LOW LEVEL LONG WAVELENGTH LASER IRRADIATION EFFECTS ON HUMAN T LEUKEMIC LYMPHOBLASTS MITOCHONDRIAL MEMBRANE POTENTIAL

I.O. DOAGĂ, E. RADU**, GYÖNGYVÉR KATONA***, TEOFILA SEREMET****, MARIA DUMITRESCU****, SÂNZIANA RADESI****, MIHAELA PISLEA****, JUDIT HORVÁTH*****, E. TANOS*****, L. KATONA****, EVA KATONA****#*

*Department of Biophysics, Dentistry Faculty, **Department of Molecular and Cellular Medicine, ***Department of Medical Biochemistry, and ****Department of Biophysics, Medical Faculty, "Carol Davila" University of Medicine and Pharmaceutics, Bucharest, Romania
*****LASEUROPA CO., Budapest, Hungary, *www.softlaser.hu*
#eva_katona@yahoo.com

Abstract. We investigated the effects of low power 680 nm far-red and 830 nm near-infrared laser light on noninjured and energy/nutrient restricted human acute T leukemic Jurkat cells mitochondrial membrane state. Nutrient restriction engendered an increase in mitochondrial membrane potential ($\Delta\Psi_m$) of the cell subpopulation with highly polarized mitochondrial membrane (HIGH), and the decrease in $\Delta\Psi_m$ of the subpopulation with low polarization mitochondrial membrane (LOW), changes induced by glucose deprivation with blockade of glycolysis being more substantial than those brought on by serum starvation. Glucose starvation also caused an increase in the relative magnitude of the HIGH subpopulation, while serum starvation resulted in a decrease in the relative magnitude of the HIGH subpopulation. Energy restriction induced by mild blockade of oxidative phosphorylation with low concentrations of cyanide caused an increase in the relative magnitude of the HIGH subpopulation, while high concentration cyanide poisoning promoted mitochondrial membrane depolarization. Low level near infrared laser irradiation caused a decrease in the HIGH subpopulation in control cell populations, partially reversed glucose starvation caused effects, while it had no significant influence on mitochondrial membrane state in serum starved cells. Low level far-red laser irradiation induced an increase in the relative magnitude of the HIGH subpopulation, and partially reversed mild and severe cyanide intoxication effects. The increase in the relative magnitude of the HIGH subpopulation caused by the intermediate level cyanide intoxication could also be enhanced by far-red laser radiation. In conclusion, our data show that soft laser irradiation significantly modulates the control and nutrient/energy restricted human T leukemia lymphoblasts mitochondrial membrane state in a dose and irradiation regime dependent manner.

Key words: AlGaInP/GaAs laser, metabolic poisoning, mitochondrial membrane polarization, JC-1, apoptosis.

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INTRODUCTION

Nowadays it is generally accepted that the mitochondria, besides their important task of energy production by oxidative phosphorylation, also play a crucial role in the control of cells death. However, in spite of plethora of high class research papers concerning assignments of various mitochondrial processes in cells physiological and pathological behavior [1, 3–5, 8, 14, 16, 31, 35, 37–38, 40–42, 44–45, 50, 52–53, 55, 60–61, 68] and excellent reviews aiming to provide an ordered vision of function regulation and mitochondrial calcium dynamics [9–10, 48–49, 54], mitochondrial cell death control [9, 34] or mitochondrial motility, morphology, fission/fusion and mitochondrial network dynamics [43, 46], particulars of the involved molecular processes, are yet not fully elucidated.

Low Level Laser Therapy (LLLT), used as part of physiotherapy in most countries, has proved to be very efficient in relieving pain, reducing inflammation and improving wound healing [12, 15, 58–59, 63–65, 67]. However, there has been little or no standardization in the application of phototherapy, studies disclosing the dependence of the expected desirable effects on well defined and reproducible irradiation parameters are scarce, and treatment procedures are so far empirical [15]. In spite of the large number of first-rate scientific papers [2, 6, 11, 17, 19, 21, 25–26, 32–33, 36, 39, 47, 51, 56–57, 62, 66], and high-class review articles and books [18, 20, 22–24] occurring in the field of biophotonics, knowledge of molecular and cellular mechanisms involved in the photosignal transduction and amplification cascade is yet fragmentary. Clearly further scientific studies on LLLT are compulsory, so that this therapy becomes more widely accepted, and be part of evidence based medicine.

