MEMBRANE EFFECTS OF LOW LEVEL INFRARED LASER IRRADIATION, AS SEEN IN METABOLICALLY INTACT AND IMPAIRED HUMAN BLOOD CELLS

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Abstract. The aim of the present work was to contribute to the understanding of the cellular and molecular mechanisms involved in low power long wavelength laser irradiation effects. Investigating the 830 nm laser light influence on the anisotropy of 1-[(4-trimethyl-amino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) fluorescence, we report low power infrared laser induced changes in the lipid order parameter in the polar head group regions of human lymphocytes plasma membrane. Using irradiation doses of therapeutic significance, we point out a sensitive dependence of the observed membrane effects on the lymphocytes actual metabolic state.

Key words: AlGaInP/GaAs, steady state fluorescence anisotropy, fluorescent lipid probe, plasma membrane fluidity.

INTRODUCTION

Given the aim to promote low power long wavelength laser therapy in the domain of evidence-based medicine, much scientific progress in this area of research was obtained in the last 30 years. Many cellular and molecular mechanisms involved were evidentiated, pointing out activation of cell metabolism through respiratory chain as basic primary mechanism of action. In spite of this, thorough knowledge of the involved signaling pathways is still missing, understanding of light regulation mechanisms of cell metabolism is still fragmentary, and the number of studies disclosing dependence of the expected desirable effects on well defined and reproducible irradiation parameters is yet few. Whenever the goal is promotion of wound healing, reduction of inflammation, and/or pain relief, low power laser therapy (LPLT) also named low

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level laser therapy (LLLT) continues to be successfully used [1, 4, 18, 22, 24–25, 28], nonetheless many short term and long term effects of irradiation have yet be thoroughly investigated before LPLT will really become a mainstream medical tool.

The aim of the present work was to contribute to the enrichment of the scientific knowledge concerning characteristics and mechanisms involved in soft laser irradiation effects at sub cellular level.

Dynamic rearrangement of membrane components at the cell surface and the changes induced in the membrane physico-chemical properties play essential role in cellular signaling [16], and in multiple cell membrane alterations involved in various human and animal pathologies [17]. Using human peripheral blood lymphocytes, and the lipid probe TMA-DPH, selected to monitor fluidity/lipid packing density in the polar head group regions of the plasma membrane lipid bilayer respectively, we report steady-state fluorescence anisotropy data revealing membrane effects of the 830 nm laser light. Data analysis is focused on revealing the modulation of the observed lipid order / membrane fluidity changes by the cells actual metabolic state.

MATERIALS AND METHODS

MATERIALS

The fluorescent lipid probe 1[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) was from Molecular Probes, Hepes buffer substance from Sigma Chemical Co., while all other chemicals were the best research grade available.

CELLS

Peripheral lymphocytes were prepared from freshly drawn human blood obtained from healthy volunteers who had not received any medical treatment within two weeks before experiment, as previously described [15]. Briefly, the blood was collected in a standard citrate buffer and lymphocytes were separated by Ficoll-Hypaque density gradient centrifugation, washed twice by centrifugation / re-suspension / centrifugation at 100 g for 5 min, and finally re-suspended in Hepes buffer HPMI (constituted of 100 mM NaCl, 5.4 mM KCl, 0.4 mM $MgCl_2$, 0.04 $CaCl_2$, 10 mM Hepes, 20 mM glucose, 24 mM $NaHCO_3$ and 5 mM Na_2HPO_4) at pH 7.4. Cell population obtained by this technique contained ~70% T cells [17]. Cell suspensions with viability level assessed by Trypan blue exclusion, higher than 95% were only used.

METABOLIC IMPAIRMENT

Metabolic impairment of cells was obtained by serum starvation at temperatures below 37 °C. Human peripheral lymphocytes were kept various time periods (1–48 hours) at room temperature (~25 °C) or at 4 °C, without any nutritional supplement (serum, amino acids or vitamins) in glucose-containing Hepes buffers HPMI. We name "fresh" the samples constituted of suspensions of lymphocytes freshly separated within 90 min after blood drawing, on which the membrane fluidity determinations were made immediately after cells re-suspension in the serum-free buffer. Metabolic intactness/impairment was appreciated checking viability and plasma membrane potential.

