Romanian Biotechnological Letters *Vol. 14, No. 5, 2009, pp. 4694-4703*

ORIGINAL PAPER

Low level long wavelength laser irradiation effects on human T leukemic lymphoblasts mitochondrial reticulum

Received for publication, February 15, 2009
Accepted, September 25, 2009

ION-OCTAVIAN DOAGĂ[1], EUGEN RADU[2], GYÖNGYVÉR KATONA[3], JUDIT HORVÁTH[4], ERVIN TANOS[§], LÁSZLÓ KATONA[5], ÉVA KATONA[**]

[1] Corresponding author: Department of Biophysics, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmaceutics, 8 Eroii Sanitari Bvd, sect. 5, 050474 Bucharest, Romania, phone/fax: +4021 3125955; e-mail: iodoaga@gmail.com
[2] Department of Molecular and Cellular Medicine, Medical Faculty, "Carol Davila" University of Medicine and Pharmaceutics, Bucharest, Romania
[3] Department of Medical Biochemistry, Medical Faculty, "Carol Davila" University of Medicine and Pharmaceutics, Bucharest, Romania
[4] LASEUROPA CO., Budapest, Hungary
[5] Department of Biophysics, Medical Faculty, "Carol Davila" University of Medicine and Pharmaceutics, Bucharest, Romania

Abstract

We investigated the effects of low power 830 nm near-infrared and 680 nm far-red laser light on Jurkat cells mitochondrial reticulum size and shape. The 1.8 μJ/cell total dose near-infrared laser irradiation induced moderate mitochondrial volume and mass decrease, and substantial fragmentation of the network. Double total dose engendered parameter changes appeared massive or mild depending on the irradiation regime. Quadruple total dose produced changes were moderate as compared to those induced by double doses. Mild cyanide intoxication caused large mitochondrial mass decrease, while higher poisoning levels did not. The 1.3 μJ/cell total dose far-red laser irradiation induced mitochondrial mass changes were sensitively dependent on cells cyanide poisoning degree. Altogether our data demonstrate significant, cell state, laser wavelength, laser dose, and irradiation regime dependent, photobiomodulation of mitochondrial volume/mass and morphology in noninjured and metabolically stressed human T leukemia lymphoblasts.

Keywords: AlGaInP/GaAs laser, mitochondrial morphology, mitochondrial fission, metabolic poisoning.

Abbreviations: $\Delta\Psi_m$ - mitochondrial membrane potential; FR – far-red laser light; NIR – near-infrared laser light; LLLT – low level laser therapy; RFI – relative fragmentation index; RMV – relative mitochondrial volume; RMM – relative mitochondrial mass.

Introduction

Mitochondria are highly dynamic organelles of great structural and functional diversity, and of great plasticity. They are strategically distributed [1, 2, 3], move along cytoskeletal tracks, and adopt a variety of different shapes ranging from small spherical compartments to tubulo-reticular networks in order to meet cellular needs and signals from outside [1, 4]. Maintenance of stability and dynamics of mitochondrial morphology is essential for proper function [5]. In healthy cells, a complex molecular machinery mediates regulated fission and fusion events ensuring the highly dynamic morphology necessary for mitochondrial integrity [4], metabolic stability [6] and fulfillment of a variety of functions [7,

8]. Mitochondrial volume homeostasis also is essential for maintaining the structural integrity, and mitochondrial volume regulation and mitochondrial biogenesis are fundamental mechanisms controlling organelle function, here including the bioenergetic capacity [5, 9, 10]. It is also known that components of the mitochondrial morphogenesis machinery possess proapoptotic functions that are independent from their recognized roles in normal mitochondrial dynamics [11, 12], and mitochondrial volume control may participate in initiation of cell death cascades [10].

There is growing body of evidence that mitochondrial dysfunction, impairment of the respiratory complexes and altered mitochondrial signaling contribute to cellular energy deficits, apoptosis, and tissue physiological failure in aging [13, 14, 15, 16]. Imbalance in fission-fusion homeostasis and related morphogenetic equilibrium may lead to apoptosis and is also involved in pathogenesis of neurodegenerative disorders [7, 14, 17, 18, 19], while defective mitochondrial biogenesis is associated to high cardiovascular risk [14, 20, 21]. Loss of the mitochondrial bioenergetic capacity underlies the glucose avidity of carcinomas [22], and retrograde regulation due to mitochondrial dysfunction may be an important mechanism for carcinogenesis [23]. Nevertheless particulars of the involved molecular processes are yet not fully elucidated.