Activation of cell metabolism through the respiratory chain is a widely accepted basic primary mechanism of long wavelength laser light action [24], and we previously reported sensitive metabolic modulation of low level far-red and near infrared laser irradiation membrane [27–29] and cellular [30] effects. In this study we focused on investigation of low level laser irradiation effects on an important parameter for mitochondrial functionality and indirect indicator of the energy status of the cell, the mitochondrial membrane potential ($\Delta\Psi_m$). Using 680 nm far-red and 830 nm near infrared laser light, we found that doses and irradiation regimes of therapeutic significance sensitively modulate control and nutrient/energy restricted human T leukemia lymphoblasts mitochondrial membrane polarization. Identifying by flow cytometry cell subpopulations with mitochondrial membranes of preponderantly high (HIGH) and low polarization (LOW), respectively, we disclosed changes induced by energy/nutrient restriction and/or by low level laser irradiation in $\Delta\Psi_m$ and in the relative magnitude of these subpopulations, as follows: (i) glucose starvation alone induced increase in $\Delta\Psi_m$ of the HIGH subpopulation, decrease in $\Delta\Psi_m$ of the LOW subpopulation, and increase in the relative magnitude of the HIGH subpopulation; (ii) serum starvation induced increase in $\Delta\Psi_m$ of the HIGH subpopulation, decrease in $\Delta\Psi_m$ of the LOW

subpopulation, and decrease in the relative magnitude of the HIGH subpopulation; (iii) energy restriction induced by mild blockade of oxidative phosphorylation with low concentrations of cyanide caused increase in the relative magnitude of the HIGH subpopulation; (iv) high concentration cyanide poisoning promoted increase in the LOW subpopulation; (v) low level near infrared laser irradiation caused a decrease in the control cells HIGH subpopulation, while low level far-red laser irradiation induced an increase in the relative magnitude of this subpopulation; (vi) low level near infrared laser irradiation partially reversed the glucose starvation caused effects, but had no effect on the serum starved cells mitochondrial membrane state; (vii) low level far-red laser irradiation induced an increase in the relative magnitude of the HIGH subpopulation, partially reversed mild and severe cyanide intoxication effects, but could also enhance intermediate cyanide intoxication caused changes.

MATERIALS AND METHODS

Chemicals, supplements and staining kits: Sterile DMSO Trypan Blue, and standard RPMI 1640 culture medium were purchased from SIGMA CHEMICAL Co., Dulbecco's Phosphate buffered Saline (PBS) 10× from BIOCHROME, while supplements (FCS, foetal calf serum, and antibiotics/glutamine) from GIBCO/ INVITROGEN. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) was from INVITROGEN/MOLECULAR PROBES (T-3168), while all other chemicals were of the best research grade available.

Stock solutions, and modified culture media: JC-1 was dissolved in DMSO, realizing stock solutions of 400μM. Modified culture media were prepared from standard RPMI medium: complete medium supplemented with 10% FCS (M), serum deprived medium (M0 – M without FCS), 2-deoxyglucose containing glucose deprived medium (D – M without D-glucose, with 5 mM 2-deoxyglucose) cyanide-containing medium (MXCN – M containing XmM NaCN).

Cell culture: The human acute T leukemic Jurkat cell line was maintained in a 5% CO_2 humidified (80%) atmosphere at 37 °C, in standard RPMI 1640 medium (SIGMA R6504) supplemented with 2 g/l sodium bicarbonate, 10% heat inactivated foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (M) or in modified media (D, MXCN), and passaged every second day.

Nutrient and/or energy restriction: Cells were deprived of particular supplies (serum, glucose) and/or exposed to conditions of blockade of specific energy production mechanisms (glycolysis, oxidative phosphorylation).

Serum starvation: Cells were resuspended in M0 medium and incubated in a 5% CO2 humidified (80%) atmosphere at 37 °C for the indicated time periods (12–24 h).

Glucose starvation (glucose deprivation with blockade of glycolysis): Cells were resuspended in D medium and incubated in a 5% CO_2 humidified (80%) atmosphere at 37°C for the indicated time periods (12–72 h).

Blockade of mitochondrial respiratory chain (hindrance of oxidative phosphorylation): Cells were cultured in a 5% CO_2 humidified (80%) atmosphere at 37 °C in 100 μM – 5 mM NaCN-containing media (MXCN) for various time periods (12–96 h).

The irradiation sources were AlGaInP/GaAs based semiconductor lasers used in the medical practice, PHILIPS CQL806D and SONY SLD202-D3, with emission wavelengths and nominal powers of 680 nm, 25 mW, and of 830 nm, 50 mW, respectively.

Sample irradiation regimes and laser irradiation doses: Sample irradiation was performed in the laminar flow, with sources placed at a 10 cm height from the upper surface of cell suspensions, in 2 consecutive positions as to cover with the expanded laser speckle the whole surface of the suspension-containing Petri dishes rotating at a speed of 0.5 s^{-1}.Duration of treatment varied between 0–600 s, giving at the average incident power densities in the expandor–increased speckle area of approximately 45 $mW.cm^{-2}$ and 100 $mW.cm^{-2}$ for the red and infrared laser beam, respectively, single incident doses equivalent with $(1\div5)\times10^{12}$ photons/cell or ~ (0.2 –1.5) μJ/cell. Irradiation regimes of once or twice per day, or every second day with these single doses gave total irradiation doses of ~ (1–15) μJ/cell.