SAMPLES

Samples were constituted by continuously stirred, thermostatted human peripheral lymphocyte suspensions of cell densities of $2x10^6$ cells/ml, prepared in HPMI buffer.

LABELING OF CELLS WITH FLUORESCENT LIPID PROBES

The $5 \cdot 10^{-4}$ M stock solution of TMA-DPH prepared in dimethylformamide, was kept at –20 °C until use within a month. Human lymphocytes were labeled with TMA-DPH by incubating them in the measuring cuvette with 1.25 μM dye at 37 °C for 3 min at a cell density of $2x10^6$ cells/ml.

FLUORESCENCE ANISOTROPY MEASUREMENTS

Steady state fluorescence and anisotropy measurements were carried out in a Perkin-Elmer MPF 44B spectrofluorimeter or in a Jobin-Yvon SpectroFluo JY3 spectrofluorimeter, equipped with thermostatted cell holders, stirring devices, Polaroid HN polarizers and connected to IBM PC computers using appropriate data acquisition software: SCOPE and EASYEST, respectively. The excitation and emission wavelengths were selected 340 nm / 425 nm. A BG12 filter placed in front of the exit slit of the sample compartment protected the detector from dispersed laser light. Steady state fluorescence anisotropy values (r) were obtained by quasi-simultaneous (within 12 s) measurements of the intensity components I_{VV} and I_{VH}, where VV and VH stand for vertical/vertical (parallel) and vertical/horizontal (perpendicular) positions of the excitation and emission polarizers respectively. A correction factor $G = \frac{I_{HV}}{I_{HH}}$ for unequal transmission by the optical

elements of the vertically and horizontally polarized intensity components also was determined and thus the steady-state fluorescence anisotropy values were calculated every 25 s according to the formula:

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} \tag{1}$$

The small auto fluorescence of cells and the unavoidable scattered light contribution, determined by measuring unlabeled controls under the same experimental conditions as samples, were substracted from each intensity component.

The lipid order parameter in the polar head group regions of the plasma membrane bilayer, $S^{\text{TMA-DPH}}$, were computed as functions of limiting initial r_o and long-time r_∞ values of the fluorescence anisotropy:

$$S^{\text{TMA-DPH}} = \sqrt{\frac{r_\infty^{\text{TMA-DPH}}}{r_0}} \tag{2}$$

where $r_o = r_o^{\text{TMA-DPH}} = 0.362$, while the limiting long-time fluorescence anisotropy values r_∞ were approximated using the empirical curve obtained by van Blitterswijk *et al*. [23] as

$$r_\infty = 1.270 \cdot r - 0.076 \quad \text{for} \quad 0.13 < r < 0.28 \tag{3}$$

$$r_\infty = 1.100 \cdot r - 0.032 \quad \text{for} \quad 0.28 < r < 0.34 \tag{4}$$

where r represents the TMA-DPH fluorescence anisotropies calculated from measured fluorescence intensities according to the formula (1).

Membrane fluidity was expressed as a reciprocal of the lipid order parameter.

Anisotropy, lipid order parameter and membrane fluidity values and changes occurred in these parameters under the influence of laser irradiation are presented as $mean \pm S.D.$ calculated from at least 3 independent measurements.

LASER SOURCE

The radiation source was an AlGaInP/GaAs based semiconductor laser used in the medical practice, Sony SLD202-D3, with continuous wave output, having emission wavelength of 830 nm, nominal power of 50 mW, elliptical beam size 2.5 mm x 7 mm with speckle area of 17.5 mm^2, divergence 5° and polarization ratio 100:1.

