LOW LEVEL LONG WAVELENGTH LASER IRRADIATION EFFECTS ON HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS VIABILITY

A. IFTIME#, E. RADU**, EVA KATONA**

*Department of Biophysics, Medical Faculty, "Carol Davila" University of Medicine and Pharmaceutics, Bucharest, Romania, #e-mail: ad_if@yahoo.co.uk
**Department of Molecular and Cellular Medicine, Medical Faculty, "Carol Davila" University of Medicine and Pharmaceutics, Bucharest, Romania

Abstract. We investigated the effects of low power 680 nm far-red (FR) and 830 nm near-infrared (NIR) laser light on control and energy restricted human peripheral blood mononuclear cells (PBMC) viability, and apoptosis rate in presence and absence of growth factors. Data obtained both by microscopy and by flow cytometry demonstrated significant changes in the explored parameters. Energy restriction induced by blockade of oxidative phosphorylation with various concentrations of cyanide caused cell microenvironment, toxicant concentration, and intoxication duration dependent viability decrease, and apoptosis induction. Multiple NIR laser irradiation of the whole PBMC population in absence of growth factors offers slight transient protection against viability decrease during mid intoxication periods, but mildly enhances apoptosis induction in intoxicated cells after longer periods of exposure. In same conditions, viability and apoptosis induction in control cells are not significantly affected. However, when the Ly subpopulation is irradiated separately alone, NIR laser light drastically reduces viability of the whole PBMC population in control samples, while still offers transient protection to cyanide intoxicated cells. Low dose FR irradiation does not significantly affect the viability of control, mildly or highly intoxicated human PBMC in absence of growth factors, but drastically enhances toxicity of mid cyanide concentrations. In presence of growth factors, either of the irradiation regimes are applied, neither NIR nor FR irradiation has any effect on control human PBMCs viability. In same conditions NIR irradiation of the whole PBMC population induces short term viability increase and long term protection against cyanide induced viability decrease. NIR irradiation of the Ly subpopulation only offers mild transient protection against cyanide toxicity caused viability decrease of human PBMC, while FR irradiation in presence of the same cyanide concentration enhances this viability decrease. Applying the same irradiation regime FR irradiation can offer mild transient protection against cyanide toxicity at much lower cyanide concentrations.

Key words: low level laser therapy (LLLT), cyanide-intoxication, photobiomodulation, apoptosis induction.

INTRODUCTION

Last decades clinical practice convincingly demonstrated efficiency of Low Level Laser Therapy (LLLT) in pain relief, wound healing, reduction of

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inflammation and of post-trauma tissue damage and neurological deficits as well as in prevention and treatment of chemo- and radiotherapy induced stomatotoxicity [6, 8, 10, 20, 24, 27, 31] Animal studies also demonstrated LLLT efficiency in overcoming intoxications and promotion of tissue repair and restoration of neural function [2, 7, 21, 25]. Furthermore, evidence gathered in *in vitro* studies demonstrates significant cellular effects of various low level long wavelength laser irradiation protocols. Enhancement of cell adhesive properties, promotion of various cells survival / proliferation, modulation of cell signalling pathways and gene expression, reversal of various agents caused toxicity and restoration of function of intoxicated neural cells [1, 3, 9, 11–16, 22–23, 28, 30, 32–35] were evidenced as beneficial effects. Nevertheless understanding of action mechanisms of low level long wavelength laser irradiation still remains fragmentary, and many details of molecular processes mediating the photosignal transduction and amplification cascade still await elucidation.

We previously reported significant changes induced by the 680 nm FR and the 830 nm NIR laser irradiation in the plasma membrane properties of human blood cells [17–19], underlining metabolic modulation of the observed changes. We also revealed low level FR and NIR irradiation induced changes in non-injured and energy/nutrient restricted Jurkat cells viability, survival/proliferation, apoptosis rate, and cell cycle progression [26] mitochondrial membrane state [4] and mitochondrial network size and shape [5], as well as significant photobiomodulation of quercetin cytotoxicity in human T leukemia lymphoblasts [29]. In this study we focused on investigation of low level laser irradiation effects on human peripheral blood mononuclear cells (PBMC) viability and apoptosis induction in control and cyanide intoxication induced stress conditions, in presence and in absence of growth factors.