Cell viability assessment: After 16–22 h of serum starvation, cells in metabolic quiescence were resuspended in various media at a density of 0.8–0.9×10^6 cells/ml, distributed in 3.5 cm diameter Petri dishes, and further cultured for 18–72 h. At indicated time points cells in aliquots of 50 μl were counted in a Buerker-Tuerk hemocytometer. Cell viability was tested by the Trypan Blue exclusion method.

Mitochondrial Membrane Potential ($\Delta\Psi_m$) *Assessment:* $\Delta\Psi_m$ was determined by flow cytometry using JC-1, a cationic dye that exhibits potential-dependent accumulation and formation of red fluorescent J-aggregates in mitochondria, while the JC-1 monomer produces green fluorescence. Changes in the plasma membrane potential do not affect the JC-1 status [7]. Increase in red fluorescence intensity indicates a higher amount of dye in aggregate form, accumulated in mitochondria with highly polarized membranes. An increase in green fluorescence indicates the release of dye from mitochondria with depolarized membranes. However, these individual fluorescence signals might be affected by other factors like mitochondrial size, shape, and density. The ratio of red to green fluorescence, not dependent on any other factor, is taken as measure of $\Delta\Psi_m$, reflecting the cells energization state.

Dye loading and flow cytometry measurements: At different time point of exposure to various nutrient/energy restriction conditions, irradiated or not irradiated, cells were incubated for 15 min at 37 °C in a 5% CO_2 atmosphere with 2 μM JC-1. Following dye loading, cells, washed twice with ice-cold PBS, and

resuspended in PBS, were immediately analyzed on a Becton Dickinson FACS Calibur flow cytometer, using Cellquest 3.0 as dedicated data acquisition software. Fluorescence was determined using excitation by a 488 nm argon-ion laser. The fluorescence of JC-1 monomer (green) and of the J-aggregates (red) was detected separately in FL1 (emission, 525 nm) and FL2 (emission, 590 nm) channels, respectively. Typically, fluorescence data on 8000–16000 single cells were collected. The freeware WinMDI 2.8 (written by Dr. Joe Trotter, Scripps Research Institute, La Jolla, CA, USA, http://www.bio.umass.edu/mcbfacs/flowcat.html) was used for data analysis.

Forward and side scattering signal analyses: Forward scatter signal (FSC) is generally related to cell size. Though this is not always true, in our case – also correlated with microscopy data – was safe to accept this simplification. Side scattering signal (SSC) intensity was correlated with cell granulosity. Subpopulations distinguished on FSC/SSC dot/density or contour plots were expressed as percentages of the total population.

JC-1 FL1 and FL2 signal analyses: Total fluorescence signals or fluorescence signals gated for cell subpopulations, defined following FSC/SSC data analysis, were analyzed. Subpopulations distinguishable / defined on FL1/FL2 dot/density or contour plots were expressed as percentages of the total analyzed population. In order to follow up changes induced in the polarization state of mitochondrial membranes in various cell subpopulations, and in relative sizes of these subpopulations, we defined two regions in the FL1/FL2 plot: (i) a region where the Red/Green fluorescence ratio FL2/FL1 > 1 – cells in region constituting a subpopulation with highly polarized mitochondrial membranes (HIGH); (ii) a region where the Red/Green fluorescence ratio FL2/FL1 < 1 – cells in a region constituting a subpopulation with mitochondrial membranes of low polarization (LOW). Average Red/Green fluorescence ratio calculated for these subpopulations was considered a measure of $\Delta\Psi_{HIGH}$ and $\Delta\Psi_{LOW}$, respectively.

Statistical Analysis: Fluorescence intensity ratios and relative cell subpopulation sizes were obtained as means calculated from at least 3 independent assessments (S.D. ≤ 12%). Unpaired analysis of data series obtained by measurements made on cells various time periods after their transfer in various modified media, irradiated and/or not irradiated, was performed by Student's t-test (two-tailed). P-values less than 0.05 were regarded as indicating statistical significance.

RESULTS

Analysis of flow cytometry data allowed identification of several cell subpopulations.

Typical forward (FSC) and side (SSC) scattering data are illustrated in Fig. 1, while the corresponding JC-1 data are shown in Fig. 2. Clearly two cell subpopulations can be distinguished on FSC/SSC plots, their relative magnitude

being dependent on the viabilities of analyzed samples, which are 97.1% and 95.1%, respectively, in the illustrated cases. Subpopulation I of increasing granulosity, shrinken cells was named "Dead", because of their failure to respond to any of applied treatments (laser irradiation or any condition of nutrient/energy restriction). Correlated data analysis shows that this subpopulation also represents the major part of cell subpopulation fluorescing very weakly in red, having mitochondrial membranes of very low polarization (cells in regions 3 and 4 on FL1/FL2 plots in Fig. 2). In subsequent analyses concerning laser and nutrient/energy restriction caused effects, JC-1 FL1/FL2 data were gated on the so-called "Live" subpopulation of responding cells (Fig. 2C and 2D).

