THE ANALYSIS OF NEUTROPHILS STRUCTURAL FUNCTIONAL STATE WITH USE OF INTERFERENCE MICROSCOPY: THE MECHANISMS OF LOW INTENSITY LASER THERAPY EFFECT

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Abstract. The aim of the work was established as to study the effect of low-intensity laser therapy (LILT) on the morphology of neutrophils by the method of interference microscopy in correlation with metabolic parameters in the use of modulators which permits to assess the contribution of various structures of neutrophils to the realization of LILT effect. There was in vitro research. The neutrophils of rats served as the object of research. The neutrophils were incubated with adrenalin, cortisol, and β-blockers of adrenoreceptors. The effect of LILT in combination with a preliminary incubation of these substances was studied. Autonomous laser shower MarsiK (R&D company "Petrolaizer," Saint Petersbourg), a laser therapeutic complex for animals, was used as a laser irradiation source. The wavelength of the irradiation was 830 nm, and the total power was 90 MW. The phasometry of neutrophils was studied by the laser modulation interference microscopy method, the processes of lipid peroxidation were analyzed by the malondialdehyde concentration, and the ATP concentration was defined by spectrophotometry. LILT provoked unidirectional changes in indices in the groups where the neutrophils were incubated with adrenalin, cortisol, and adrenoreceptor blockers. It manifested in the intensification of lipid peroxidation, increase in ATP concentration, and on the other hand, an increase in phase diameter of neutrophils regarding indicators without the impact of LILT. It is shown that the LILT effect intensity may be modified depending on stress realizing hormones (adrenalin and cortisol), β-blocker of adrenoreceptors. It permits us to suppose that the LILT effects may be realized in different ways depending on the state of the cells.

Keywords: low intensity laser therapy (LILT), neutrophils, interference microscopy.

List of Abbreviations

LILT – low intensity laser therapy
MDA – malondialdehyde
ATP – adenosinetriphosphate

Introduction

Low intensity laser therapy as a method of treatment is used in various areas of medicine: in urology, neurology, stomatology, pediatry, otorhinolaryngology, gynecology (Moskvin, 2017). Various mechanisms of low intensity laser therapy were suggested. It's shown that the background of mechanism of LILT effect is the absorption by the molecules of chromophores (Silveira *et al*., 2008). Besides, the target of LILT effect is water environment of biological organelles as well as of laser-induced dimerization of some receptors and then their phosphorylation. As result the increase in intracellular calcium takes place (Duan, 2001). It's sug-gested to treat by LILT with various signaling systems (Shefer *et al*., 2003; Braun *et al*., 2005).

Based on the above it's possible to suppose that LILT effect may be realized through signaling ways of cells and make any modeling impact on their morphologic and metabolic properties. This supposition encouraged us to study at the same time metabolic and morphologic parameters of neutrophils treated by LILT after a preliminary incubation of cells with various compounds which permits to analyze the role of various cell structures in the reaction to LILT treatment. Morphometrical changes taking place in neutrophils was controlled by interference method (Deryugina *et al*., 2019). The interference microscopy method permits to receive high contrast pictures without use of any colorant and to quantify to a high precision the phase height (the phase height is proportional to the product of the sample thickness by its re-

fractive index) (Deryugina *et al*., 2021). It makes these technics a "hands free" analogue of probe microscopes (for example, of atomic force microscopy quantifying the value of interaction between a specimen with a probe) (Zagubizhenko *et al*., 2011). It's supposed that phase height as well as refractive index would be able to characterize the concentration and the quantity of substance in cell as well as the intensity of metabolic processes (Tychinsky *et al*., 2013). The aim of the work was established as to study the effect of LILT on the morphology of neutrophils by the method of interference microscopy in correlation with metabolic parameters in use of modulators which permit to assess the contribution of various structures of neutrophils to the realization of LILT effect.

Materials and Methods

There were in vitro researches conducted. The object of research was the neutrophils of rats. The blood was sampled from sublingual vein in the day of the experiment. Neutrophils were isolated by the standard Boyum method on a Ficoll-Paque density gradient manufactured by Amersham Biosciences (Sweden). The aliquot from 45 μl packed and washed neutrophils was used for morphologic and metabolic analysis.

There were two series of the experiments. In the first series the neutrophils were incubated with adrenalin ($1 \cdot 10^{-9}$ g/ml), cortisol ($1 \cdot 10^{-7}$ g/ml), β-blocker (propranolol) ($1 \cdot 10^{-9}$ g/ml). The adrenalin and cortisol concentration was equal with that in stress. 2 series – the effect of analogic compounds on neutrophils with further treatment by LILT.