SAMPLE IRRADIATION

Irradiation of the samples was performed using a dedicated experimental set-up and/or directly in a fluorimeter, both equipped with thermostatting and stirring facilities. Due to beam divergence of the used laser source, the incident power density values were source-sample distance dependent. At a 2 cm distance from source the measured incident power density in the speckle area was 363.8 mW/cm^2. This resulted in an estimated average incident power density on the 1 cm^2 upper surface of the continuously stirred cell suspension of ~550 W/m^2, which lies in the upper limit region of intensities typically used in low power long wavelength laser therapy. Moving the source 6 cm away from sample, beam divergence caused increase in speckle size and in consequence an ~48% loss in the magnitude of power incident on the sample. In these conditions the estimated average incident power density on the 1 cm^2 upper surface of the continuously stirred cell suspension became ~260 W/m^2.

The minimum irradiation time of the continuously stirred cell suspension was 20 s, equal with the time needed to measure all four fluorescence intensities in order to calculate one fluorescence anisotropy value, according to formula (1). With the laser source placed at the 6 cm distance from sample, the estimated average incident energy density per experimental point gathered in presence of laser radiation, was ~5.2 kJ/m^2.

Duration of irradiation – realized previous to or during steady state fluorescence anisotropy measurements – varied between 12 – 600 s, resulting at a given power density in energy densities in the range of 3.2– 156 kJ/m^2.

RESULTS

At 37^{o}C the positively charged lipid probe, TMA-DPH, reported in the head group region of the plasma membrane lipid bilayer of human peripheral blood lymphocytes an average fluorescence anisotropy of 0.28 ± 0.01. The corresponding values of the second rank order parameter and fluidity of membrane lipids are 0.87 ± 0.02 and 1.15 ± 0.02 respectively. The values of the same parameters at 25°C are 0.30 ± 0.02, 0.91 ± 0.03 and 1.10 ± 0.03 respectively.

The positively charged lipid probe, TMA-DPH, is known to localize in the polar head group region of the plasma membrane of intact cells. It reaches relatively rapidly the inner surface of the plasma membrane of human blood cells (within 20 min) and it remains localized there for a sufficiently long time [8]. TMA-DPH fluorescence anisotropy measurements performed in the time interval 3-10 min after dye loading always resulted in higher fluorescence anisotropy values: 0.29 ± 0.03 for human peripheral lymphocytes at 37 °C. We considered this

parameter characteristic for the lipid order in the outer surface region of the plasma membrane. Hence we considered that the TMA-DPH fluorescence anisotropy measurements performed in the time interval 20–40 min after dye loading report on the average lipid packing density proportional to the average equivalent membrane micro viscosity in the lipid head group region on the inner surface of the plasma membrane. In our hand the fluorescence anisotropy calculated in these conditions for peripheral blood lymphocytes at 37°C was 0.26 ± 0.02. Thus the obtained values document higher lipid order in the human peripheral lymphocytes plasma membrane outer surface region as compared to the inner surface region.

Under the influence of the 830 nm laser irradiation apparently significant changes occur in the lipid order parameters in both regions of the lymphocytes plasma membrane (Fig. 1).

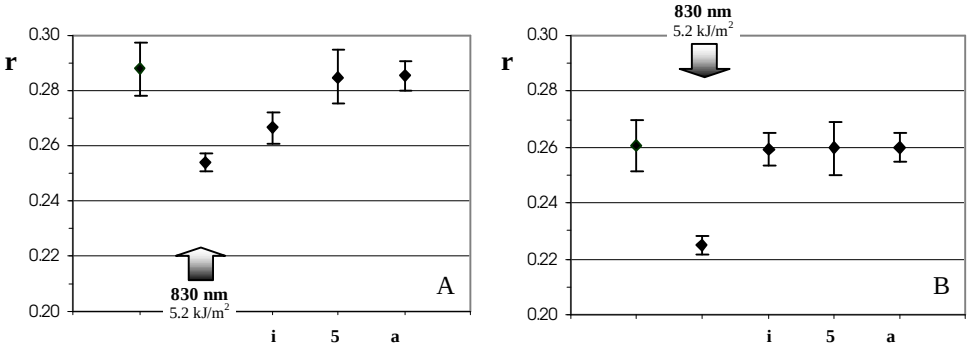

Fig. 1. – TMA-DPH fluorescence anisotropy (*r*) data, demonstrating apparently significant laser irradiation effects in human peripheral lymphocytes both in the outer (A) and in the inner surface polar head group region of plasma membrane (B).