Fig. 3. illustrates typical LOW and HIGH subpopulations defined in order to easily analyze treatment effects on "Live" cells.

Figures 4–7 illustrate nutrient/energy restriction caused effects on individual fluorescence signals, Red/Green ratios, and subpopulations relative sizes.

Glucose starvation caused decrease in the average green fluorescence, increase in the average red fluorescence of the HIGH subpopulation, and increase in the average Red/Green fluorescence ratio of this subpopulation (Fig. 4A and 4C). At the same time average green fluorescence of the LOW subpopulation increased, while the average red fluorescence, and the Red/Green fluorescence ratio of this subpopulation decreased (Fig. 4B and 4C). This treatment also caused increase in the relative size of the HIGH subpopulation (Fig. 4C). Serum starvation induced increase in the Red/Green fluorescence ratio of the HIGH subpopulation, decrease of the same parameter of the LOW subpopulation, and decrease in the relative magnitude of the HIGH subpopulation (Fig. 4C).

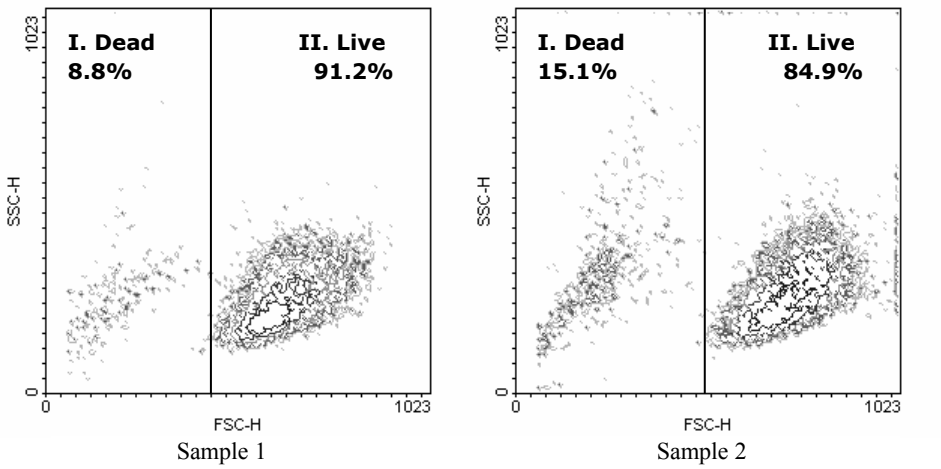

Fig. 1. Typical forward scatter (FSC)/side scatter (SSC) contour plots. Two different samples of Jurkat cells with Trypan Blue method given viabilities of 97.1% (Sample 1), and 95.1% (Sample 2), respectively. The subpopulation named "Dead" represents the major part of cell subpopulation with mitochondrial membranes of very low polarization, occurring in regions 3 and 4 in Fig. 2.