MATERIALS AND METHODS

CHEMICALS, SUPPLEMENTS AND STAINING KITS

Sterile DMSO, the standard RPMI 1640 culture medium (R6504, lyophilized powder, 1 vial/L), NaCN, the colorant Trypan Blue (TB, T8154, GM 960.81, 0.4% solution in 0.81% NaCl and 0.06% Na_2HPO_4) were purchased from SIGMA CHEMICAL Co., Dulbecco's Phosphate buffered Saline (PBS) 10× from BIOCHROME, while the supplements (FCS, foetal calf serum, EU tested, and antibiotics/glutamine) from GIBCO/INVITROGEN. The Annexin V-FITC kit was from BECKMAN COULTER (IM 3546), while 7-aminoactinomycin D (7-AAD (559925, 0.5 mg/mL suspension in PBS with protein stabilizer and 0.09% sodium azide) were purchased from BD PHARMINGEN. All other chemicals were of the best research grade available.

STOCK SOLUTIONS

Annexin V binding buffer 10× was provided within the Annexin V-FITC kit, while the NaCN stock solution (20 N) was prepared in 0.1 NaOH.

CULTURE MEDIA

The standard RPMI 1640 medium was supplemented with 2 g/L sodium bicarbonate, 10% heat inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and pH was adjusted to 7.2 (complete medium, M). Modified culture media were prepared from the complete medium FCS (M), adding growth factors: medium with growth factors (M_gf – M with 5 ng/mL IL4 and 10 ng/mL GMCSF and/or 1 ng/mL IL2). The cyanide containing media were prepared from the complete medium (M), adding the appropriate quantity of NaCN stock solution (MX – M containing XmM NaCN).

CELLS

Human Peripheral Blood Mononuclear Cells (PBMC) were obtained from venous blood collected in a PCD buffer, of drug-free healthy volunteer donors. All donors signed an informed consent, and protocols were approved by the University Ethics Committee. PBMC were separated from the venous blood by the standard Ficoll-Hypaque density gradient centrifugation, obtaining ~ 15% adherent cells constituted of monocytes (Mo), and ~ 65–70% CD3+ T cells (CD3+Ly). Monocyte-derived dendritic cells (DC) occurred spontaneously or under the influence of growth factors/cytokines/T cells.

CELL CULTURE

Human PBMCs were cultured in Petri dishes of variable surfaces, in standard conditions (37 ºC, 80% humidity, 5% CO_2) in complete medium supplemented (M_gf) or not (M) with growth factors, and containing (MX or M_gf-X) or not various concentrations of NaCN. Non-adherent cells constituted mainly of human peripheral blood lymphocytes (Ly) and adherent cells, constituted of monocytes/dendritic cells (MoDC), were cultured together or virtually alone.

ENERGY RESTRICTION

Energy restriction was realized by hindrance of oxidative phosphorylation through cytochrome C oxidase inhibition caused blockade of the mitochondrial respiratory chain, incubating cells in media containing various concentrations

(0.1–10 mM) of NaCN (MX) in the humidified (80%) 5% CO_2 atmosphere at 37 °C for various time periods.

CELL SAMPLES

Cell samples were suspensions constituted of PBMC, Ly and/or MoDC cells cultured in standard media with (control_gf) or without growth factors (control), in absence (controls) or presence of various concentrations (XmM) of NaCN, with (CNX_gf) or without growth factors (CNX), irradiated or not.

IRRADIATION SOURCES

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 (far-red source, FR), and of 830 nm / 50 mW (near-infrared source, NIR), 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 2–3 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 1–600 s, giving single incident doses equivalent with $(1–5)\times10^{12}$ photons/cell or ~ (0.2–1.5) μJ/cell. Irradiation regimes of once per day, or every second day with these single doses gave total irradiation doses of ~ (1–15) μJ/cell. Various cell samples were irradiated together (PBMC) or separately (Ly or MoDC).

CELL VIABILITY ASSESSMENT THROUGH THE TB EXCLUSION METHOD

Viabilities of cells cultured in various media, irradiated or not, were assessed every day or in every second days. At indicated time points cells in aliquots of 50 μL were stained with the TB colorant (final concentration ≤ 0.2%), visualized using a Zeiss Axiovert 25CFL or 40CFL inverted microscope. Live (TB excluding) and dead (TB stained) cells were counted in a Buerker-Tuerk hemocytometer. Percentage viabilities (v, %) were calculated as number of live cells out of total number of cells ($v = n_{TB-} / (n_{TB+} + n_{TB-})$, while relative viabilities were calculated as ratio of viabilities at moment t related to their initial values (v_t/v_i).

