STUDY OF THE EFFECT OF UV LASER PULSE DURATION AND WAVELENGTH ON FIBROBLASTS

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Abstract – We report the cytotoxic effect of pulse duration and wavelength of UVB laser radiation from Li(Lu,Y)F_4:Ce laser on human skin fibroblasts. When performing the MTT test 24 hours after irradiation, an increase in the viability parameter was observed. Flow cytometry showed that 80.8 ± 6.9% of cells have a damaged membrane and only 6.2 ± 2.2% reach the stage of late apoptosis, due to which they have the potential to restore the cytoplasmic membrane and subsequently divide.

INTRODUCTION

Vitiligo is the depigmentation disease characterized by loss of epidermal melanocytes [1]. One of the most effective methods of vitiligo treatment narrowband ultraviolet B (NB-UVB) [2] was first used in vitiligo in 1997 [3]. The used wavelength was 310-315 nm with peak emission at 311 nm. This unique wavelength is effective possibly because it can stimulate non-active epidermal melanocytes and modulate cutaneous immune system [4]. However, simultaneously with “treatment” of skin melanocytes most of other cells are negatively affected by UVB-irradiation. Reactive oxygen species (ROS) are formed in fibroblasts, that causes various damages [5]. In this work we studied new perspective methods of vitiligo treatment which will cause less or no side effects.

MATERIALS AND METHODS

Cell Culture

Human skin fibroblasts obtained from healthy donor according to the conventional protocol [10.1385/1-59259-940-0:083] (HSF) are obtained from Russian collection of vertebrate cell cultures, Russian Academy of Sciences, St. Petersburg, Russia and cultured with Dulbeco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Australia), 4 mM L-glutamine (ReagentPlus, ≥99% (HPLC)), 100 U/ml streptomycin and penicillin, and grown in a

CO_2 incubator at 37°C in 5% CO_2. Logarithmic phase cells were seeded into a 96-well plate (SPL Lifesciences) at a rate of 1 × 104 cells/well and incubated for 24 hours.

UVB irradiation

Monolayer of HSF cells (50 confluence) were irradiated using the laser radiation of Li(Lu,Y)F_4:Ce+Yb active medium, which provides generation of sub-nanosecond pulses and wavelength tuning in UVB range [6,7] with different parameters: wavelength 310 nm, 325 and 300 nm (from pump source), pulse duration 1 and 10 ns, exposition 5 and 15 minutes (0.15 mJ/cm^2). Viability of irradiated cells were measured: 1) immediately after; 2) after 24 hours. To better understand the biological effects of irradiation, we assessed the viability of HSF cells immediately after treatment. The cells were irradiated with a 325 nm laser for 15 min, then the cell suspension was stained with DiOC6 and PI and cytometric analysis was performed.

Experimental Setup

Laser was assembled based on the Li(Lu,Y)F_4:Ce+Yb active medium [6]. The characteristic length of the cavity was reduced to a size of 2.5 cm in order to reduce the photon lifetime. To change the pulse duration we changed the reflection coefficient of couplers, (Fig. 1a) and (Fig. 1b). To change the radiation wavelength, we included a dispersive prism in the cavity (Fig. 1c).

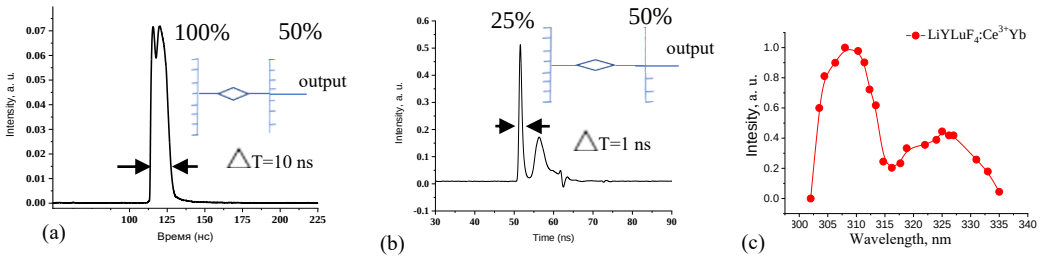


Fig. 1 Pulse signal at wavelength λ = 310 nm and duration t = 10 ns (a) Pulse signal at wavelength λ = 310 nm and duration t = 1 ns (b) Wavelength tuning (c)

Cellular Viability Assay MTT and Flow Cytometry

Cell viabilities were measured based on conversion of yellow tetrazolium salt 3-(4,5-dim-ethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, (Sigma-Aldrich, USA)) to dark blue formazan by viable cells [7]. Cells in approximately 10.000 units were plated onto each well of 96-well plates. In all variants of the study, the viability of cells not subjected to treatment was taken as 100%. Percentage of live and dead cells was also examined by flow cytometry. The cell monolayer with 50%

confluence was trypsinized, The cell suspension was precipitated by centrifugation (1500 rpm, 5 min). The supernatant was removed, the cells were resuspended in a complete DMEM medium at a concentration of 10^6 cells/ml. The propidium iodide (PI) and 3,3'-Dihexyloxacarbocyanine Iodide ($DiOC_6(3)$) were added to each sample [8].

Results and Discussion

The effect of pulse duration on cells viability

We have discovered relation in cells viability depending on time of irradiation, pulse duration and timing of the MTT Assay. Cells viability right after irradiation at wavelength 310 nm is shown in (Fig. 2a), the viability of HSF cells in the tested samples has decreased in comparison with the control by 8±1,5%, 11±0,5% and 20±1,5% for 10 ns (5 min), 10 ns (15 min) and 1 ns (15 min). However, viability 24 h after irradiation was increased: by 4±1,5% for10 ns (5 min), 3±1,5% for 10 ns (15 min) and 1±2,5% for 1 ns (15 min) (Fig. 2b).