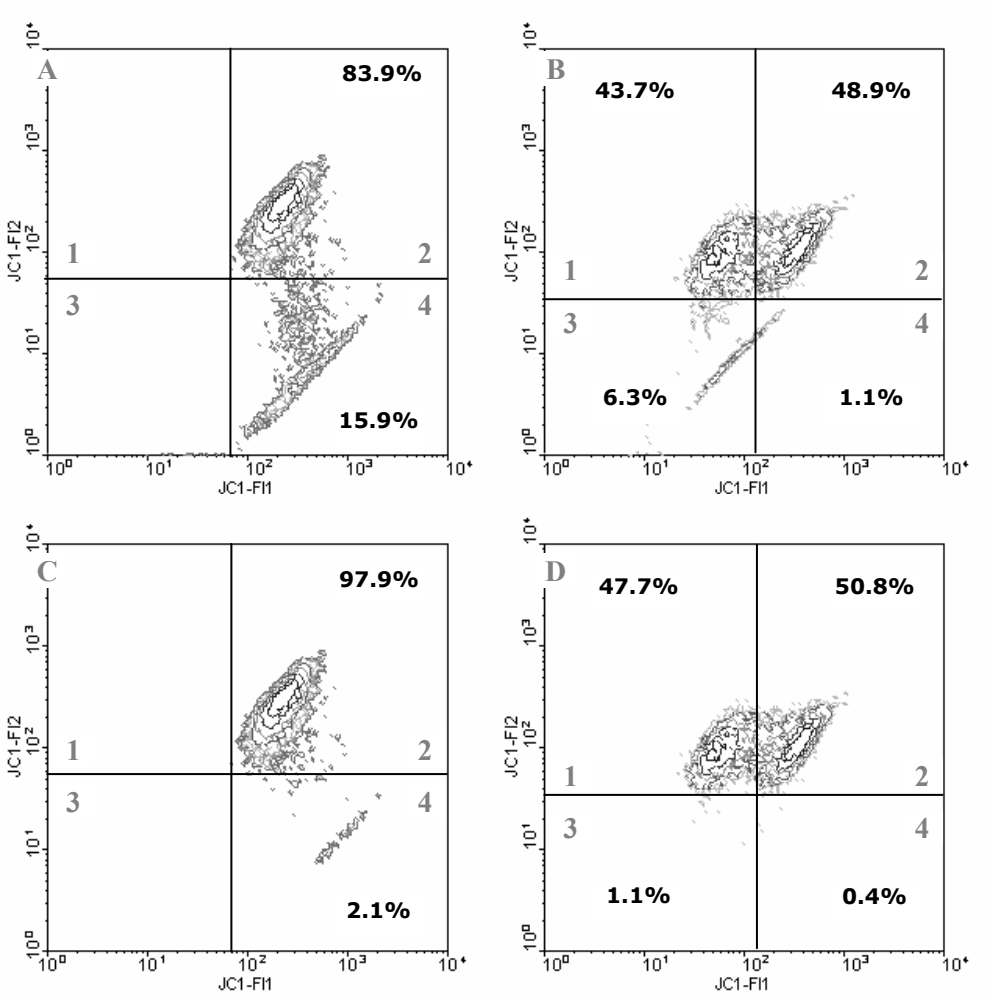

Fig. 2. Typical JC-1 FL1/FL2 contour plots. Two different samples of Jurkat cells with Trypan Blue method given viabilities of 97.1% (Sample 1), and 95.1% (Sample 2), respectively. All cells are included in the A and B plots. Cell subpopulations of low level red fluorescence in regions 3 and 4 mainly correspond to the subpopulation I named "Dead" on the FSC/SSC plots in Fig. 1C and D plots are gated on subpopulation II in Fig. 1 named "Live" of cells responding to various treatments.

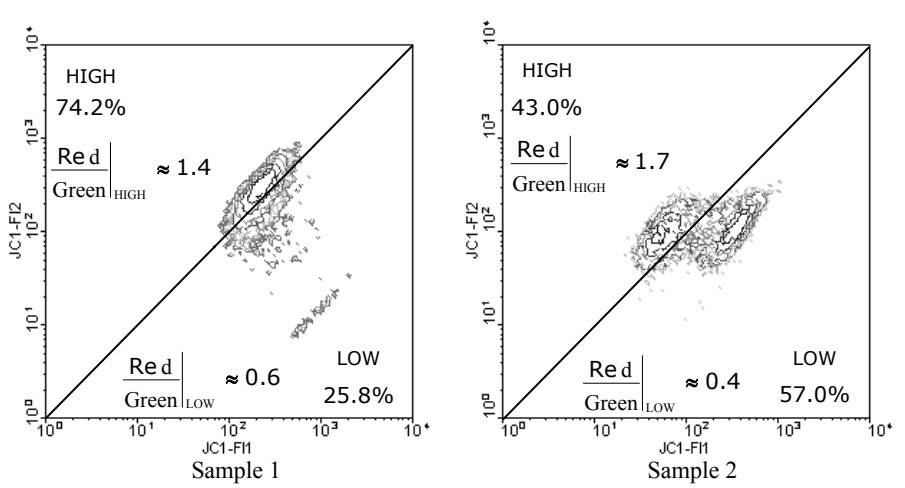

Fig. 3. Definition of subpopulations HIGH and LOW on typical JC-1 FL1/FL2 contour plots gated on "Live" cell populations. Two different samples of Jurkat cells with Trypan Blue method given viabilities of 97.1% (Sample 1), and 95.1% (Sample 2), respectively. Plots are gated on subpopulation II in Fig. 1 named "Live", consituted of cells responding to various treatments. The ratios Red/Green are giving the subpopulations characteristic $\Delta\Psi_m$ in arbitrary units.

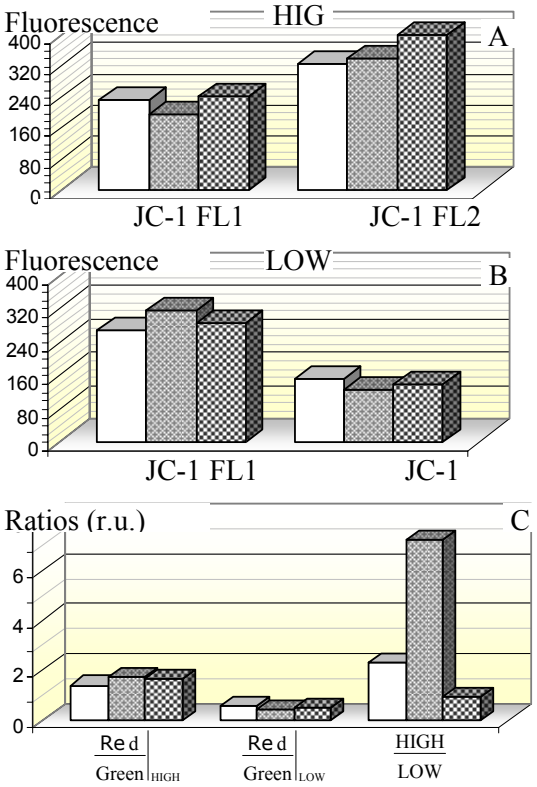

Fig. 4. Nutrient restriction induced changes in Jurkat cells mitochondrial membrane state. Changes in JC-1 fluorescences of HIGH (A) and LOW (B) subpopulations, in Red/Green fluorescence ratios (C), and in relative sizes of HIGH/LOW subpopulations (C) in control (□), glucose starved (▨), and serum starved (▩) cells.