APOPTOSIS DETECTION

At different time point of exposure to various treatments (energy restriction / laser irradiation) cells were counted. 8×10^5 cells containing suspensions were pulse centrifuged at 4 ºC and resuspended in 1 mL ice-cold PBS. After a new pulse centrifugation washed cells were resuspended in 100 μL ice-cold Annexin V binding buffer (5×10^6 cells/mL). After adding 1 μL Annexin V-FITC and 2.5 μL 7-AAD (0.125 μg), samples were incubated 15 minutes in darkness, on ice. Following vigorous stirring of samples completed with 400 μL Annexin V binding buffer, samples (~10^6 cells/mL) were kept in darkness on ice, and analyzed using a BD FACS Calibur flow cytometer within 2–3 h. Alternately cells were fixed in 2% paraformaldehyde (25 μL PFA 40% in 500 μL suspension) 30 minutes in darkness on ice, transferred in BD tubes and kept at 4 ºC until their analysis by flow cytometry within 2–3 days. Using for excitation the 488 nm line of the Ar ion laser, fluorescence intensities were measured at 520 nm (Annexin V-FITC), at $\lambda >$ 660 nm (7-AAD). Cells negative for Annexin V and 7-AAD/PI staining were considered live, those stained by Annexin V-FITC, but not by 7-AAD/PI, early apoptotic (EA), while those stained both by Annexin V-FITC, and by 7-AAD, late apoptotic or necrotic (LA+N). Routinely 5000–15000 cells/sample were analyzed by flow cytometry. Percentages of live, EA and LA+N cell subpopulations were identified in density plots obtained through row cytometry data analysis using the WinMDI 2.8 freeware.

STATISTICAL ANALYSIS

Cell viabilities were obtained as means calculated from at least 3 independent assessments (standard deviation S.D. ≤ 15%). 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

Cyanide intoxication alone causes microenvironment, cyanide concentration and intoxication duration dependent decrease in viability of human PBMCs, as seen 24–120 h after their re-suspension in cyanide-containing media (Fig. 1). Short term exposure (up to 48 h) to cyanide concentrations up to 5 mM causes no significant viability decrease. Longer exposure of human PBMC to 1 mM NaCN results in significant viability decrease both in absence and in presence of growth factors (Figs. 1a and 1b), however in presence of growth factors the cyanide caused viability decrease is transient (Fig. 1b). Exposure of human PBMC to higher cyanide concentrations in absence of growth factors results in concentration

dependent progressing viability decrease after time periods longer than 48 h. In presence of 10 mM NaCN the progressing viability decrease starts after 24 h (Fig. 1c).