The blood of 6 animals was studied in each series. The researches were made in accordance with rules of execution of works and use of experimental animals established by European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes of March 18, 1986; and by the Local Ethics Committee of Institute of Biology and Biomedicine «Lobachevsky State University of Nizhny Novgorod». The blood of animals treated by LILT without either preliminary membrane modification or impact served as a control specimen (intact cells).

Autonomous laser shower MarsiK (R&D company "Petrolaizer", Saint Petersbourg), a laser therapeutic complex for animals, was used as a laser irradiation source. The total power was 90 MW. The wavelength of the laser was 830 nm. Neutrophils were irradiated in Petrie dish, diameter 3 cm. The source of irradiation was at distance of 1cm from the cell membrane surface. The LILT effect in 15 minute exposure was studied.

The neutrophils complex phasemetry was studied by laser modulation interference microscopy method with use of laser interference microscope MIM-340 (Ekaterinburg, Russia). The laser with 532 нм wavelength equipped with the 20× objective was used as the source of coherent radiation. The surface resolution is up to 15 nm, the vertical resolution is 0.1 nm. The technical characteristics of this microscope permit to study the tools which relief depth is up to 600nm. The morphology of native cells was studied without any preliminary fixation. It permits to visualize the modification of cells in on-line regime and to study the cell morphology and the dynamics of intracellular processes (Deryugina *et al*., 2019).

The state of oxidative stress was assessed by the malondialdehyde concentration (Livshits & Sedelnikova, 2007). The concentration of malondialdehyde (MDA) was defined by colored trimethine complex formation with the maximum of absorption at 530 nm when reacting with thiobarbituric acid. To calculate the concentration of MDA the molar extinction coefficient $E = 1.56 \times 10^{-5}$ $M^{-1}см^{-1}$ was used.

The activity of metabolic processes was studied by the level of ATP in neutrophils with nonenzymic method defining by the concentration of inorganic phosphate (Deryugina *et al*., 2018). It was defined by registering the coloration density with photometer KFK 3 in wavelength 660nm. Inorganic phosphorus was defined by calibration curve using a standard KH2PO4 solution.

For statistical processing of the results Microsoft Excel 2013 and BIOSTAT Statistica 6.0 were used. After proving the belonging of ex-

perimental data to normal distribution with the use of Shapiro-Wilk criterion, the values of arithmetical average and standard deviations were defined. To compare two groups Student's t-criterion was used. The confidential interval for stastistical significance $p < 0.05$.

Results

The research results of optical-geometric parameters generating the phase portrait: phase diameter, altitude (thickness) of neutrophils showed that the phase diameter of neutrophils incubated with adrenalin and cortisol was lower than that of the intact animals (Table 1). The incubation with propranolol predetermined the increase in the phase diameter of neutrophils regarding adrenaline and cortisol.

The phase diameter of neutrophils treated by LILT without any preliminary incubation increased relative to the intact cell value. After the treatment by LILT of the cells incubated with cortisol the phase diameter of the cells was restored up to the intact group value. In the adrenalin group the phase diameter kept lowered relative to the intact cell even after the LILT treatment. The LILT did not provoke any change in the index in the cells fixed with β-blocker of adrenoreceptors relative to the phase diameter of neutrophils which did not treat by LILT.

The phase altitude of neutrophils incubated with cortisol was lower than that of the intact group (Table 1). The phase altitude was higher in the adrenalin group. But it did not change after the impact of β-blockers of adrenoreceptors. The LILT did not change the dynamics which had been being discovered in the groups which was not treated by LILT.

The study of MDA concentration under the impact of the substances used in the experiment showed that the MDA concentration in the cells suffered the adrenalin impact was higher by

Table 1

Effect of the studied substances in vitro on phase diameter (μm) and phase altitude (μm) of neutrophils

Studied substances	Phase diameter of intact, (M ± m)	Phase altitude of intact, (M ± m)
Intacts	10.55 ± 0.05	2.71 ± 0.04
LILT	10.69 ± 0.04 *	2,76±0,05
Adrenalin	10.19 ± 0.06 * **	2.82 ± 0.04 *
Adrenalin + LILT	10.28 ± 0,04 * **	2.63 ± 0.04 **
Cortisol	10.37 ± 0.03 * **	2.57 ± 0.06 * **
Cortisol + LILT	10.49 ± 0.04 **	2.45 ± 0.06 * **
β-blocker of adrenoreceptors	10.43 ± 0.05 * **	2.69 ± 0.04
β-blocker + LILT	10.50 ± 0.05 **	2.66 ± 0.05

Note: * – statistically significant difference from intact group, $p < 0.05$; ** – relative to the LILT group

Table 2

Influence of different factor on the malondialdehyde concentration (μmol/ml) in neutrophils

