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BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

Impact of Tightly Focused Femtosecond Laser Pulses on Nucleolus-Like Bodies of Mouse GV Oocyte and the Ability of Mouse Oocytes to Mature

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Abstract—Using femtosecond laser radiation, nucleolus-like bodies (NLBs) of mouse oocytes were locally dissected without damage to zona pellucida, cytoplasmic membrane, nuclear membrane, and nucleoplasm surrounding NLB. It was found that, after dissection of 2.7×10^{-11} cm^3 of NLB material, which is approximately 5.2% of 10 μm NLB volume, the probability of germinal vesicle oocyte development to metaphase II stage of meiosis decreased 3–7 times compared to the non-treated oocytes. This result indicates that NLB material organization is significant for mouse oocyte maturation.

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A characteristic feature of the germinal vesicle (GV) of oocytes of all mammals is the presence of large (up to 10 μm in diameter) organelles that are called the "nucleolus-like bodies" (NLBs) or "post-nucleoli" [1–5, 14]. In less mature GV of oocytes, NLBs can transcribe ribosomal genes (rDNA) [6]. However, before ovulation, rRNA synthesis in NLBs stops and NLBs acquire a homogeneous fibrillar structure and increase in size up to 10 μm [7]. Immunocytochemical studies showed that NLBs contain rDNA transcription factors (RNA polymerase I, UBF) and rRNA processing factors (fibrillarin, NPM1/nucleophopmin, and nucleolin) [2, 7–9]. It was reported that the NLB material plays an important role in the embryo development after the egg fertilization [6]. However, the currently available data on the role of NLB in the developmental biology of oocytes do not allow to understand in detail their role in mammalian oogenesis [8].

To elucidate the role of NLB in the maturation of oocyte GVs, in this study we for the first time used femtosecond laser nanosurgery. The unique advantage of this technique is the possibility to influence NLBs without damaging the zona pellucida, the cytoplasmic membrane, the nuclear envelope, and the oocyte nucleus contents.

To obtain oocytes, we used 1.5- to 2.5-month-old female hybrid mice (CBA × C57BL/6)F1 (Pushchino animal breeding facility, Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Pushchino Branch, Russian Academy of Sciences). The oocyte isolation protocol is described in [9]. Cumulus-free oocytes were placed in 50 μL of M2 medium on a coverslip. Oocytes of the control group (without laser irradiation) were placed nearby in the same volume of the medium. The time of manipulations with the sample during laser irradiation did not exceed 1 min.

Irradiation procedure. Femtosecond laser radiation (duration 100 fs, wavelength 80 nm, pulse repetition frequency 80 MHz) with a Gaussian mode is focused with a lens of 60×, NA = 0.7 μm in a spot with the following parameters: the laser beam waist $w_0 = 0.61\lambda$, NA = 0.68 μm, Rayleigh parameter $z_0 = kw_0^2/2 =$ 1.86 μm ($k = 2\pi/\lambda_0$, wave number, Fig. 1). Femtosecond laser radiation was focused to the central region of NLBs in the form of a pulse train with a train repetition frequency of 80 MHz. The pulse train duration was regulated with a Thorlabs SH05 electromechanical shutter and was 15–60 ms. NLBs were exposed to laser radiation 5 times. Changes in morphology of the object after laser irradiation were recorded as a video file using a Thorlabs DCC1545M camera (Thorlabs Inc., United States). Quick temporal resizing of the disturbance area, generated by the femtosecond laser,

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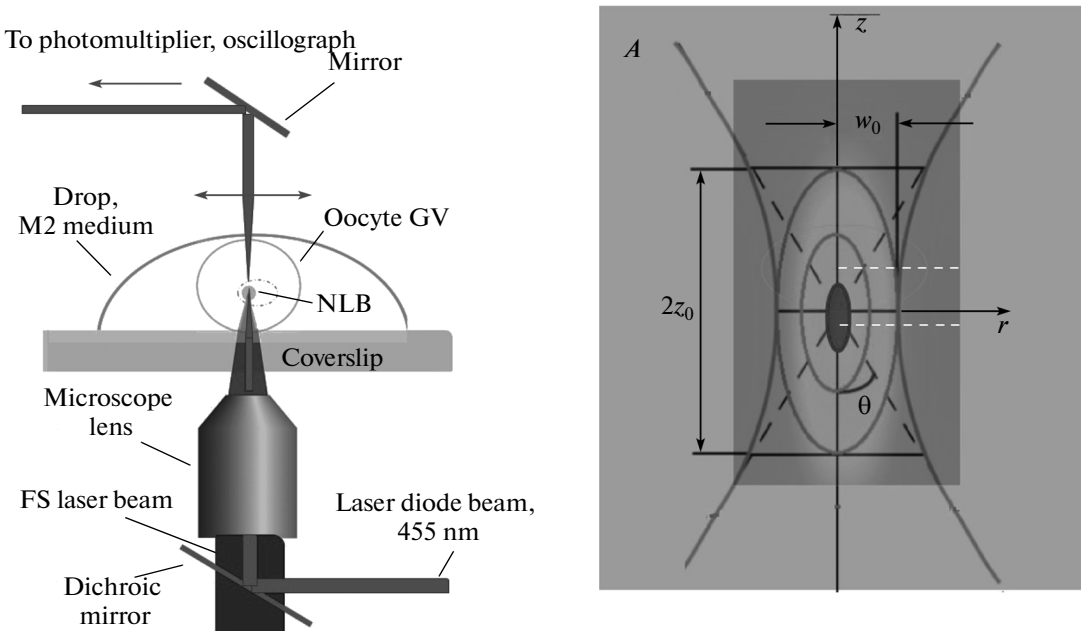

Fig. 1. Principal optical scheme. The distribution of the intensity of a laser beam with a Gaussian mode, which was focused with a lens.