Energy restriction induced by mild blockade of oxidative phosphorylation with low concentrations of cyanide (0.1–1 mM), causing an increase in the relative magnitude of the HIGH subpopulation, produced apparent hyperpolarization (Fig. 5), while higher concentrations of cyanide (5 mM) promoted an increase in the LOW subpopulation, causing mitochondrial depolarization (see also Fig. 8).

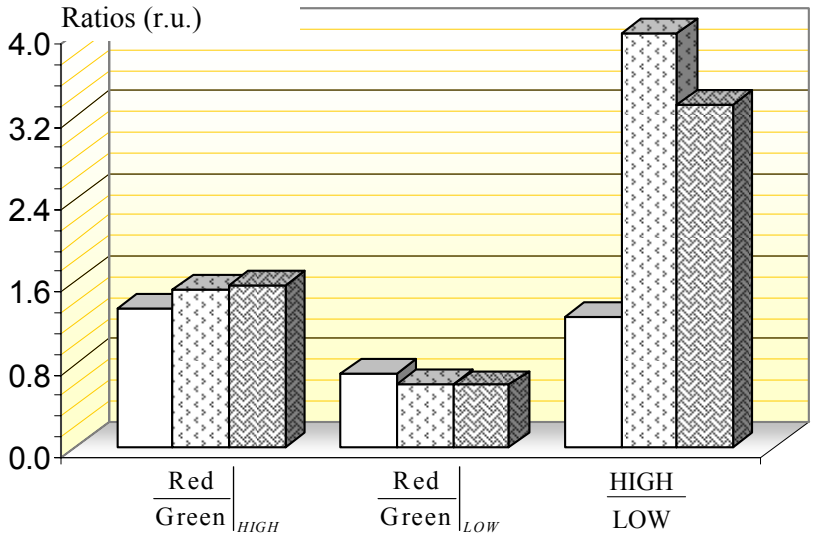

Fig. 5. Energy restriction induced changes in Jurkat cells mitochondrial membrane state. Changes in JC-1 fluorescences of HIGH (A) and LOW (B) subpopulations, in Red/Green fluorescence ratios (C), and in relative sizes of HIGH/LOW subpopulations (C) in control (□), 0.1 mM NaCN treated (▨), and 1 mM NaCN treated (▩) cells. Treatment period: 46 h, after previous 22 h serum starvation.

Figures 6–8 illustrate low level FR (680 nm) and NIR (830 nm) laser irradiation effects on subpopulations JC-1 Red/Green fluorescence ratios and relative sizes in control and nutrient/energy restriction caused stress exposed cells.

The low level near infrared laser irradiation caused a decrease in the control cells HIGH subpopulation, partially reversed the glucose starvation caused effects, but had no effect on serum starved cells mitochondrial membrane state (Fig. 6).

The low level far-red laser irradiation induced an increase in the relative magnitude of control cells HIGH subpopulation, partially reversed mild and severe cyanide intoxication effects, but could also enhance intermediate cyanide intoxication caused changes (Fig. 7 and Fig. 8).

