A POSSIBLE MECHANISM OF LOW LEVEL LASER-LIVING CELL INTERACTION

R. Lubart,* Z. Malik,† S. Rochkind‡ and T. Fisher§

*Department of Physics, Bar-Ilan University, Ramat Gan, Israel. †,§Department of Life Sciences, Bar-Ilan University, Ramat Gan, Israel. ‡Department of Neurosurgery, Tel Aviv Medical Center, Ichilov Hospital, Israel

A qualitative E.P.R. (Electron Paramagnetic Resonance) study has been performed to determine the action of Low Level Lasers on living cells. Singlet oxygen ($^1\text{O}_2$) was found to be generated by Friend Erythroleukaemic Cells (FELC) after irradiating them with a HeNe laser. The production of $^1\text{O}_2$ in the cell during irradiation was proved by an E.P.R. technique. No singlet oxygen is photoproducted by irradiating either cytochrome C or hemin.

Singlet oxygen, in small amounts, is very significant in biochemical processes and hence can be important in biostimulation. It is proposed that singlet oxygen is photoproducted by the natural porphyrins in the cell.

KEY WORDS LLLT (Low Level Laser Therapy) E.P.R. FELC Singlet oxygen Porphyrins

Introduction

Low Level Laser (LLL) irradiation has been used for more than a decade in clinical practice causing biostimulation. There are numerous examples where this method has been successful in dermatology, gynaecology, otorhinolaryngology and other medical areas.¹⁻⁵

Our group has been studying the effects of LLL irradiation on nervous system regeneration. We have found that laser irradiation affects the compound action potential of the nerve directly. Its effect being both preventive and therapeutic, is not thermal and is limited to certain wavelengths and to a specific energy range.⁶⁻⁹ A major problem, tackled by many researchers but with no conclusive results, is the question of how LLL affects living tissue. Olson¹⁰ believes that there is a primary absorption of light by mitochondrial enzymes, resulting in local heating following an immediate change in membrane conductivity. Karu¹¹ suggests that at doses causing biostimulation, visible light is absorbed by the respiratory chain components such as flavins and cytochromes, causing an acceleration of the electron-transfer in the redox pairs in some sections of the respiratory chain. Klima¹² compares laser therapy to oxygen therapies. In oxygen therapies it is the light (632 nm) emitted by $^1\text{O}_2$ which has a therapeutic effect. Klima assumes that HeNe laser light (632 nm) plays the same role as singlet oxygen emission in immune regulation.

Singlet oxygen has been recently shown to be a significant biochemical intermediate in biochemical

processes¹³ and hence can be important in biostimulation. The production of $^1\text{O}_2$ in the cell during irradiation was proved by E.P.R. (Electron Paramagnetic Resonance) technique according to the method of Moan.¹⁴

In the present work we show that LLL light used for biostimulation does not replace the emission of singlet oxygen but actually generates small amounts of singlet oxygen ($^1\text{O}_2$) in the irradiated cells. The singlet oxygen is photoproducted by the endogenous porphyrins produced by the cells which are photo-excited by the HeNe light.

Materials and Methods

We used Moan's¹⁴ technique: In this technique singlet oxygen, photosensitized by porphyrin, reacts with a trap molecule to produce a stable free radical which is then detected by E.P.R. spectroscopy. Potential advantages of this technique include: (1) high sensitivity, (2) simplicity, since it is not necessary to isolate the singlet oxygen product, and (3) the ability to study optically opaque systems. In the present work we used the sterically hindered amine 2,2,6,6-tetramethyl-4-piperidone (TMP) (Sigma) as the trap molecule. The TMP reacts with singlet oxygen to give the stable radical 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TAN) which is easily detected by an E.P.R. technique.

Photosensitization of Deuteroporphyrin (DP)

TMP dissolved in water 4 mg/ml was added to an aqueous solution of DP 2.5 mg/ml in an E.P.R.

Addressee for correspondence: Dr R. Lubart, Physics Department, Bar-Ilan University, Ramat Gan 52100, Israel.

tube. The tube was then introduced into the E.P.R. instrument, *X* band, (Varian/E-101) with 100 kHz field modulation. An optical fibre was inserted into the tube and the sample was cooled to 77° K. The light source was a HeNe laser ($\lambda = 632$ nm). The light intensity at the output of the fibre was 100 J/cm². The E.P.R. spectrum was measured before and during the irradiation. The spectrometer was operated at: gain, 8×10^3 ; time constant, 0.3 s; microwave power, 3.5 mW; scan time 4 min; field set 3200 G; scan range, 1000 G; time constant, 0.3 s; modulation amplitude, 16 g and microwave frequency, 9.05 GHz.

Photosensitization of Friend Erythroleukaemic cells (FELC)

FELC line 745, subclone 21, were isolated by the soft agar technique. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (Gibco) in a humidified incubator enriched with 10% CO₂ at 37°C. Cells grown for 3 days were collected and washed in 0.1 M phosphate-buffered-saline, pH 6.6. Then they were resuspended in fresh media without serum to a concentration of 5×10^6 cells/ml. Cells were counted in a Neuhauser camera.

