width 180 nsec. Pulse frequency min. 200 Hz - Max. 4000 Hz. Output beam divergence 70 m Rad. Number of diodes = 5

2- I.R. Handles: Peak power = 10 W. Average output power = 3 mW (min.). Pulse duration 180 nsec. Pulse frequency 4000 Hz. Output beam divergence 70 mRad.

**Peripheral blood mononuclear cells (PBMC) isolation:**

10ml of heparinized venous blood samples were used for isolation of peripheral blood mononuclear cells (PBMC) by density separation over 10 ml ficoll solution in 50 ml falcon tubes then the tubes were centrifuged 20 min at 1600rpm and we took the ring with white blood cells (buffy coat) without touching the ficoll using a sterile pipette tips. Cells were used twice in 6 ml phosphate buffer saline PBS and centrifugate again at 1600rpm for 20 minutes to get cell pellets that were used for RNA isolation (Byum, 1968).
RNA was extracted by simply P spin column total RNA extraction Bioer kit package, cat. no. BSC52S1. 100μl/2×10^6cells were transferred to a new 1.5 ml tube and 100 μl of solution R1 were added, and incubated at room temperature for 1 minute, 600μl of solution R2 were added and incubated at the room temperature for 3-5 minutes. The supernatant was transferred into a spin column and centrifuged for 30 minutes. Wash buffer (600 ml) was added into the spin column and the spin column was centrifuged for 30 seconds then was transferred to 1.5ml micro centrifuge tube followed by addition of 20-50μl elution buffer to the central of the membrane and was incubated at the room temperature for 1 minutes and then was centrifuged for 30 second. The total RNA was used for the determination of the telomerase reverse transcriptase gene expression. All RNA isolated was quantified spectrophotometrically and the optical density (OD) 260/280 nm ratio was determined.

**Determination of telomerase reverse transcriptase (hTERT) activity:**

Determination of Telomerase reverse transcriptase (hTERT) activity was carried out by a telomerase reverse transcriptase (hTERT) mRNA one step RT-PCR quantification kit package Cat. No. BSB04M1, for the determination of gene expression (Hang Zhou Bioer Technology Co., Ltd.).

The PCR mixture (total volume, 36μl), which was prepared from 20 μl RT-PCR mix, 2 μ ml Mn2+, 4μl rTERT probe and 10μ DEPC H2O. The amplification protocol for cDNA on the Light Cycler was a 10 min denaturation step at 95°C for polymerase activation, a ‘touch down’ PCR step of 10 cycles consisting of 10s at 95°C, 10 s at 65-C, and 30 s at 72°C, followed by 40 cycles consisting of 10 s at 95°C, 10 s at 55°C, and 30 s at 72°C. After slow heating (0.1°C s^-1) of the amplified product from 65 to 95°C to generate a melting temperature curve, which serves as a specificity control, the PCR samples were cooled to 40°C.

**RESULTS AND DISCUSSION**

**Fig: 1.** represents that human telomerase transcriptase (hTERT) activity in patients received anti-inflammatory protocols as well as those received cold laser irradiation beside the anti-inflammatory protocols as compared to control. The right plots are those of patients did not receive cold laser as a complementary drug, gene needed excess cycles to be expressed. The middle plots are those of patients received cold laser irradiation beside the normal anti-inflammatory protocols. Left plots represent the normal controls. Gene expression level was lower in patients did not receive cold laser than those of patients received cold laser as a complementary drug. Both groups of RA patients revealed lower gene expression as compared to control.

**Fig. 1:** Telomerase reverse transcriptase (hTERT) activity in patients received anti-inflammatory drugs with and without cold laser irradiation as compared to control. The x-axis denotes the cycle number of a quantitative PCR reaction. The y-axis denotes the log of fluorescence intensity over the background.
Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that may affect many tissues and organs, but principally attacks synovial joints. The process produces an inflammatory response of the synovium (synovitis) secondary to hyperplasia of synovial cells, excess synovial fluid, and the development of pannus in the synovium. The pathology of the disease process often leads to the destruction of articular cartilage and ankylosis of the joints. Rheumatoid arthritis can also produce diffuse inflammation in the lungs, pericardium, pleura, and sclera, and also nodular lesions, most common in subcutaneous tissue. Although the cause of rheumatoid arthritis is unknown, autoimmunity plays a pivotal role in both its chronicity and progression, and RA is considered a systemic autoimmune disease (Allsopp et al., 1995).