Systematic decrease of anisotropy coefficients during laser irradiation is apparent in both polar head group regions of human lymphocytes plasma membrane. Laser irradiation induced changes reported by the TMA-DPH probe in the polar head group region of the plasma membrane bilayer, are more substantial in the inner surface region as compared to the outer surface region (Fig. 1.). Statistical significances of the observed fluorescence anisotropy differences are higher in the outer surface region of the plasma membrane.

Metabolically impaired cells with intact plasma membrane (more than 88% trypan blue excluding, data not shown) appear to be more sensitive to laser irradiation (Fig. 2. – Fig. 4.), as compared to metabolically intact (less than 20% altered membrane potential, higher than 92% viability, data not shown) controls.

Use of cell populations homogeneous as concerns the metabolic status and treatment parameters, allows disclosure of significant differences between the characteristics of groups exposed to different serum starvation periods. As a

general rule, the 830 nm laser induced late effects are more substantial and significant in the outer surface region of lymphocytes plasma membrane as compared to the inner surface – at least at the time scale covered by us.

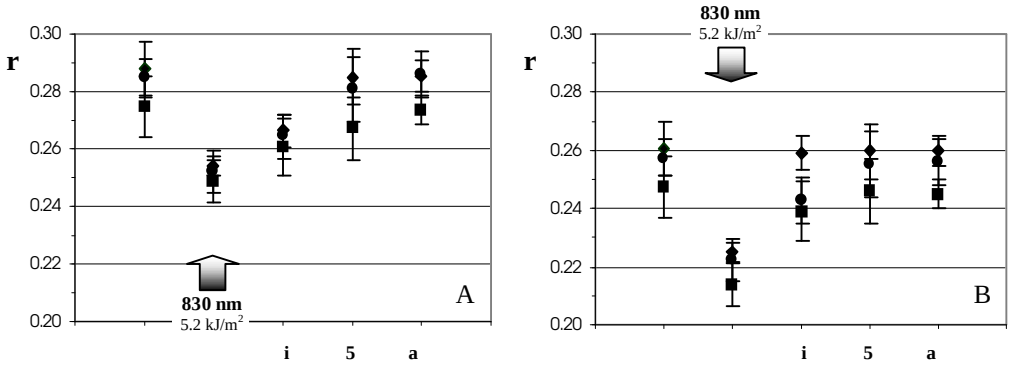

Fig.2. TMA-DPH fluorescence anisotropy (r) data, demonstrating apparently significant laser irradiation effects in the outer (A) and inner (B) polar headgroup region of human peripheral lymphocytes exposed to previous serum starvation periods of various lengthes (♦ - fresh samples, measurements made immediately after the transfer of cells in serum-free buffer; ●, ■ - cells kept 1h, respectively 4h at room temperature in serum-free buffer); parameters determined before, during, immediately after (i), 5' later (5), and 5-15' after (a) laser irradiation

Furthermore, mild metabolic impairment due to shorter periods of serum starvation, makes cells more sensitive to infrared laser radiation (Fig. 2), while serious metabolic impairment due to long period serum starvation (≥ 24h) causes loss of cells sensitivity to laser irradiation in all plasma membrane regions of human lymphocytes (Fig. 3).

