Monitoring of the Effect of Low-Intensity Laser Radiation with Constant Pulse Generation on Neutrophil Granulocytes *in Vitro*

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We studied the effect of low-intensity laser radiation with constant pulse generation on bactericidal activity of neutrophilic granulocytes, in particular, on their capacity to form extracellular structures, so-called extracellular neutrophil traps. It was found that exposure to low-intensity laser radiation with constant pulse generation enhanced bactericidal activity of neutrophilic granulocytes, which manifested in the increase of the percent of neutrophils forming extracellular neutrophil traps.

Key Words: *neutrophil granulocytes; neutrophil extracellular traps, low-intensity laser radiation with constant pulse generation*

Neutrophil granulocytes (NG) are the main cells playing the leading role in anti-infectious defense of the organism due to their capacity to absorb pathogens and to release a wide spectrum of antimicrobial components [2,4-6]. A mechanism of antimicrobial defense consisting in their capacity to release nets, so-called neutrophil extracellular traps, where microorganisms are entrapped (and probably, neutralized) and then killed, was recently described. Neutrophil extracellular traps (NET) consist of nucleic acids and enzymes secreted by neutrophils in response to microbial and non-microbial stimuli [7,8].

Studies performed by a research group headed by I. I. Dolgushin, Corresponding Member of the Russian Academy of Medical Sciences, on the basis of Chelyabinsk State Medical Academy, showed that NET are effective against pathogenic and opportunistic microorganisms (*S. aureus*, *E. coli*, and *Candida* fungi). It was found that the number of dead bacteria in NET is comparable with the number of microorganisms killed by neutrophil over its life [7].

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Since NET formation is an important component of bactericidal activity of NG, the means improving the efficiency of their formation are an important problem. This can be achieved by exposure to some physiotherapeutic factors, *e.g.* low-intensity laser radiation (LILR) with constant pulse generation. Experimental and clinical studies performed over the recent decades demonstrated the effect of LILR on cell membrane energetics and conformation, rearrangements of the nuclear apparatus, and activation of the major enzyme systems [1,3]. These findings drove us to an assumption that laser radiation can modulate the process of NET formation.

Here we performed monitoring of NG viability and the number of NET depending on the time of NG exposure to LILR with constant pulse generation *in vitro*.

MATERIALS AND METHODS

Neutrophilic granulocytes were isolated from the peripheral blood of healthy donors aged 17-35 years without bad habits (n=40).

For isolation of NG suspension, 4 ml heparinized (10-15 U/ml heparin, Gedeon Richter) peripheral ve-

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nous blood was mixed (1:1) with 0.9% NaCl. Neutrophils were isolated by centrifugation on sterile Ficoll-verografin double density gradient (Pharmacia, Schering). The densities of the upper and lower gradients were 1.075-1.077 and 1.093-1.095 g/ml, respectively, the volume of each gradient was 2 ml. The cells were centrifuged for 40 min at 1500 rpm in siliconized tubes. The ring at the interface between the gradients containing 90-95% NG was carefully collected, transferred into sterile centrifuge tubes, and washed with sterile 0.9% NaCl at 1500 rpm (2×10 min). The neutrophil suspension was adjusted to a concentration of 5×10^6 cells per ml (the cells were counted in a Goryaev chamber).

Neutrophil viability was evaluated by exclusion of 1% trypan blue. To this end, 0.2 ml neutrophil suspension in physiological saline (5×10^6 per ml) was mixed with 0.02 ml 1% trypan blue. The cells were placed into a Goryaev chamber and examined under a light microscope. Viable cells were transparent (trypan-negative) and dead cells were stained violet (trypan-positive). We determined the number of viable and dead cells per 100 cells; the result was expressed in percents.

For the experiment, 1 ml neutrophil leukocyte suspension in 40-mm silicon-coated shaded disposable plastic Petri dishes (Leningrad Plant of Medical Polymers, 1 mm monolayer thickness) was irradiated using a URAL-I low-intensity laser operated in a continuous mode (λ=632 nm, 50 W laser power). Irradiation was performed at 36.6°C. Control samples were incubated in shaded disposable plastic Petri dishes without irradiation with He-Ne laser with constant pulse generation.

For luminescent microscopy, the neutrophil suspension was applied onto slides (degreased with Nikiforov mixture) and the preparation was dried on air, fixed in 96% ethanol, and stained with 0.04% acridine orange. The percentages of neutrophils with segmented nucleus, neutrophils with non-differentiated nucleus, and NET were determined on smears. The time of incubation and laser exposure was 0, 2, 5, and 10 min.