It was found that UVB treatment led to disruption of the integrity of the cytoplasmic membrane of cells and, to a lesser extent, the mitochondrial membrane. The proportion of cells with uncompromised cytoplasmic and intact mitochondria after irradiation was 0.4 ± 0.1%, while in the variant without treatment the value of this indicator was 94.8 ± 5.3%. The proportion of cells with a disrupted cytoplasmic membrane but high mitochondrial potential immediately after treatment was 80.8 ± 6.9% ((Fig. 2d). However, the results of the MTT test do not demonstrate a dramatic decrease in viability in the wells where the cells were exposed to irradiation, which suggests the restoration of membrane systems damaged by UV treatment.

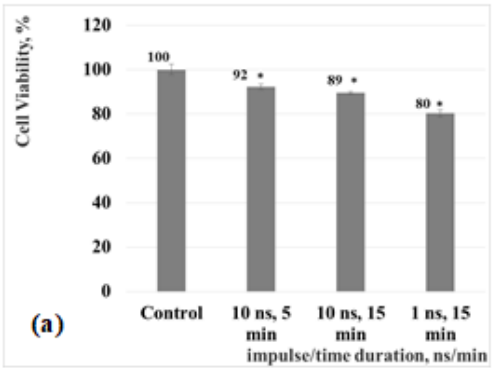
The effect of various wavelengths on HSF cells viability

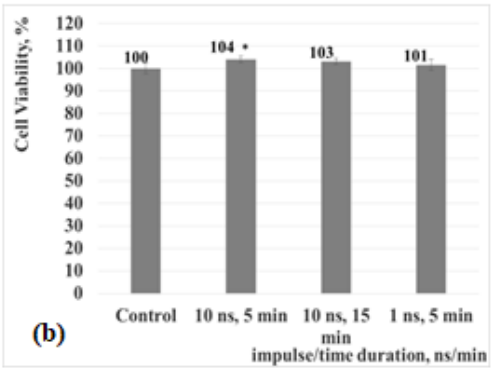
As shown in (Fig. 2c) viability of cells has changed over time and in dependence on wavelength. After 24 h viability of HSF cells that were irradiated at wavelength 300 and 310 nm and 10 ns pulse duration was less than control, and was equal to 97±3% and 99±1%. Whereas, in the last sample (325 nm) viability was increased quite a lot and was equal to 110±3%.

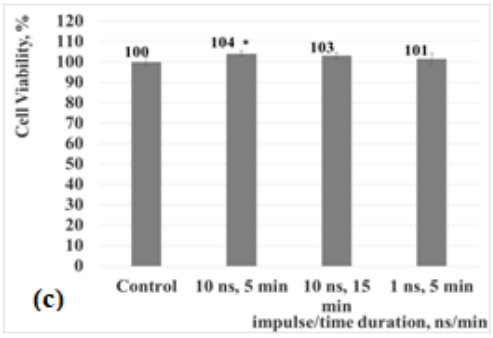
Flow Cytometry

For better understanding of the 24h viability increase we have investigated the 325 nm irradiation during 15 minutes effect. From data we have received, 85,6 %

of cells have damaged membrane and about 69,9% have healthy mitochondria compared with control sample (Fig. 2d) what goes together with increasing of viability after 24 h in MTT assay. The significance of differences between data groups was determined by the nonparametric Mann-Whitney test. Differences were considered significant at $p \leq 0.05$.







Population	Irradiation, 325 nm, 15 min	Control
DiOC$_6$+/PI-	0,4 ± 0,1 %	94,8 ± 5,3 %
DiOC$_6$-/PI-	12,6 ± 3,8 %	2,8 ± 0,9 %
DiOC6-/PI+	6,2 ± 2,2 %	2,1 ± 1,4 %
DiOC6+/PI+	80,8 ± 6,9 %	0,3 ± 0,1 %

(d)

Fig.2 Cell viability right after irradiation * - $p<0.05$ (a) Cell viability 24 hours after irradiation * - $p<0.05$ (b) Effect of different wavelength to HSF cells viability 24 hours after irradiation * - $p<0.05$ (c) Table of the results of flow cytometry, DiOC6+PI- (alive) I, DiOC6-PI- (with damaged mitochondria) II, DiOC6-PI+ (dead, late apoptosis) III, DiOC6+PI+ (perforated cytoplasmic membrane) IV (d)

Conclusion

In this work, the effect of UV laser radiation of different wavelengths, pulse durations and irradiation times on human skin fibroblast cells HSF was investigated. The results obtained from the MTT test immediately after irradiation show a decrease in the viability of HSF cells, depending on the wavelength, pulse duration and irradiation time, which are significantly different from the sample without treatment. When performing the MTT test 24 hours after irradiation, an increase in the viability parameter was observed. From flow cytometry investigation directly after irradiation. It was found that 80.8 ± 6.9% of cells have a damaged membrane and only 6.2 ± 2.2% reach the stage of late apoptosis, due to which they have the potential to restore the cytoplasmic membrane and subsequently divide. We observed confirmation of this hypothesis 24 hours after irradiation using the MTT test for cells irradiated with a

laser with a wavelength of 325 nm for 15 minutes; cell survival was 106 ± 4%, which is significantly different from the sample without treatment.

Acknowledgments

Authors are thankful for RSF 20-73-10029 project of SPbSU in the frame of which cells investigation was carried out. The UV laser was elaborated within FZSM-2022-0021 project.

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