Part of physiotherapy in most countries, Low Level Laser Therapy (LLLT) uses low power long wavelength monochromatic light in the red – near infrared region to treat in a non-destructive and non-thermal fashion various soft-tissue and neurological conditions. LLLT proved to be very effective in healing infected, ischemic or hypoxic wounds, relieving pain, reducing inflammation, mitigating mechanical injury or after-stroke tissue damage, exercise-induced skeletal muscular fatigue and neurological deficits. Nevertheless knowledge of molecular and cellular mechanisms involved in LLLT beneficial effects is yet fragmentary [24].

The mitochondrial respiratory chain terminal enzyme, cytochrome c oxidase, was recognized as photoacceptor of laser light in the red – near infrared region, and the existence of a cellular signaling pathway mitochondria $\Rightarrow$ cytoplasm $\Rightarrow$ [plasma membrane $\Rightarrow$ cytoplasm] $\Rightarrow$ nucleus, named photosignal transduction and amplification chain, was proposed more than 20 years ago [review: 24, 25]. This signaling pathway was recently recognized as retrograde mitochondrial signaling [24, 26, 27] indicating mitochondrial membrane potential ($\Delta\Psi_m$), reactive oxygen species (ROS), calcium ions Ca^{2+}, nitric oxide radicals NO•, intracellular pH_i, and fission-fusion homeostasis of mitochondria, as key photosensitive elements of the defined information channel [26].

Influence of a short time He-Ne laser irradiation on chondriome ultrastructure in successive generations of yeast cells [28, 29, 30] and formation of giant mitochondria in He-Ne laser irradiated human blood lymphocytes [31, 32] were persuasively evidenced.

Recent in vitro [33, 34, 35] and in vivo [36, 37] studies convincingly demonstrated connection between the up-regulation of cytochrome c oxidase by irradiation with the 680 nm far-red laser light (FR) and/or the 830 nm near-infrared laser light (NIR) and protection of intoxicated retina or neurons [33, 34, 35, 36, 37]. Light-induced rescue of breathing after spinal cord injury [38], transcranial NIR therapy engendered improvement of motor function following embolic [39] and ischemic strokes [40], and protection and rescue from hypoxia and reoxygenation injury, offered to cardiomyocytes by near-infrared light therapy [41] were also proved. Other investigators revealed that LLLT in proper dosage can induce improvement of electrical stimulation induced mitochondrial dysfunction [42]. Furthermore promotion by LLLT of cellular homeostasis and of cell proliferation [43, 44, 45] and attenuation of cholinergic hyper-reactivity / adrenergic hypo-responsiveness in airway inflammation [46] were evidenced. In all these studies up-regulation / restoration of

mitochondrial function or of cellular redox mechanisms or activation of various cell signaling pathways constituted the revealed or hypothesized action mechanisms involved in the observed protection or rescue.

Nevertheless LLLT may induce beneficial effects – namely acute and chronic pain relief - via a reversible blockade of fast axonal flow and mitochondrial transport, decrease of $\Delta\Psi_m$, and reduction in ATP availability, resulting in conduction block and neurotransmission failure of nerve fiber nociceptors [47]. High fluency low-power laser irradiation can also induce reactive oxygen species mediated apoptosis [48], and appropriate LLLT may facilitate enhanced tumor cell kill [49].

We previously reported sensitive metabolic modulation of low level FR and NIR membrane [50] and cellular [51] effects, and also disclosed significant photobiomodulation of quercetin cytotoxicity in human T leukemia lymphoblasts [52]. In this study we focused on investigation of low level laser irradiation effects on Jurkat cells mitochondrial network size and morphology.