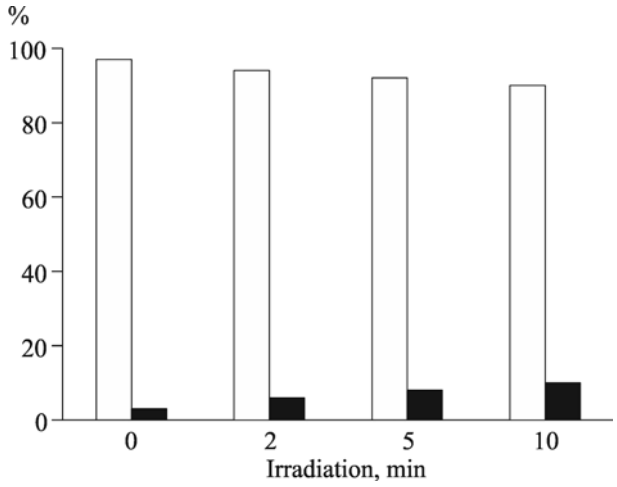

Fig. 1. Viability of NG after incubation for 0, 2, 5, and 10 min at 36.6°C without LILR exposure. Here and on Fig. 2: open bars: viable NG; dark bars: dead cells.

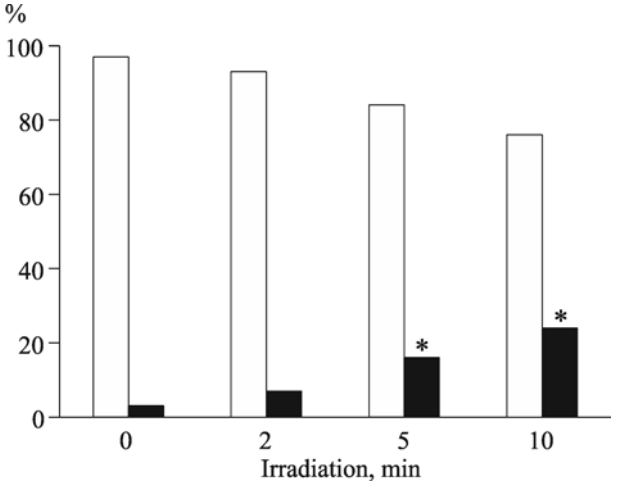

Fig. 2. Viability of NG after LILR exposure for 0, 2, 5, and 10 min.

TABLE 1. Effect of Exposure to LILR for 0, 2, 5, and 10 min on NG

LILR exposure, min	Neutrophils, %		
	with segmented nucleus	with non-differentiated nucleus	NET
0	86.59±2.72	9.19±2.29	4.22±1.05
2	77.93±5.24	14.44±4.77	7.63±1.04*
5	61.69±9.32	20.42±9.19	17.85±2.69
10	52.37±10.01	36.67±9.77*	10.96±1.95*

Note. *$p<0.00001$ compared to the corresponding parameter after 5-min exposure.

The data were processed using Statistica 6.0 software and expressed as $M \pm m$. Nonparametric Mann–Whitney test was also used for the analysis.

RESULTS

Experiments revealed a decrease in NG viability after 5-min irradiation with LILR with constant pulse generation. The number of dead cells in the fraction of neutrophils exposed to laser radiation for 10 min 2-fold surpassed the control (Figs. 1 and 2).

The maximum number of NET was formed after 5-min exposure to LILR with constant pulse generation (Table 1). Longer exposure led to a decrease in the number of NET. On the contrary, the number of neutrophils with non-differentiated nucleus increased with time. The maximum value was attained after 10-min laser irradiation of neutrophils.

Thus, exposure to LILR with constant pulse generation for 5 min and longer reduced neutrophil viability. The maximum NET formation was observed after 5-min exposure of NG to LILR: their number by 4 and 2 times surpassed the corresponding parameter in the control and after 2-min laser irradiation. The 2-fold decrease in the number of NET after 10-min exposure to LILR on NG suspension was noted.

REFERENCES

1. Yu. A. Vladimirov, *Efferent Medicine*, Ed. S. Ya. Chikin [in Russian], Moscow (1994), pp. 23-35.
2. I. I. Dolgushin and O. V. Bukharin, *Neutrophils and Homeostasis* [in Russian], Ekaterinburg (2001).
3. G. I. Klebanov, *Byull. Eksp. Biol. Med*., **123**, No. 4, 395-399 (1997).
4. A. N. Mayanskii and O. I. Pikuza, *Clinical Aspects of Phagocytosis* [in Russian], Kazan' (1993).
5. I. V. Nesterova, *Rus. J. Immunol*., **4**, 22-29 (1999).
6. I. S. Freidlin, *Immune System and Its Defects* [in Russian], St. Petersburg (1998).
7. T. A. Fuchs, U. Abed, C. Goosmann, *et al., J. Cell. Biol*., **176**, No. 2, 231-241 (2007).
8. C. F. Urban, U. Reichard, V. Brinkmann, and A. Zychlinsky, *Cell Microbiol.*, **8**, No. 4, 668-676 (2006).