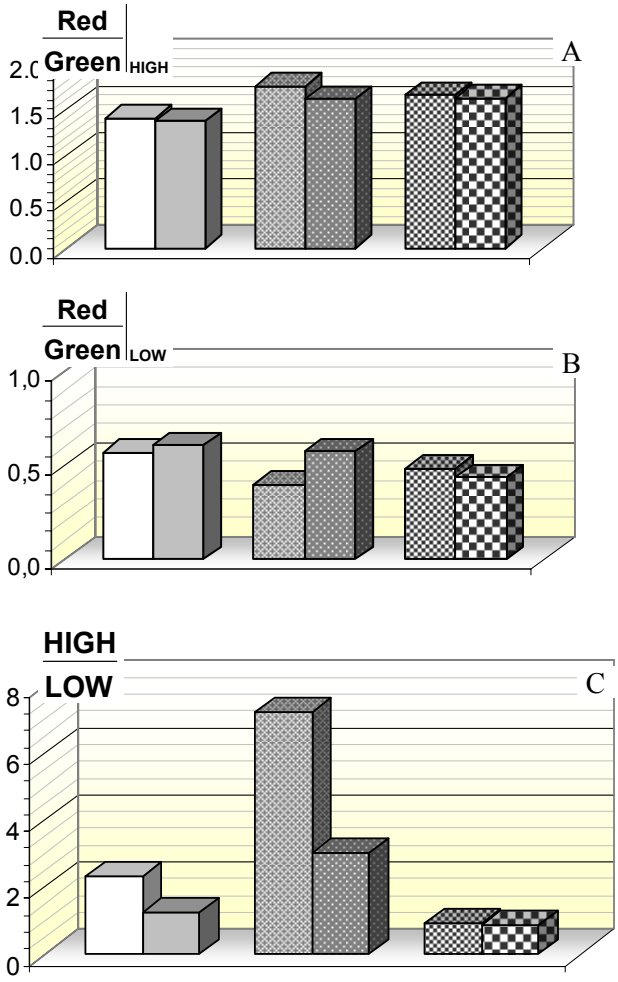

Fig. 6. Laser effects on mitochondrial membranes, seen in control and nutrient restricted Jurkat cells. Changes induced by a dose of ~ 1 μJ/cell of 830 nm laser light in the JC-1 Red/Green fluorescence ratios of HIGH (A) and LOW (B) subpopulations, and in relative sizes of HIGH/LOW subpopulations (C), observed in control (□, ▩), glucose starved (▨, ▩), and serum starved (▩, ▩) cells. Samples exposed to 10 h nutrient restriction conditions, irradiated or not, and analyzed after 20 h of subsequent culturing in the same modified media.

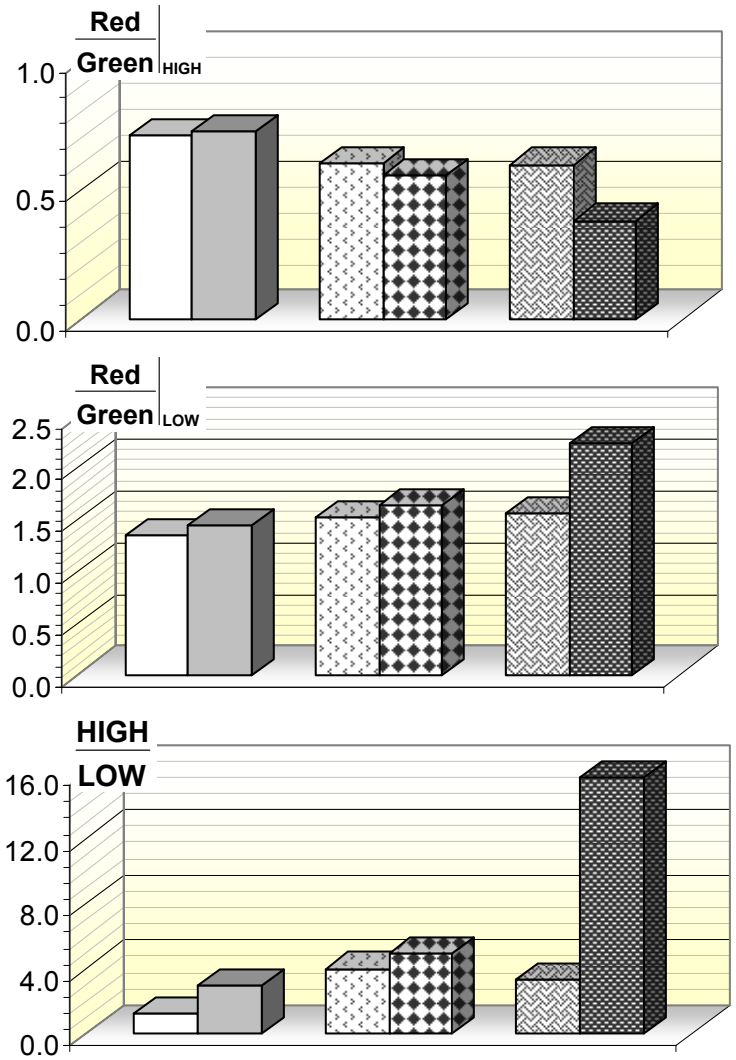

Fig. 7. Laser effects on mitochondrial membranes, seen in control and energy restricted Jurkat cells. Changes induced by a dose of ~ 0.9 μJ/cell of 680 nm laser light in the JC-1 Red/Green fluorescence ratios of HIGH (A) and LOW (B) subpopulations, and in relative sizes of HIGH/LOW subpopulations (C), observed in control (□), 0.1 mM NaCN (▨), and 1 mM NaCN treated (▩) cells. Samples exposed to energy restriction caused by blockade of the respiratory chain 22 h, irradiated or not, and analyzed after 24 h of subsequent culturing in the same modified media. Represented entities are averages of values obtained in 3 independent measurements; S.D. ≤ 12%.

The low level near infrared laser irradiation caused a decrease, while the low level far-red laser irradiation induced an increase in the relative magnitude of

control cells HIGH subpopulation, partially reversed mild and severe cyanide intoxication effects, but could also enhance intermediate cyanide intoxication caused changes (Fig. 8).