Materials and Methods

Chemicals, supplements and staining kits: Sterile DMSO, the standard RPMI 1640 culture medium (R6504, lyophilized powder, 1 vial/l), and NaCN (S3296) were purchased from SIGMA CHEMICAL Co., the fluorophore MitoTracker Green (M-7514, $\lambda_{ex}/\lambda_{emm}$=490nm/516nm) was from Molecular PROBES / INVITROGEN, foetal calf serum from Biochrom (FCS, EU tested, S0115), while the other supplements (antibiotics/glutamine) from GIBCO/INVITROGEN. All other chemicals were of the best research grade available.

Stock solutions: MitoTracker Green stock solution (100 μM) was prepared in DMSO, while the NaCN stock solution (20N) 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/mlmL penicillin, 100 μg/mlmL streptomycin, 2 mM L-glutamine, and pH was adjusted to 7.2 (complete medium). The serum free medium was prepared in the same way without adding FCS. The cyanide containing media were prepared from the complete medium, adding the appropriate quantity of NaCN stock solution.

Cell culture: The human acute T leukemic Jurkat cells (ATCC® TIB-152™) were cultured in flasks or Petri dishes of variable surfaces, in humidified (82%) 5% CO2 atmosphere at 37°C, in complete medium, and passaged every 2/3 days through dilution of cell suspensions with fresh media to a concentration of 6 – 9 ×x 10^5 cells/ml.

Serum starvation: Cells were cultured for 16 – 26 h in serum-free medium, so as to establish metabolic quiescence at the beginning of experiments.

Energy restriction was engendered by culturing cells in complete media containing 100 μM – 5 mM NaCN for various time periods (44–128 h), so as to establish various degrees of hindrance of the oxidative phosphorylation through cytochrome c oxidase inhibition caused blockade of the mitochondrial respiratory chain.

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/ and 25 mW, and of 830 nm/ and 50 mW, respectively.

Sample irradiation regimes and laser irradiation doses: Samples were irradiated in sterile conditions. Individual incident doses of ~ (0.4 —1.8) μJ/cell, equivalent with (0.3÷1.5) x 10^{13} photons/cell, were used. Irradiation regimes of once per day, or two groups of 2 individual

doses given on consecutive days, separated by a two days rest period, were applied, as to realize total irradiation doses of ~ (1.3 – 7.2) μJ/cell. Control unirradiated samples were handled in the same way as the irradiated ones.

Sample preparation: At different time point of exposure to various nutrient/energy restriction conditions, irradiated with certain doses or not irradiated, cells were loaded with MitoTracker Green by culturing them in presence of 100 nM probe for 30 min. Though we have to note the mild mitochondrial membrane potential sensitivity of MitoTracker Green accumulation in Jurkat cells mitochondria, this in our hand never exceeded several percentages. For confocal microscopic measurements, following dye loading, 2% formaldehyde was introduced in the culture medium for 15 min, in order to fix the cells. Fixed cell samples, washed twice and resuspended in PBS, were stable for days. Before actual measurements fixed cells were immobilized on Poly-L-Lysine treated cover glasses. For flow cytometric measurements stained live cells were washed twice with ice-cold PBS, and resuspended in PBS.

Confocal scanning microscopy measurements: Stained, fixed, immobilized cells were analyzed using a Nikon PCM2000 laser scanning confocal system (3 lasers) mounted on a Nikon Eclipse E200 inverted fluorescence microscope. Experiments were repeated thrice. Confocal stack images were acquired at room temperature, using the 60x oil objective with 1.40 numeric aperture. The excitation and emission wavelengths were 490 and 525 nm, respectively. The same scanning parameters were used for all presented measurements (voxel size = 0.227/0.227/1 μm^3). Image processing was performed using the Image Pro Express 6.0 with 3D Constructor plug-in (MediaCybernetics Inc.). The dye intensity level for volume selection was normalized using the histogram of averaged image on each stack. After intensity normalization, stacks were loaded for 3D reconstruction and processing. A median 3D convolution filter (3x3x3, pass 1) was applied to eliminate noise and artifacts. The program automatically pointed out the structures volumes of the given (normalized) dye intensity level. The mean mitochondrial volume was computed by dividing the sum of all measured volumes in the stack to the number of stained cells. Cell counting was made on the averaged stack image after applying a best fit contrast. The cell volume was separately visualized in stacks by increasing the residual signal of the membrane. The mean cell volume was quantified measuring cells diameters in 3D and averaging the computed volume of cells assuming ellipsoidal shape. Currently 150-600 cells were analyzed for each sample. The ratio of the mean mitochondrial volume over the mean cell volume was computed. Additionally we defined the mean mitochondrial fragmentation index, calculated using Microsoft® Office Excell 2003, as ratio of number of stained mitochondrial fragments over the number of stained cells. Finally relative parameters were computed in proportion to those of control samples: the relative mean mitochondrial volume (RMV) and the relative mean fragmentation index (RFI).