The factors that allow an abnormal immune response, once initiated, to become permanent and chronic, are becoming more clearly understood. The genetic association with HLA-DR4, as well as the newly discovered associations with the gene PTPN22 and with two additional genes (Plenge et al., 2007), all implicate altered thresholds in regulation of the adaptive immune response. It has also become clear from recent studies that these genetic factors may interact with the most clearly defined environmental risk factor for rheumatoid arthritis, namely cigarette smoking (Padyukov et al., 2004). Other environmental factors also appear to modulate the risk of acquiring RA, and hormonal factors in the individual may explain some features of the disease, such as the higher occurrence in women, the not-infrequent onset after child-birth, and the (slight) modulation of disease risk by hormonal medications. Exactly how altered regulatory thresholds allow the triggering of a specific autoimmune response remains uncertain. However, one possibility is that negative feedback mechanisms that normally maintain tolerance of self are overtaken by aberrant positive feedback mechanisms for certain antigens such as IgG Fc (bound by RF) and citrullinated fibrinogen (bound by ACPA) (Rubio et al., 2009).

Once the abnormal immune response has become established (which may take several years before any symptoms occur), plasma cells derived from B lymphocytes produce rheumatoid factors and (anti-circullinated protein antibody) ACPA of the IgG and IgM classes in large quantities. These are not deposited in the way that they are in systemic lupus. Rather, they appear to activate macrophages through Fc receptor and perhaps complement binding. This can contribute to inflammation of the synovium, in terms of edema, vasodilatation and infiltration by activated T-cells (mainly CD4 in nodular aggregates and CD8 in diffuse infiltrates). Synovial macrophages and dendritic cells further function as antigen presenting cells by expressing (myosin heavy chain) MHC class II molecules, leading to an established local immune reaction in the tissue. The disease progresses in concert with formation of granulation tissue at the edges of the synovial lining (pannus) with extensive angiogenesis and production of enzymes that cause tissue damage. Modern pharmacological treatments of RA target these mediators. Once the inflammatory reaction is established, the synovium thickens, the cartilage and the underlying bone begin to disintegrate and evidence of joint destruction accrues (Stefan et al., 2003).

In RA patients, telomere attrition in CD4 T cells is accelerated (Koetz et al., 2000 and Schonland et al., 2003) by either proliferative stress or insufficient telomeric repair. Telomeres are not critically short and should not force T cells into cell cycle arrest or cell death. Nevertheless, defects in telomeric maintenance could affect broader cellular functions. An important consequence of telomere shortening is the induction of replicative senescence, a state in which the cell is viable but prohibited from further cell divisions (Aubert and Lansdorp, 2008). Telomeric lengthening and maintenance is facilitated by telomerase, an enzyme composed of a catalytic protein unit known as human telomerase reverse transcriptase (hTERT) and an RNA template complementary to the telomeric DNA (hTR) (McEachern et al., 2000). In most tissues, telomerase is strongly suppressed, but in T cells telomerase activity is dynamically regulated and coincides with periods of cellular expansion (Weng et al., 1996 and Hodes et al., 2002). Telomerase induction allows for telomere elongation, translating into lengthening of life span (Luiten et al., 2003; Roth et al. 2003 & 2005 and Rufer, 2001). How ongoing telomeric maintenance affects T cell proliferation and function before the state of a short and dysfunctional telomere is reached is currently not understood completely.

Hiroshi et al. (2009) reported that in RA, naïve CD4 T cells fail to up-regulate telomerase when primed through the TCR. Knockdown of hTERT in primary human T cells revealed a direct effect on cell survival, with telomerase insufficiency rendering T cells apoptosis susceptible. Naïve RA CD4 T cells were prone to die when driven into clonal expansion, impairing their clonal size. Apoptosis during this early phase in the T cell life cycle was Fas independent and mediated through the mitochondrial pathway. Ectopically expressed hTERT repaired apoptotic propensity of RA T cells. In essence, the enzyme telomerase is critically involved in determining life/death decisions in proliferating CD4 T cells. Telomerase insufficiency in RA T cells may lead to a defect in the homeostatic regulation of the T cell pool.

Telomerase insufficient T cells died from excessive apoptosis despite stability in telomeric length. By reinstating lost DNA telomeric sequences, telomerase protects cells from replicative senescence (Bodnar et al., 1998) and may confer cellular immortality (Stewart and Weinberg, 2006). Transfer of hTERT into human mammary epithelial cells promotes spontaneous growth (Beliveau et al., 2007). In a recent report (Beliveau et al., 2007), the growth promoting ability of telomerase was p53 dependent, suggesting that the cell recognizes a shortened telomere as damaged DNA. 53BP1/phosphorylated histone H2AX foci appeared at chromosome ends long before telomeres were critically shortened indicating that telomeric dysfunction precedes erosion of
telomeric sequences and functionally affects the cell long before senescence. Gamma-H2AX has a role in early signaling of DNA damage (Rogakou et al., 1998) and, again, has been observed in foci at the telomeric ends (Hao et al., 2004). The detailed structure of telomeres in T cells as they pass through different life-cycles is unknown. Besides the premature loss of telomeric ends, telomere structure in RA T cells may be sufficiently disturbed to physically disrupt binding sites for essential proteins of the shelterin complex (De Lange, 2005) or may interrupt T-loop formation (Blackburn, 2001).