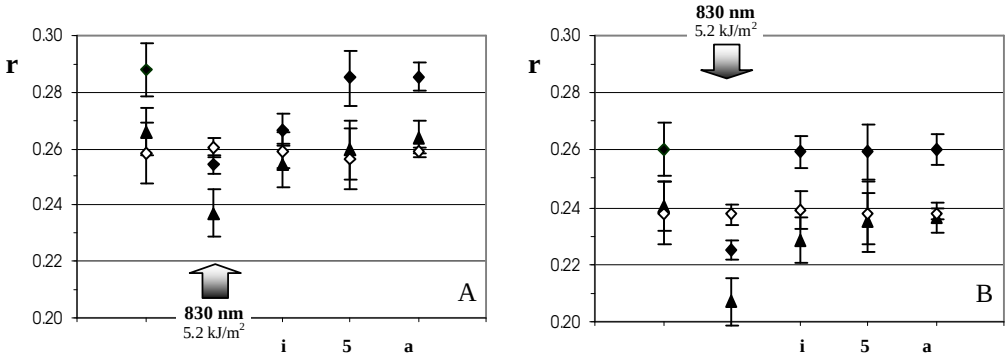

Fig.3. TMA-DPH fluorescence anisotropy (r) data, illustrating yet significant laser induced changes (▲) and apparent loss of laser irradiation effects (◊) in the outer (A) and inner (B) polar headgroup region of human peripheral lymphocytes exposed to previous long-term serum starvation (♦ - fresh samples, measurements made immediately after the transfer of cells in serum-free buffer; ▲, ◊ – cells kept 8h, respectively 24h at room temperature in serum-free buffer); parameters determined before, during, immediately after (i), 5' later (5), and 5-15' after (a) laser irradiation

Data concerning the corresponding lipid order parameter and membrane fluidity changes (Fig. 4.) illustrate the same increased sensitivity to laser irradiation of the lipid head group region of the plasma membrane.

Early effects are more important in the outer head group region of lymphocytes plasma membrane as compared to the inner one. One hour of metabolic does not lead to significant changes in the followed plasma membrane parameters in peripheral lymphocytes. In contrast sensitivity to laser irradiation of these lymphocytes is higher. After 4h of serum starvation changes in membrane parameters are already significant and sensitivity to infrared laser irradiation is additionally increased.

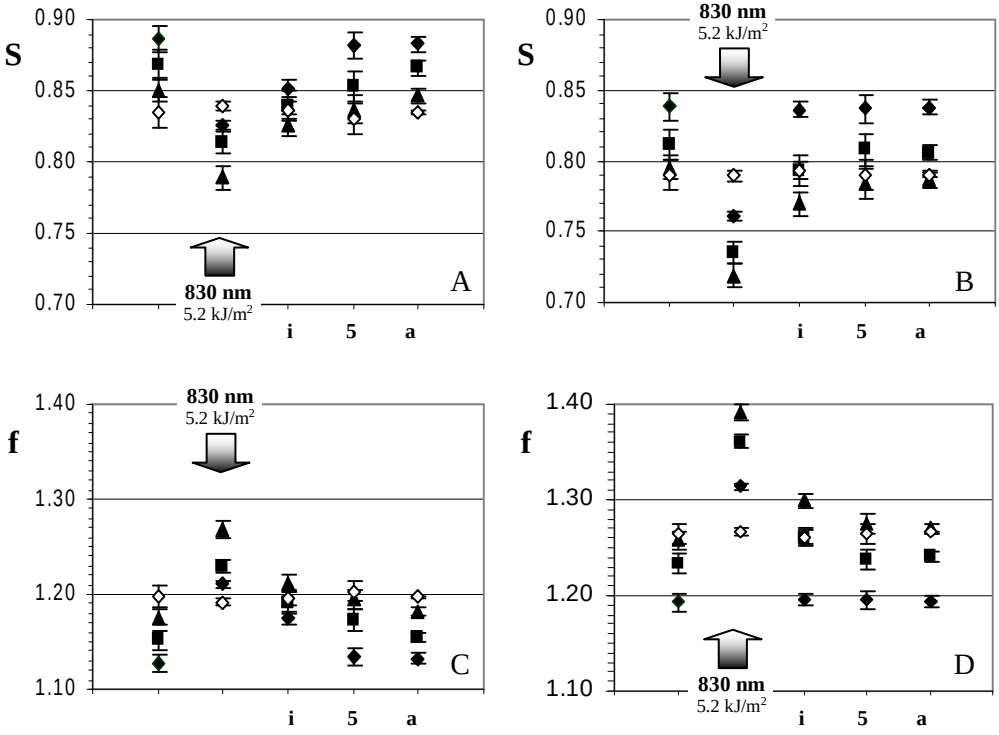

Fig.4. Data concerning membrane lipid order (S) and fluidity (f) in the outer (A,C) and inner (B,D) headgroup region of the plasma membrane lipid bilayer of peripheral lymphocytes exposed to various periods (♦ - 0, ■ – 4h, ▲ – 8h, ◊- 24h) of previous serum starvation, demonstrating metabolic modulation of laser irradiation effects; parameters determined before, during, immediately after (i), 5' later (5), and 5-15' after (a) laser irradiation