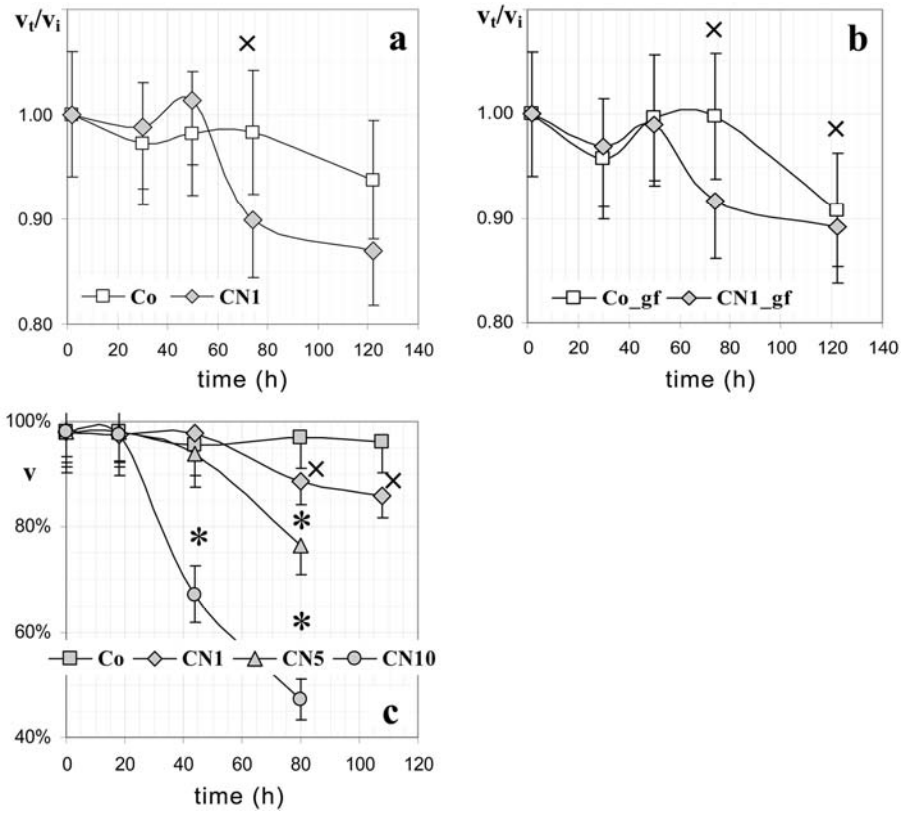

Fig. 1. Cyanide caused energy restriction effects on human PBMCs viability in absence and in presence of growth factors. v and v_t/v_i – percentage and relative cell viabilities, respectively, determined by the TB exclusion method, counting TB excluding and TB stained cells in a Neubauer-Tuerk hemocytometer, using a Zeiss Axiovert 25CFL microscope; Co and Co_gf – control PBMCs in absence and presence of growth factors; CN1, CN5, and CN10 – PBMCs in media containing 1, 5, and 10 mM NaCN, respectively; CN1_gf – PBMCs in media containing 1 mM NaCN, in presence of growth factors (10 ng/mL GMCSF, 5 ng/mL IL4); × – $p < 0.05$; * $p < 0.01$.

In absence of growth factors multiple NIR irradiation (830 nm, daily single doses of 0.9 μJ/cell) of non-injured human PBMCs does not cause significant viability change when the whole PBMC population is irradiated. In same conditions separate irradiation of the Ly subpopulation alone causes no significant effects in the first 48 h, while it induces drastic decrease in the viability of all PBMCs in co-culture in time periods longer than 48 h (Fig. 2a). In presence of growth factors none of the above irradiation regimes results in significant short or long term changes in the viability of exposed human PBMCs (Fig. 2b). In energy restriction caused stress conditions, in absence of growth factors, both NIR

irradiation regimes offer only transient protection against viability decrease of the exposed human PBMCs in the time interval of 60–90 h after cells transfer in 1 mM NaCN containing medium (Fig. 2c). In same conditions, in presence of growth factors, Ly irradiation alone offers only transient protection against viability decrease of cyanide intoxicated human PBMCs, as in absence of growth factors, while NIR irradiation of the whole PBMC population induces short term viability increase and long term protection against 1 mM NaCN caused viability decrease of human PBMCs (Fig. 2d).