Flow cytometry measurements: Within 30 min – 2 h after fluorophore loading, currently 8000-16000 stained cells were analyzed by flow cytometry using a FACSCalibur cytometer (Becton-Dickinson), and Cellquest 3.0 as dedicated data acquisition software. The fluorescence excited by the 488nm line of the argon-ion laser was detected in the FL1 channel (525 nm). 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) and Microsoft® Office Excell 2003 were used for data analysis. FL1 histograms obtained in measurements made on cells various time periods after their transfer in various media, and 17 – 22 h after the last irradiation or not irradiated, were used to calculate area ratios considered as indicative of relative mitochondrial mass (RMM).

Statistical Analysis: Experiments were repeated thrice. The represented values are means (standard deviation S.D. ≤ 15%). Unpaired analysis of data series was performed by Student's t-test (two-tailed). P-values less than 0.05 were regarded as indicating statistical significance (*).

Results and Discussion

Figure 1 summarizes the results obtained by analysis of confocal scanning microscopy data, illustrating changes induced by various NIR doses and irradiation regimes, in morphological parameters of cells mitochondrial reticulum, namely in RMV and RFI in human T leukemia lymphoblasts.

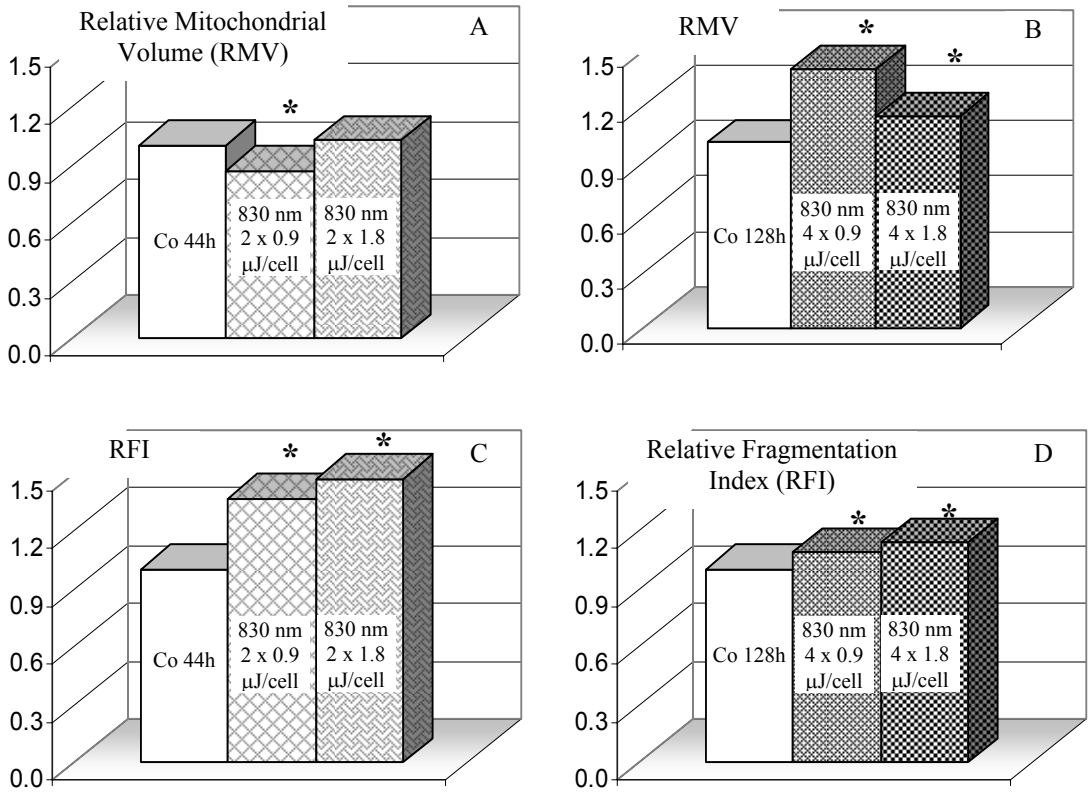

Figure 1. 830 nm Near Infrared (NIR) Laser Irradiation Induced Changes in the Mitochondrial Reticulum Volume and Morphology, Revealed by Confocal Scanning Microscopy Data Analysis, in Mitotracker Green Loaded Human T Leukemia Lymphoblasts.

□ - control samples; ▨▨▨▨ - samples exposed to NIR laser light; individual daily irradiation doses: 0.9 μJ/cell (□, ▨) or 1.8 μJ/cell (▨, ▩); total irradiation doses: 1.8 (□), 3.6 (▨), and (▨), and 7.2 (▩) μJ/cell, respectively; samples taken 12h after the last irradiation, 44h (A,C) and 128h (B, D) after metabolically quiescent cells resuspension in complete medium, respectively. ▨, ▩ - 4 individual doses regimes, applied as 2 groups of 2 individual doses, separated by a 2 days rest period.

Jurkat cells - cultured in standard conditions, irradiated or not, loaded with 100 nM MitoTracker Green, fixed in 2% formaldehyde, immobilized on Poly-L-Lysine treated cover glasses, and analyzed using a Nikon PCM2000 laser scanning confocal system mounted on a Nikon Eclipse E200 inverted fluorescence microscope with a 60x and 1.40 numeric aperture oil objective.