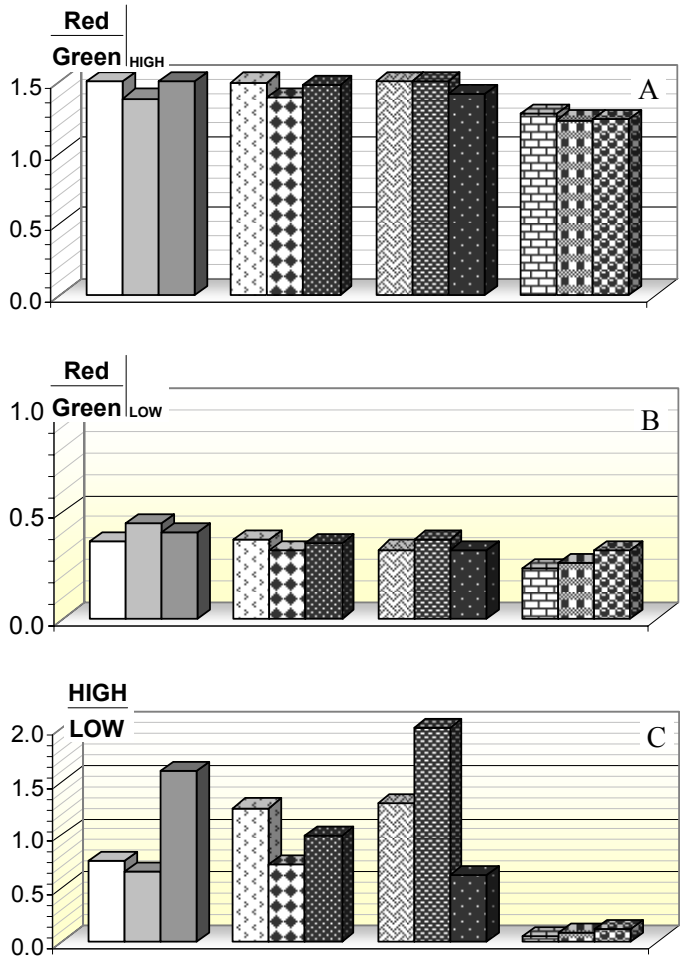

Fig. 8. Laser effects on mitochondrial membranes, seen in control and energy restricted Jurkat cells. Changes induced by doses of ~ 0.88 μJ/cell ( ) and ~1.32 μJ/cell ( ) of 680 nm FR laser light, in the JC-1 Red/Green fluorescence ratios of HIGH (A) and LOW (B) subpopulations, and in relative sizes of HIGH/LOW subpopulations (C), observed in control ( ), 0.1 mM NaCN ( ), 1 mM NaCN ( ), and 5 mM NaCN treated ( ) cells. Samples exposed to 2 h energy restriction caused by blockade of the respiratory chain, irradiated every day ( ) or every second day ( ) with single doses of ~0.44 μJ/cell or not irradiated ( ), and anlyzed after 39 h of subsequent culturing in the same modified media. Represented entities were calculated as averages of values obtained in 3 independent measurements; S.D. ≤ 12%.

DISCUSSION AND CONCLUSIONS

The 680 nm and 830 nm lights are recognized to be absorbed by the oxidized forms of the so-called Cu_B and Cu_A redox active centers of the mammalian mitochondrial respiratory chain terminal enzyme cytochrome C oxidase [13, 18, 20, 24]. The suspected mechanism of action is further oxidation of this respiratory chain terminal enzyme due to the electronic excitation of its absorbing chromophores, and following enhancement of the electron transfer rate [24], which may lead to increase in $\Delta\Psi_m$.

Absorbing entities in the plasma membrane of eukaryote cells are not known, however cellular membranes are thought to be part of the photosignal transduction and amplification chain constituting secondary mechanisms of laser actions at cellular level [18, 24]. We [27–29], and others [23, 26], repeatedly reported significant, cellular redox/energetic state dependent membrane effects of low power red and/or infrared laser light of various wavelengths in various cells.

Mitochondrial membranes electrical potential is a key feature whose maintenance in the normal range, as well as alterations, are deeply involved in mitochondrial functions and dysfunction.

The flow cytometric JC-1 data presented here document significant soft laser irradiation induced changes both in the mitochondrial membrane potential and in the relative size of cell subpopulations characterized by high and low electrical polarization, respectively, both in control and in nutrient/energy restricted Jurkat cells. Though disclosure of the exact dependence of the effects on fluence rate, frequency of irradiation, and individual and total doses, needs more investigation, cell state dependence of laser effects is evident. At our best knowledge our data are the first providing sound evidence for changes occurring in the mitochondrial membrane state under the influence of low power 680 nm far-red and 830 nm near infrared laser light in metabolically intact and nutrient/energy restricted human T leukemia lymphoblasts *in vitro*.

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