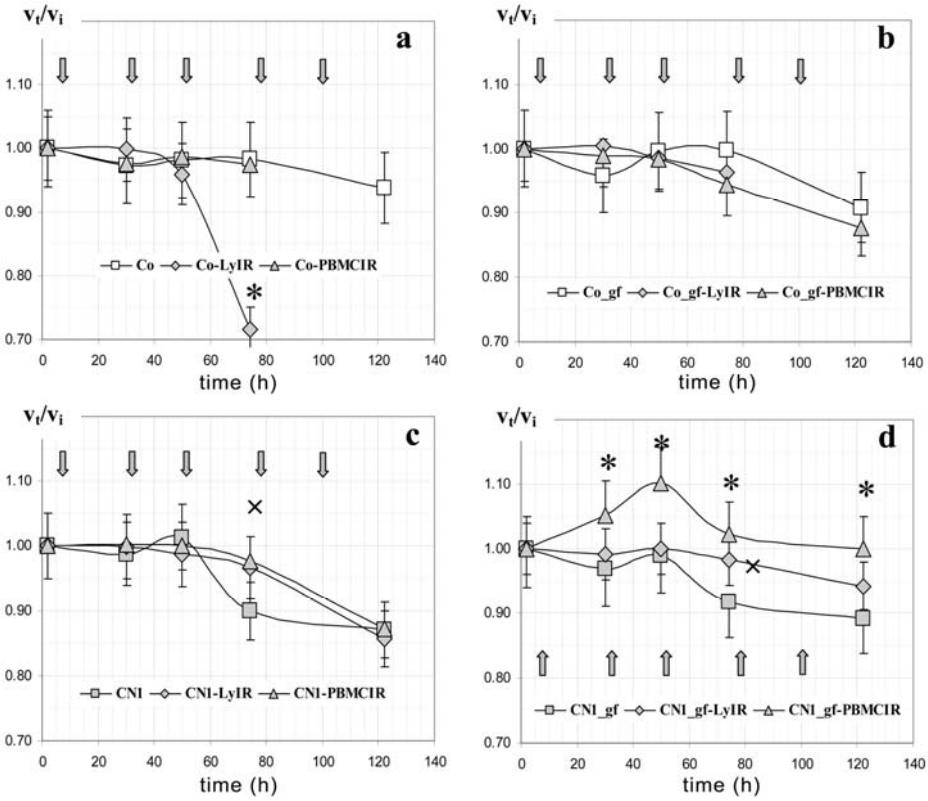

Fig. 2. Multiple NIR laser irradiation effects on control and cyanide intoxicated human PBMC in absence and in presence of growth factors. Relative cell viabilities determined by the TB exclusion method, counting TB excluding and TB stained cells in a Neubauer-Tuerk hemocytometer, using a Zeiss Axiovert 25CFL microscope; Ly and Mo/MoDC subpopulations in co-culture; mitochondrial respiratory chain blockade related energy restriction induced by presence of 1 mM NaCN in the culture medium; Co and Co_gf – control PBMCs in absence and presence of growth factors (10 ng/mL GMCSF, 5 ng/mL IL4); CN1 and CN1_gf – PBMCs in media containing 1 mM NaCN, in absence and presence of growth factors, respectively; NIR source: 830 nm / 50 mW semiconductor laser; total dose: 4.5 μJ/cell; daily applied single doses: 0.9 μJ/cell; irradiation regimes: Ly subpopulation irradiated separately alone and cultured together with the Mo/MoDC subpopulation (LyIR) and irradiation of the whole PBMC population in co-culture (PBMCIR); × $p < 0.05$; * $p < 0.01$.

In absence of growth factors single dose FR laser irradiation (680 nm, 0.6 μJ/cell) does not significantly affect the viabilities of human PBMC in control and low (1 mM) and high (10 mM) cyanide concentration induced mitochondrial respiratory blockade related stress conditions (Fig. 3). In presence of 5 mM NaCN enhancement of cyanide toxicity induced viability decrease can be observed at time periods longer than 72 h after cells transfer in cyanide containing medium.