Image processing: Image Pro Express 6.0 with 3D Constructor plug-in (MediaCybernetics Inc.); relative mean mitochondrial volume (RMV) and the relative mean fragmentation index (RFI) calculated using Microsoft® Office Excell 2003; number of analyzed cells: 150-600 for each sample; S.D. ≤ 15%; * - statistically significant difference as compared to untreated control at level $p < 0.05$

The 1.8 μJ/cell total dose of 830 nm NIR, applied as 2 equal individual doses in 2 consecutive days, caused a 13% decrease in RMV and a 37% increase in RFI. Irradiation with a double total dose, applied as 4 equal individual irradiation doses with 2 days rest period after the first 2 doses, resulted in 40% increase in RMV and less fragmentation (only 9% increase in RFI). However the same 3.6 μJ/cell total dose, applied as double individual doses in 2 consecutive days, did not induce volume changes, but did induce massive fragmentation of mitochondria (47% increase in RFI). Irradiation with a quadruple total dose, applied as 4 equal double individual doses with the same 2 days rest period after the first 2 doses, resulted in 14% increase in both RMV and in RFI.

The total dose dependency of the observed NIR effects is obvious. Our results resemble those obtained by Karu and co-workers [28, 29, 30] in studies concerning He-Ne laser irradiation effects on ultrastructure of yeast cells chondriome. Though comparison of data is not straightforward, the remarkable dose-dependency is similar.

At the same time, the irradiation regime dependency of the 830 nm NIR irradiation induced parameter changes revealed in our experiments is striking.

In order to check whether the observed volume changes imply or not mitochondrial mass changes, we also performed flow cytometry measurements.

Figure 2 illustrates changes induced by various FR and NIR doses in RMM of human T leukemia lymphoblasts. Jurkat cells cultured in standard conditions in standard media and/or in presence of various concentrations of NaCN, as to realize various levels of metabolic stress through hindrance of mitochondrial respiratory chain, were irradiated or not under sterile conditions. Parameters of noninjured and metabolically stressed cell samples irradiated with various total doses of FR or NIR were compared to those of control unirradiated samples.