Impact	LILT	Without LILT
Intacts		2.90 ± 0.45
LILT	3.45 ± 0.29 *	
Adrenalin	4.36 ± 0.28 *	3.75 ± 0.38*
Cortisol	3.69 ± 0.27 * **	2.64 ± 0.44
β-blocker of adrenoreceptors	4.22 ± 0.31 * **	2.66 ± 0.39

Note: * – statistically significant difference from intact group, $p < 0.05$; ** – relative to the LILT group

Table 3

Influence of different factors on ATP concentration (μmol/ml of neutrophils) in neutrophils

Effect	LILT	Without LILT
Intacts		1.82 ± 0.23
LILT	3.11 ± 0.45*	
Adrenalin	2.72 ± 0.23* **	1.19 ± 0.17*
Cortisol	2.51 ± 0.67* **	1.59 ± 0.27
β-blocker of adrenoreceptors	2.67 ± 0.58*	1.62 ± 0.27

Note: * – statistically significant difference from intact group, $p < 0.05$; ** – relative to the LILT group

29% relative to the intact group value, whereas in the β-blocker groups as well as in the cortisol group its value was unchanged relative to the intact group (Table 2).

LILT provoked the increase in MDA concentration in all the impacts relative to its value in the respective groups without LILT treatment. So, after the treatment of the intact neutrophils by LILT the MDA concentration index was higher by 19% relative to the intact group value, after the treatment by LILT of the neutrophils incubated with adrenalin it was higher by 16%. In the group of neutrophils incubated with cortisol it was higher by the factor of 1,3, with β-blocker – by the factor of 1,5 relative to this index in the respective groups of animals not treated by LILT.

The study of ATP concentration in neutrophils showed that the incubation of neutrophils with adrenalin provoked the decrease in this index relative to the intact group value (Table 3).

The treatment of neutrophils by LILT predetermined the increase in ATP concentration. An analogical dynamic were discovered in the groups treated by LILT and preincubated with adrenalin, cortisol and β-blocker of adrenoreceptors but less pronounced.

Discussion

The results of the phase portrait suggest that there are signs of functional insufficiency under the action of adrenaline and cortisol.

It has been shown that the action of adrenaline increases the content of cAMP, increases the content of Ca^{2+} ions in the cytosol, inhibits superoxide formation and elastase release, i.e. it reduces the functional activity of neutrophils as participants of immunity. According to these authors, the activation effect was blocked by propranolol (Tintinger, 2001). Whereas the genomic effects of glucocorticoids usually manifest themselves within hours, glucocorticoids within seconds to minutes also cause biological changes that are not the result of changes in gene transcription. Intercalation (incorporation) of glucocorticoids into membranes involves a mechanism independent of glucocorticoid receptors to alter cation transport across cytoplasmic membranes. Cortisol exerts an immunosuppressive effect by reducing phagocytosis. The mechanism of suppression of oxygen-dependent killing is associated with insufficient energy supply of phagocytes.

Discussing the mechanisms of LILT effects it should be noted that LILT provokes unidirectional changes in indices in the groups where the neutrophils were incubated with adrenalin, cortisol and adrenoreceptor blockers. It manifested in intensification of lipid peroxidation, increase in ATP. The analysis of neutrophils morphology makes evident that LILT provokes the increased the phase diameter.

Porphyrins have been shown to be LILI acceptors (Lebedev *et al*., 2005). The excited triplet state of porphyrins formed from the adsorption of laser photons generates ROS by the transmission of energy to the atoms of oxygen in ground state (Castano *et al*., 2005). The intensification of lipid peroxidation processes is registered under the influence of LILT (Verma *et al*., 2007). However the effect of stress realizing hormones (adrenalin and cortisol) and β-blocker of adrenoreceptors did not annulled the LILT effects, it modified it. Discussing the

LILT effect it's possible to suppose that LILT predetermines the effects associated with the action of protein kinases. In particular, the impact on α reseptors by the system of G proteins activates phospholipase C and further increase in concentration of diacylglycerol (DAG) and Inositol trisphosphate (IP3) in cytoplasm. IP3 stimulates the increase in Ca^{2+} which cooperates with DAG for the activation of protein kinases C (Braun *et al*., 2005). The stimulation of β- adrenoreceptors by the activation of intracellular adenylyl cyclase forms a complex with G-protein, under the influence of which the processes of cyclic AMP formation and of the stimulation of cAMP depending protein kinase become more intensive (Wu *et al*., 2013). So, the LILT effect may be realized by various way when the membrane component and cell signaling cascades are involved. The presented results demonstrate the capabilities of the computer phase morphometry method to detect real-time changes in the phase parameters of lymphocytes related directly to their activation.

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