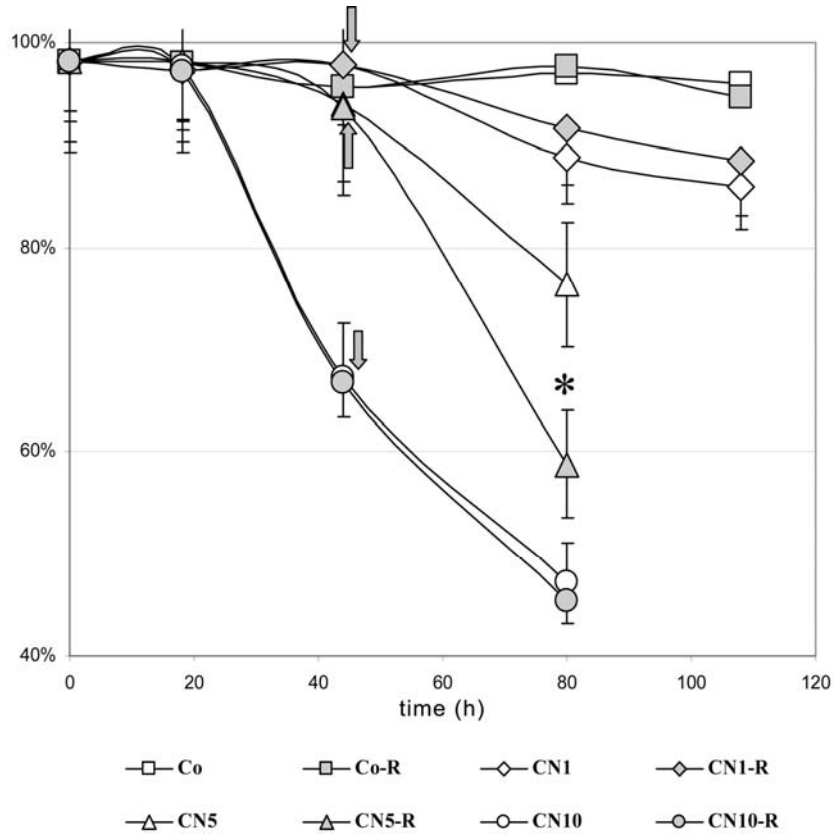

Fig. 3. Single dose FR laser irradiation effects on control and cyanide intoxicated human PBMC in absence of growth factors. Percentage cell viabilities (v) determined by the TB exclusion method, counting TB excluding and TB stained cells in a Neubauer-Tuerk hemocytometer, using a Zeiss Axiovert 25CFL microscope; Co – control PBMCs in absence of growth factors; CN1, CN5, and CN10 – PBMCs in media containing 1, 5, and 10 mM NaCN, respectively; FR source: 680 nm / 25 mW semiconductor laser; applied single dose: 0.6 μJ/cell; irradiation regime: irradiation of the whole PBMC population in co-culture (X-R); * $p < 0.01$.

In presence of growth factors multiple FR laser irradiations (830 nm, 0.9 μJ/cell daily single doses) does not significantly affect the non-injured human PBMCs viabilities (Fig. 4a). Significant protection against viability decrease of mildly intoxicated human PBMC can be observed 70–120 h after cells transfer in

the 0.1 mM cyanide containing medium when the Ly subpopulation, maintained in co-culture with the MoDC subpopulation, is irradiated separately alone (Fig. 4b). In same conditions, applying the same irradiation regime, the same FR light enhances the human PBMCs viability decrease in the time interval of 48–96 h after cells transfer in an 1 mM NaCN containing medium (Fig. 4c).