Eight hours of serum starvation effects are similar in both polar head group regions of lymphocytes plasma membrane (Fig. 4), however decrease in membrane lipid order is less substantial on the inner surface. After 24 hours of serum starvation lymphocytes membrane parameters are significantly changed, and infrared laser effects are not statistically significant at the level $p = 0.05$.

DISCUSSION AND CONCLUSIONS

The 830 nm light is recognized to be absorbed by the cytochrome C oxidase molecule in one of its mixed valence form The suspected mechanism of action is the change of the oxidation state of this respiratory chain terminal enzyme following the electronic excitation of its absorbing chromophore Cu_A [5–7]. Near infrared laser light is supposed to induce change in the of this redox-active center, thus promoting an enhancement of the electron transfer rate [5]. Absorbing entities in the plasma membrane of eukaryote cells are not known, however the cellular membrane is thought to be part of the photo signal transduction and amplification chain constituting secondary mechanisms of laser actions at cellular level [11–13]. 820 nm laser light induced enhancement of cell membranes adhesive properties was described [10, 14], and our previous data revealed metabolically modulated significant fluidity changes in various regions of human platelets and peripheral blood lymphocytes plasma membrane under the influence of the 680 nm laser light [15].

The TMA-DPH fluorescence anisotropy data presented here document significant changes in the average lipid packing density in the polar head group region of the human peripheral blood lymphocytes plasma membrane, induced by the 830 nm laser light. Though disclosure of the exact dependence of the effects on fluence rate, frequency of irradiation, and individual and total absorbed doses needs more investigation, modulation by the actual metabolic state of cells is evident. At our best knowledge our data are the first providing sound evidence for membrane fluidity changes under the influence of low power 830 nm laser light in human peripheral lymphocytes *in vitro*.

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REFERENCES

1. BAXTER, G.D., *Therapeutic lasers: theory and practice*, Churchill Livingstone, Edinburgh, 1994.
2. DYSON, M., Cellular and subcellular aspects of laser therapy. In: Progress in Laser Therapy, T. Ohshiro & G. Calderhead eds., John Wiley & Sons, Chichester 1990, pp. 221–224.
3. GEIGER, J., C. NOLTE, E. BUTT, S. O. SAGE, U. WALTER, Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonist-evoked calcium elevation in human platelets, Proc. Natl. Acad. Sci. USA, 1992, 89, 1031–1035.
4. KAMATA, H., H. HIRATA, Redox regulation of cellular signalling, Cell. Signal., 1999, 11, 1–14.
5. KARU, T.L., Photobiology of low power laser therapy, In: Laser Science Technology, an International Handbook, Vol 8, Letokov V.S. et al., eds., Harwood Academic Publishers, London, 1989.
6. KARU, T.L., The Science of Low Power Laser Therapy, Gordon and Breach, London, 1998.