The question remains of how impaired telomerase induction and apoptotic hypersensitivity in naïve CD4 T cells is related to RA. Most important to consider is the impact of chronic T cell attrition on homeostatic control of the T cell compartment. Notably, the major risk factor for RA is advanced age; most patients are diagnosed during the second half of life. During this life period, naïve T cells are not replenished through thymic production but rather through autoproliferation (Slijepcevic, 2006). Failure to reach appropriate clonal size during priming must inevitably lead to smaller clonal sizes of memory cells. This scenario predicts that RA patients have difficulties maintaining a filled T cell pool and expose memory T cells to more and more replicative turnover. In essence, the entire T cell pool in RA is overaged, forcing the patient to generate immune responses with T cells that have essentially reached the end of their life span. Interestingly, senescent CD4 T cells acquire apoptotic resistance (Schirmer et al., 1998), endowing them with a survival advantage. If such enddifferentiated memory T cells stay alive, they will compete for space and further disadvantage incoming new cells. T cell senescence has also been identified as a pathway in atherosclerosis, particularly the unique inflammatory response precipitating plaque instability (Pryshchep, 2006 and Sato et al., 2006). Cardiovascular complications are now recognized as extra-articular manifestations of RA (Giles et al., 2006). Finally, dysfunction of naïve CD4 T cells puts the patient at risk for inadequate anti-pathogen responses, a complication well recognized within the spectrum of the rheumatoid syndrome (Doran et al., 2002). Implicating telomerase in T cells as an element of the pathogenic network in RA provides novel and exciting opportunities for therapeutic approaches in this chronic and as yet incurable disease.

Low level laser therapy (LLLT) is a light source treatment that generates light of a single wavelength. LLLT emits no heat, sound, or vibration. Instead of producing a thermal effect, LLLT may act via nonthermal or photochemical reactions in the cells, also referred to as photobiology or biostimulation, photobiomodulation, cold laser therapy. Laser radiation and monochromatic light may alter cell and tissue function. Laboratory studies suggest that irradiation stimulates collagen production, alters DNA synthesis, and improves the function of damaged neurological tissue (Cochrane Database, 2007). Red and near-infrared laser irradiation is reported to have a range of biological effects on cultured cells and different tissues, leading to the hypothesis that laser light can affect energy metabolism. Increased adenosine triphosphate (ATP) synthesis has been reported in cultured cells and rat brain tissue after irradiation at 632.8 nm and 830 nm, respectively (Kuo et al., 2010).

Cold laser is the type used in physical medicine as its depth of penetration is sufficient to produce a biological effect on deeper tissues without damaging them. The helium neon depth of penetration is up to 0.8 mm directly and from 10 to 15 mm indirectly. Direct penetration refers to the characteristic properties of laser that have not been altered. In direct penetration, the light is transmitted into the deep tissues through hyperscopic absorption properties of the surrounding tissue. Once this occurs the coherent and non divergent properties of laser are altered. Therefore, the difference between the two field’s depths in due to the dispersion of light is tissue. It is therefore a superficial physical agent (more than 50 % of the energy is absorbed by tissue located less than 1 cm below the skin surface (Karen Adams et al., 2009).

Laser action on cell proliferation may be explained by the direct effects of laser to increase the low oxygen concentration and necessary nutrients at the injured site. Light provides proliferative stimulus, having some effect on the system that are known to regulate cellular proliferation mainly (CAMP). The cyclic adenosine monophosphate system has been demonstrated to control biosynthesis of DNA and RNA (Rubio et al., 2009).

Regarding the present work, cold laser irradiation was used as complementary therapy for the regular anti-inflammatory drugs protocols applied for rheumatoid arthritis patients in the rheumatology department of Air Force Hospital in Cairo. Human telomerase reverse transcriptase gene expression was measured to investigate to what extend cold laser may affect the proliferation/apoptosis T cells decision.

Both patients groups either treated with anti-inflammatory drug protocols only or treated with the regular protocols plus cold laser irradiation session revealed lower human telomerase reverse transcriptase (hTERT) as compared to non rheumatoid arthritis patients. Gene expression enhancement was revealed in patients received cold laser irradiation session as compared to those did not receive cold laser irradiation.

In conclusion, establishing a better gene activity in patients received cold laser sessions as compared to those did not receive laser irradiation guaranteed a better telomere ends repairing process, that in role, save better proliferation to apoptosis balance of T cells in rheumatoid arthritis patients.
Declaration of interest:
The author reports no conflicts of interest. The author alone is responsible for the content and writing of this paper.

REFERENCES