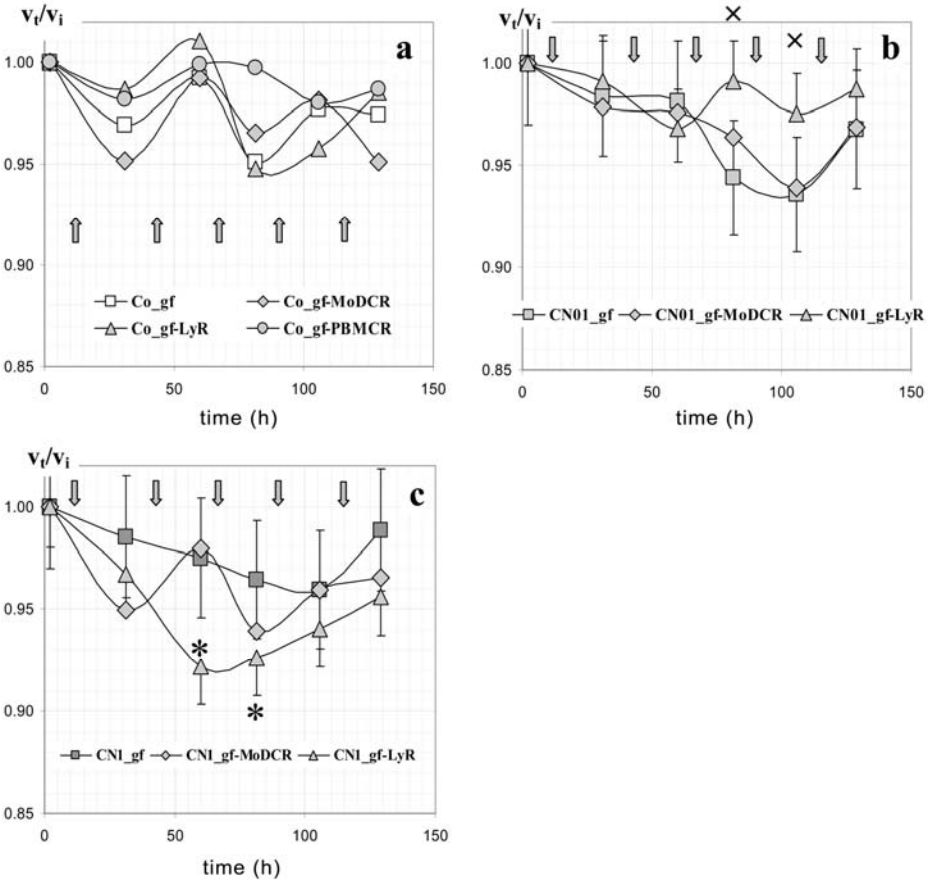

Fig. 4. Multiple FR laser irradiation effects on control and cyanide intoxicated human PBMC in presence of growth factors. Relative cell viabilities determined by the TB exclusion method, counting TB excluding and TB stained cells in a Neubauer-Tuerk hemocytometer, using a Zeiss Axiovert 25CFL microscope; Ly and Mo/MoDC subpopulations in co-culture; mitochondrial respiratory chain blockade related energy restriction induced by presence of 0.1 mM and 1 mM NaCN in the culture medium; Co_gf – control PBMCs in presence of growth factors (10 ng/mL GMCSF, 5 ng/mL IL4); CN01_gf and CN1_gf – PBMCs in media containing growth factors, in presence of 0.1 mM and 1 mM NaCN, respectively; FR source: 680 nm / 25 mW semiconductor laser; total dose: 4.5 μJ/cell; daily applied single doses: 0.9 μJ/cell; irradiation regimes: MoDC subpopulation irradiated separately alone and cultured together with the Ly subpopulation (MoDCR), Ly subpopulation irradiated separately alone and cultured together with the Mo/MoDC subpopulation (LyR) and irradiation of the whole PBMC population in co-culture (PBMCR); × $p < 0.05$; * $p < 0.01$.

Flow cytometric assessment of percentage subpopulations of live, dying (early apoptotic, EA) and dead (late apoptotic and necrotic, LA+N) cells discloses significant photobiomodulation of human PBMCs viability. Long term hindrance of oxidative phosphorylation through blockade of mitochondrial respiratory chain with cyanide (1 mM NaCN, 108 h) results in significant decrease of live cell subpopulation with apoptosis induction in human PBMCs cultured in absence of growth factors (Fig. 5). Multiple NIR laser irradiation (830 nm, 4 × 0.9 μJ/cell) of the whole PBMC population mildly enhances apoptosis induction in cyanide intoxicated cells in absence of growth factors, as seen 12 h after the last irradiation. In same conditions apoptosis induction in control cells is not significantly affected (Fig. 5).