7. KARU, T., Primary and secondary mechanisms of action of visible-to-near IR radiation on cells, Photochem. Photobiol., B, Biology, 1999, 49, 1–17
8. KARU T., Mechanisms Of Low-Power Laser Light Action On Cellular Level, In: Lasers in Medicine and Dentistry, Z. Simunovic ed., Vitgraf: Rijeka, 2000, pp. 97–125.
9. KARU, T.I., L.V. PYATIBRAT, G.S. KALENDO, Studies into the action specifics of a pulsed GaAlAs laser (λ = 820 nm) on a cell culture. I. Reduction of the intracellular ATP concentration: dependence on initial ATP amount, Lasers Life Sci., 2001, 9, 203–210.
10. KARU, T.I., L.V. PYATIBRAT, G.S. KALENDO, Studies into the action specifics of a pulsed GaAlAs laser (λ = 820 nm) on a cell culture. II. Enhancement of the adhesive properties of cellular membranes: dependence on the dark period between pulses, Lasers Life Sci., 2001, 9, 211–217.
11. KARU, T.I., Low-power laser effects, In: Lasers in Medicine, R.Waynant, Boca Raton eds. CRC Press, 2002, pp. 171–209.
12. KARU, T.I., Cellular mechanism of low power laser therapy: new questions., In: Lasers in Medicine and Dentistry, Vol. 3., Z. Simunovic ed., Vitgraf: Rieka, 2003, Ch.IV., pp.79–100.
13. KARU, T.I., Low power laser therapy, In: Biomedical Photonics Handbook, Tuan Vo-Dinh ed., CRC Press, Boca Raton 2003, Ch. 48, pp 48-1–48-25.
14. KARU, T.I., L.V. PYATIBRAT, G.S. KALENDO, Photobiological modulation of cell attachment via cytochrome c oxidase., Photochemical and Photobiological Sci. 2004, 3, 211–216.
15. KATONA, E., GY. KATONA, A. CĂPLĂNUȘI, I.O. DOAGĂ, DIANA IONESCU, R. MATEI, JUDIT HORVÁTH, E. TRUȚIA, E. TANOS, L. KATONA, Low power red laser irradiation effects, as seen in metabolically intact and impaired human blood cells, Romanian J. Biophys., 2003, 13, 2–17.
16. MATKO, J., J. SZÖLLÖSI, L. TRÓN, S. DAMJANOVICH, Luminescence spectroscopic approaches in studying cell surface dynamics, Quarterly Rev. Biophys., 1988, 21, 479–544.
17. MATKO, J., P. NAGY, Fluorescent lipid probes 12-AS and TMA-DPH report selective, purinergically induced fluidity changes in plasma membranes of lymphoid cells, J. Photochem. Photobiol. B., 1997, 40, 120–125.
18. MOORE, K., G. CALDERHEAD, The clinical application of low incident power density 830nm GaAlAs diode laser radiation in the therapy of chronic intractable pain: A historical and optoelectronic rationale and clinical review, J. Optoelectronics, 1991, 6, 503–520.
19. OKUMURA, M., T. OKUDA, T. NAKAMURA, YAJIMA M., Acceleration of wound healing in diabetic mice by basic fibroblast growth factor, Biol. Pharm. Bull., 1996, 19, 530–535.
20. POGREL, M.A., J.W. CHEN, K. ZHANG, Effects of low-energy gallium-aluminum-arsenide laser irradiation on cultured fibroblasts and keratinocytes, Lasers Surg. Med. 1997, 20, 426–432.
21. SZÖLLÖSI J., Fluidity/viscosity of biological membranes, In "Mobility and Proximity in Biological Membranes" Damjanovich, S., J. Szöllösi, L. Trón, M. Edidin eds., CRC Press, Boca Raton, 1994, pp. 137–208.
22. TUNER, J., L. HODE, Laser Therapy, Prima Books, Grängesberg, 2002.
23. VAN BLITTERSWIJK, W.J., R. P. VAN HOEVEN, B. W. VAN DER MEER, Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements, Biochim. Biophys. Acta, 1981, 644, 323–332.
24. WHEELAND, R.G., History of lasers in dermatology, Clin. Dermatol., 1995, 13, 3–10.
25. WHEELAND, R.G., Lasers for the stimulation or inhibition of wound healing, J. Dermatol. Surg. Oncol., 1993, 8, 747–752.
26. YU, W., J.O. NAIM, R.J. LANZAFAME, The effect of laser irradiation on the release of bFGF from 3T3 fibroblasts, Photochem. Photobiol. 1994, 59, 167–70.
27. YU, H.S., K.L. CHANG, C.L. YU, J.W CHEN, G.S. CHEN, Low-energy helium-neon laser irradiation stimulates interleukin-1 alpha and interleukin-8 release from cultured human keratinocytes, J. Invest. Dermatol., 1996, 107, 593–596.
28. YU, W., J.O. NAIM, R.J. LANZAFAME, Effects of photostimulation on wound healing in diabetic mice, Lasers Surg. Med., 1997, 20, 56–63.