Another cell sample was prepared as described and then incubated for 30 min in the phosphate buffer containing 10 µg/ml of DP (pre-dissolved in 20 µl of 1 N NaOH and diluted in the phosphate buffer).

The cell samples were transferred into the E.P.R. tube containing the solution of TMP and their E.P.R. spectra were measured before and during the irradiation.

Photosensitization of Cytochrome C and Hemin

Cytochrome C was dissolved in water and brought to a final concentration of 2.5 mg/ml.

Hemin was dissolved in a small volume of 1 N NaOH and diluted in the phosphate buffer to a concentration of 2.5 mg/ml.

The cytochrome C and the hemin were incubated with TMP. Their E.P.R. spectra were recorded before and during irradiation.

The illumination parameters and the E.P.R. spectral measurements in all samples were the same.

Results and Discussion

In Figures 1b, 1c and 1d one can see the E.P.R. spectrum of the photoproduct radical TAN which is produced by the reaction of ¹O₂ with the TMP. Singlet oxygen (¹O₂) is in all cases photoproducted by porphyrins. It is known that the efficiency of

Figure 1. EPR. spectra at 77° K of: (a) All samples before the irradiation. (b) An aqueous solution of DP and TMP during irradiation. (c) A cell sample of FELC containing exogenic DP, incubated in TMP during irradiation. (d) A cell sample of FELC incubated in TMP during irradiation. (e) An aqueous solution of cytochrome C and TMP during irradiation. (f) An aqueous solution of Hemin and TMP in a phosphate buffer during irradiation

this photoprocess depends on the nature of the porphyrins side chains, the wavelength of the irradiation and its energy density. Incorporation of metal ions into the porphyrin molecule depresses or even inhibits the formation of ¹O₂.¹⁵ In Figure 1b TAN radical is formed by an aqueous solution of DP. The formation of TAN radical in Figure 1c shows that cells enriched with exogenic porphyrin generate ¹O₂. The formation of radicals in Figure

1d shows that $^1\text{O}_2$ can be photoproduced by the natural porphyrins in the cell. It is known that protoporphyrin is biosynthesized by almost all living cells.^{16,17} The FELC in our experiment is known to produce protoporphyrin constitutively.¹⁸

It is thus conceivable that $^1\text{O}_2$ can be generated by light irradiation of living cells.

It was previously suggested by Karu¹⁹ that $^1\text{O}_2$ can be the clue to LLL biostimulation as it can be photoproduced by cytochrome C. This work demonstrates the appearance of $^1\text{O}_2$ photoproduced by HeNe irradiation of cells due to the presence of porphyrins in them. Figure 1d. Singlet oxygen was not produced by illumination of either cytochrome C or hemin as is indicated in Figures 1e and 1f. This result is obvious as Fe complexes of porphyrins do not generate $^1\text{O}_2$ when being irradiated.¹⁵ This is also the reason why hemin does not produce $^1\text{O}_2$ (Figure 1f).

Singlet oxygen ($^1\text{O}_2$) at $^1(\Delta g)$ is a highly reactive intermediate. It has a sufficiently long half-life in the biological environment (of the order of 1 μs) to react chemically. Singlet oxygen at large amounts rapidly oxidizes a large variety of biological molecules,¹¹ damages DNA and is responsible for cell destruction.

At low concentration, on the other hand, singlet oxygen can modulate biochemical processes taking place in the cell and can trigger immune regulation.¹⁰ Recently several biochemical systems have been shown to produce singlet oxygen.²⁰ Human eosinophil,²¹ for example, was shown to generate detectable amounts of singlet oxygen. These recent studies suggest that singlet oxygen is a significant biochemical intermediate which may have an important role in biostimulation. We believe that by irradiating damaged tissues with LLL, small amounts of singlet oxygen are produced and are responsible for tissue regeneration.

From our previous work^{8,9} we know that LLLT is dependent on wavelength, is not thermal and is limited to a specific energy density. There is a minimum threshold of energy flux below which there is no effect, and a maximum threshold beyond which the effect is reversed. We also know from Karu's¹¹ work that irradiation of cells with visible light of one and the same wavelength but with different energy doses has a positive effect, acceleration of cell division at low doses, and a negative effect, death of cells at high doses. We are now able to explain these results. At low doses of laser irradiation small amounts of $^1\text{O}_2$ are produced which have a positive effect on the cells. At high doses of energy large amounts of $^1\text{O}_2$ are produced and photodynamic damage occurs.^{22,23}

The action spectrum of porphyrins agrees well with our previous results⁹ as to the biostimulation dependence on the laser wavelength. Porphyrins have an intense absorption in the violet region

around 440 nm and four additional absorption bands with decreasing intensity between 500 and 630 nm. In LLLT and in Photodynamic Therapy (PDT) only the 630 nm is used because of its good penetration.

If indeed porphyrins are responsible for LLLT, addition of porphyrins, in the appropriate amounts, to the damaged cells would improve their regeneration.

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