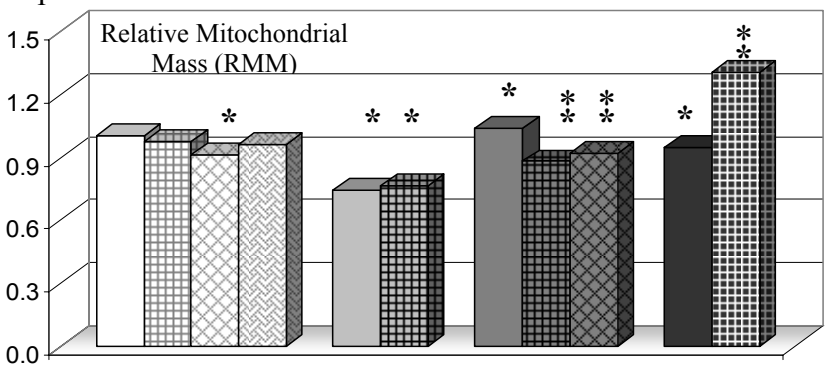

Figure 2. Laser Irradiation Induced Changes in the Mitochondrial Reticulum Mass, Revealed by Flow Cytometry Data Analysis, in MitoTracker Green Loaded Control and Energy Restricted Human T Leukemia Lymphoblasts.
□ - control samples; ▫, ▪, and ■ - samples exposed to energy restriction caused by blockade of the respiratory chain in presence of 0.1 mM, 1 mM, and 5 mM NaCN, respectively; ⊞, ⊞, ⊞, and ⊞ - cells exposed to action of the 680 nm laser light (dose: ~1.3 uJ/cell, 3 individual doses of ~0.44 μJ/cell); ⊠, ⊠, and ⊠ - cells exposed to action of the 830 nm laser light, individual daily doses of ~0.9 μJ/cell, total doses of ~1.8 (⊠, ⊠) and ~2.7 uJ/cell (⊠), respectively).
Jurkat cells - cultured in standard conditions or in energy restriction caused stress conditions, irradiated or not, loaded with 100 nM MitoTracker Green, and analyzed using a FACSCalibur cytometer (Becton-Dickinson), and Cellquest 3.0 as dedicated data acquisition software.
Fluorescence excited by the 488nm line of the argon-ion laser - detected in the FL1 channel (525 nm)
FL1 histogram analysis: WinMDI 2.8; relative mean mitochondrial mass (RMM) calculated using Microsoft® Office Excell 2003; number of analyzed cells: 8000-16000 for each sample; S.D. ≤ 15%; */* - statistically significant difference at level $p < 0.05$, as compared to untreated control and to the treated unirradiated one, respectively

The 1.3 μJ/cell total dose of 680 nm FR was applied as 3 equal individual doses in 3 consecutive days, and apparently did not cause change in RMM in control unimpaired samples. The 1.8 J/cell total dose of 830 nm NIR, applied as 2 equal individual doses in 2 consecutive days, caused a 9 % decrease in RMM in these control samples. In same conditions irradiation with a dose of 2.7 J/cell, applied as 3 equal individual irradiation doses, resulted in an apparent 4 % decrease in RMM. Mild blockade of oxidative phosphorylation in presence of 0.1mM NaCN in the culture medium resulted in 26 % decrease in RMM. The 1.3 μJ/cell total dose of 680nm FR did not modulate significantly the mild poisoning induced changes. Intermediate level cyanide intoxication caused no statistically significant modification of RMM in exposed samples. Irradiation of exposed cells with the 1.3 μJ/cell total FR dose or with the 1.8 μJ/cell total NIR dose induced 12% and 8% RMM decrease, respectively. High level mitochondrial respiratory chain poisoning in presence of 5 mM NaCN apparently engendered only 5% decrease in RMM. Irradiation with the 1.3 μJ/cell total FR dose induced a 30% RMM increase in these conditions.