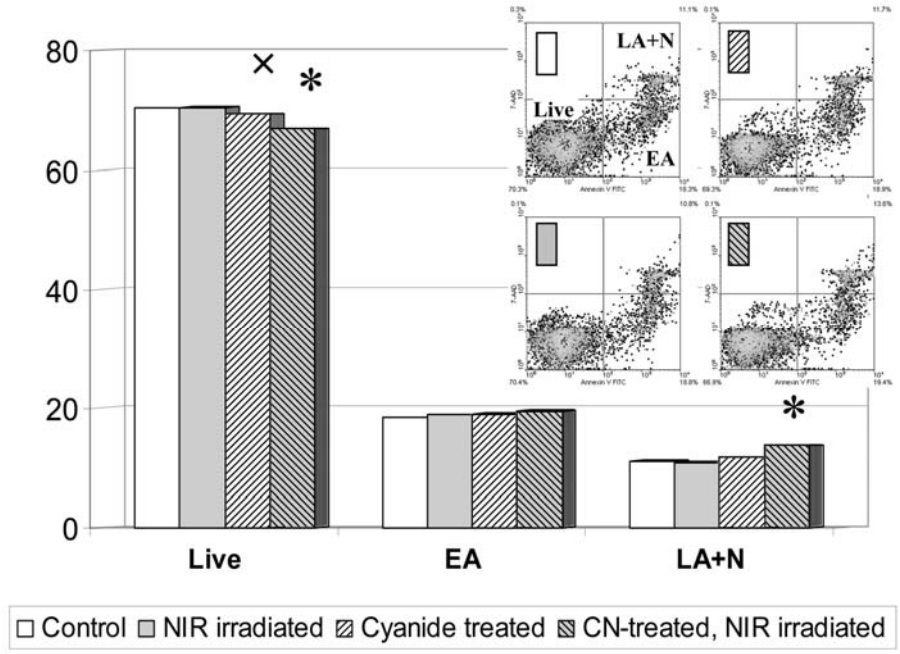

Fig. 5. Multiple NIR laser irradiation influence on 1 mM NaCN death promoting effects on human PBMC in absence of growth factors. Percentage subpopulations of live, early apoptotic (EA) and late apoptotic and necrotic (LA+N) cells, revealed by flow cytometric analysis of cell suspension samples stained with AnnexinV-FITC and 7-AAD 12 h after the last irradiation, using a BD FACS Calibur instrument with CellQuest 3.0 and WinMDI 2.8 as acquisition and analysis software respectively;NIR source: 830 nm / 50 mW semiconductor laser; total dose: 3.6 μJ/cell; daily applied single doses: 0.9 μJ/cell; irradiation schedule of the cyanide intoxicated sample: first irradiation before cells transfer in cyanide containing medium; 7-AAD – nuclear stain for late apoptotic and necrotic cells; AnnexinV-FITC – phosphoserine-specific cell surface stain for early and late apoptotic and necrotic cells. × $p < 0.05$; * $p < 0.01$.

DISCUSSION AND CONCLUSIONS

Last decades research convincingly indicated the endogenous mitochondrial enzyme, cytochrome c oxidase, as the most important biological photoacceptor of long wavelength laser radiations in the red – far-red – near infrared (R – FR – NIR) range [12, 14, 16]. Absorption of laser light by the copper chromophores of cytochrome c oxidase, with subsequent enzyme reduction, stimulation of intramolecular electron transfer and of cellular energetics, are generally accepted as most important primary mechanisms mediating photobiomodulation [14, 33]. As concerns further steps of the photosignal transduction and amplification chain – recently recognized as retrograde mitochondrial signaling [15, 28] – changes occurring in cell membrane properties, and in cell cytoplasm characteristics sustaining pathways leading to cell survival, proliferation or death, constitute important intermediary mechanisms [11, 13–15]. Up-regulation / restoration of mitochondrial function or of cellular redox mechanisms or activation of various cell signaling pathways by low FR/NIR irradiation doses were repeteadly indicated/demonstrated as important mechanisms involved in protection or rescue of intoxicated or damaged cells [2, 7, 32–33]. However, high fluency low-power laser irradiation was also shown to induce reactive oxygen species mediated apoptosis [34].

In the present study, investigating by microscopy and by flow cytometry impact on human PBMC of exposure to various concentrations (0.1–10 mM) of sodium cyanide (NaCN) and/or to low doses (0.5–5 μJ/cell) of FR / NIR radiations, we disclosed specifics of photobiomodulation of energy restriction caused effects occurring in human PBMCs, as follows: (i) hindrance of oxidative phosphorylation through blockade of mitochondrial respiratory chain caused cell microenvironment, cyanide concentration and intoxication duration dependent decrease in viability of human PBMCs and apoptosis induction; (ii) in absence of growth factors multiple NIR laser irradiation (830 nm, daily single doses of 0.9 μJ/cell) of the whole PBMC population did not affect viability and apoptosis induction in control cells, offered slight transient protection against viability decrease induced by 1 mM NaCN during mid intoxication periods, but mildly enhanced apoptosis induction in intoxicated cells after longer periods of exposure; (iii) in absence of growth NIR irradiation of the Ly subpopulation separately alone drastically reduced viability of the whole PBMC population in co-culture in control samples, while still offered transient protection to cyanide intoxicated cells; (iv) in absence of growth factors low dose FR irradiation (680 nm, 0.6 μJ/cell) did not affect the viability of control, mildly or highly intoxicated human PBMC, but drastically enhanced toxicity of mid cyanide concentrations; (v) in presence of growth factors, no matter which irradiation regime was applied, neither NIR nor FR laser light had any effect on control human PBMCs viability; (vi) in presence of growth factors multiple NIR irradiation (830 nm, daily single doses of 0.9 μJ/cell) of the whole PBMC population engendered short term viability increase and long term protection

against 1 mM NaCN induced viability decrease, while only offered mild transient protection when the Ly subpopulation was irradiated alone; (vii) in presence of growth factors multiple FR irradiation (680 nm, daily single doses of 0.9 μJ/cell) of the Ly subpopulation alone enhanced the 1 mM NaCN induced viability decrease of human PBMC, while offered transient protection against cyanide toxicity at much lower cyanide concentrations (0.1 mM).

In sum, significant, cell microenvironment, toxicant concentration, intoxication duration, laser wavelength, irradiation dose and irradiation regime dependent changes were observed in viability and apoptosis rate of human PBMCs. Further investigations into this issue are ongoing in our laboratories.

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