Mitochondrial mass changes are currently interpreted as signs of increase or decrease in mitochondrial biogenesis [53]. Apparently neither FR irradiation nor intermediate level mitochondrial respiratory chain poisoning impeded mitochondrial biogenesis, at least not in our experimental conditions. Inquisitive observations are the large mitochondrial mass decrease in mildly poisoned samples, and the very modest diminution of this parameter in case of the highly intoxicated cells. The former is difficult to explain as impairment of mitochondrial biogenesis is currently associated with mitochondrial membrane depolarization [14], while these mildly poisoned samples occurred in our hand highly hyperpolarized, while in the latter case, in view of the observed deep mitochondrial membrane depolarization [50] more substantial mitigation were expected. Clearly this subject deserves further investigations. The remarkable FR induced stimulation of biogenesis in highly poisoned cells is not surprising as the FR induced mitochondrial membrane repolarization might be considered as trigger for biogenesis and recovery [50]. The 1.8 μJ/cell total doses NIR irradiation caused RMM decrease can be explained as consequence of mitochondrial membrane depolarization affected mtNOS activity and defective signaling induced decrease in mitochondrial biogenesis [14] and/or of offence caused increased mitochondrial autophagy [54]. The fact that this decrease is not manifest at higher irradiation doses and longer times of exposure is not surprising as recovery with increased biogenesis following injury caused increased mitochondrial autophagy was demonstrated in renal proximal tubule cells [54].

Considering the flow cytometry data the 1.8 μJ/cell total doses NIR induced RMV decrease, revealed by confocal scanning data analysis, can largely be explained as impaired mitochondrial biogenesis and/or increased mitochondrial autophagy caused mass decrease. The RFI increase in these samples may be taken as sign of programmed cell death, as mitochondrial fission resulting in mitochondrial fragmentation is currently considered an early event during apoptosis [17]. Swelling and less fragmentation, observed in case of further irradiation of these samples may be associated with cell recovery in these conditions, as activation of respiratory chain with increase in ATP production, engendered by increase in matrix volume was demonstrated [10], and the irradiation regime applied in these experiments (3.6 μJ/cell total dose, applied as 2 groups of 2 individual doses with 2 days rest period in between) could allow recovery with further stimulation, as seen with other insults in other cells [54]. The fact that RFI is still 9 % is not in contradiction with this interpretation, as fragmentation of the mitochondrial network is not necessarily related to cell damage or dysfunction [55].

Following this way of reasoning, we can consider that the huge fragmentation degree increase observed under the influence of the same total dose NIR irradiation applied as 2 double individual doses, indicates cell apoptosis. Doubling the total irradiation dose, applying again the 2 amid rest days including irradiation regime, appears to result once more in cell recovery, though much less impressive as compared to that observed using lower individual doses. This is not unusual, as greater efficiency in cells stimulation or protection of lower irradiation doses as compared to higher ones was often noticed [24, 25].

Conclusions

Using laser scanning confocal microscopy with 3D constructor image analysis and flow cytometry, we disclosed particulars of Jurkat cells chondriome photobiomodulation in normal and stress conditions, as follows: (i) irradiation with a total dose of 1.8 μJ/cell of 830 nm NIR induced mitochondrial volume and mass decrease, as well as substantial fragmentation of the network; (ii) a 2.7 μJ/cell dose NIR produced no mass change; (iii) the 3.6 μJ/cell total dose of the same laser light engendered massive fragmentation, but no volume change, when applied daily in 2 individual doses, and caused large volume increase and mild fragmentation, when applied in 4 individual doses with amid rest period; (iv) the 7.2 μJ/cell total dose of 830 nm light brought about moderate mitochondrial volume and fragmentation increase; (v) mild blockade of the mitochondrial respiratory chain in presence of 0.1mM NaCN caused large mitochondrial mass decrease, while higher cyanide intoxication levels did not brought on significant mass changes; (vi) irradiation with a total dose of 1.3 μJ/cell of 680 nm FR did not induce mitochondrial mass changes in control and mildly cyanide poisoned samples, while caused moderate decrease and substantial increase of mitochondrial mass in intermediate and high concentration cyanide poisoned samples, respectively.

Taken together, our data document significant, laser wavelength, laser dose, and irradiation regime dependent FR and NIR induced changes in human T leukemia lymphoblasts mitochondrial volume, fragmentation grade, and mass. The revealed effects are clearly dependent on cells state, and kind, level and duration of stress exposure. Further investigations into this topic are currently underway in our laboratories.

Acknowledgments: Partial financial support of the Ministry of Education, Research and Innovation of Romania (Grant CNCSIS 924) is gratefully acknowledged.

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