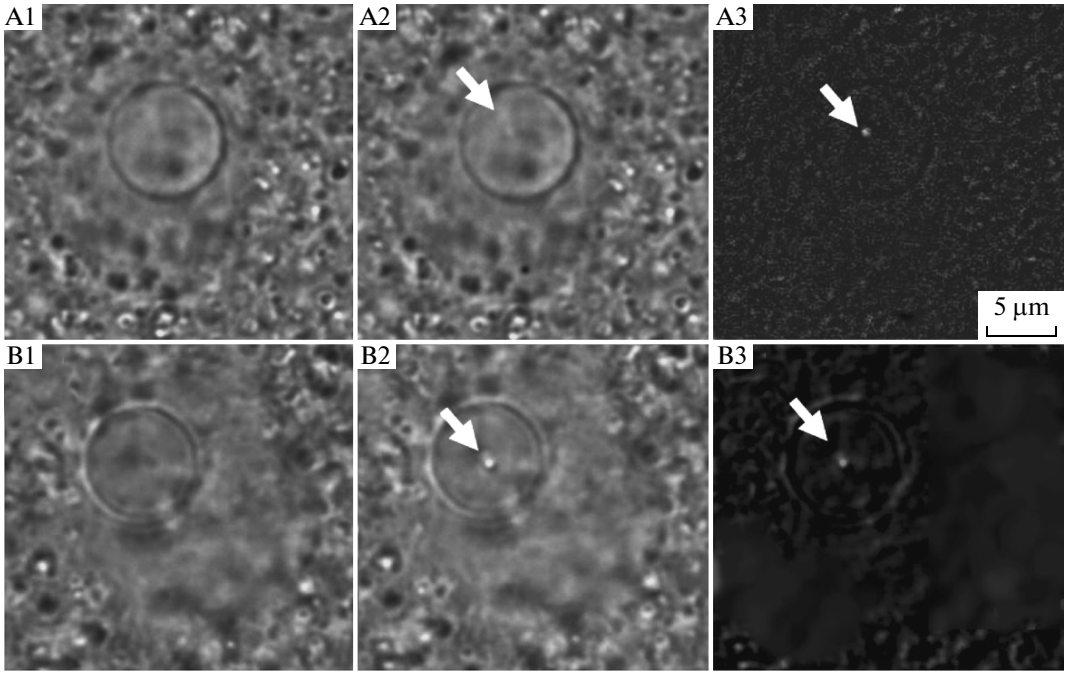

Fig. 2. NLB images before (A1 and B1) and after the exposure to femtosecond laser radiation (A2 and B2) at a pulse energy of (A) 0.5 and (B) 2 nJ. The pulse train duration was 60 ms. Iimages A2 and B2 were obtained 15 s after irradiation. The difference image A3 and B3 was obtained by numerical subtracting from the NLB image after exposure (A2, B2) of the image taken before exposure (A1, B1), respectively.

was measured by the light scattering intensity of the laser diode (445 nm, 100 μW), which was focused on the waist area of the femtosecond laser using a photomultiplier and a WaveSurfer MSO 64MXs-B digital oscilloscope (Teledyne LeCroy, United States). The scheme of the instrument was described in detail in [11].

After irradiation, the oocytes were cultured in a CO_2-incubator (5% CO_2, 37°C) [10]. The effect of

Dependence of oocyte development on the parameters of femtosecond laser irradiation of NLBs

No.	Pulse energy; pulse train duration	Exposure—the energy of the incident laser light on NLB	Number of oocytes in group	NLB preserved	NLB dissolved, the polar body is not observed	Polar body separation	Oocyte degradation
1	0.5 nJ, 15 ms	0.6 mJ	20	5/20 (25%) $P = 0.023$	8/20 (40%) $P = 0.31$	7/20 (35%) $P = 0.009$	0
2	0.5 nJ, 30 ms	1.2 mJ	22	5/22 (23%) $P = 0.049$	13/22 (59%) $P = 0.013$	4/22 (18%) $P = 0.0001$	0
3	0.5 nJ; 60 ms	2.4 mJ	22	5/22 (23%) $P = 0.049$	14/22 (64%) $P = 0.006$	3/22 (13%) $P = 2.9 \cdot 10^{-5}$	0
4	1 nJ, 15 ms	1.2 mJ	22	6/22 (27%) $P = 0.021$	10/22 (45%) $P = 0.11$	5/22 (23%) $P = 0.0005$	1/22 (5%) $P = 1$
5	1 nJ, 30 ms	2.4 mJ	22	6/22 (27%) $P = 0.021$	11/22 (50%) $P = 0.057$	5/22 (23%) $P = 0.0005$	0
6	1 nJ, 60 ms	4.8 mJ	20	5/20 (25%) $P = 0.023$	7/20 (35%) $P = 0.48$	6/20 (30%) $P = 0.003$	2/20 (10%) $P = 0.48$
7	2 nJ, 15 ms	2.4 mJ	20	6/20 (30%) $P = 0.028$	9/20 (45%) $P = 0.17$	5/20 (25%) $P = 0.001$	0
8	2 nJ, 30 ms	4.8 mJ	22	10/22 (45%) $P = 0.0005$	3/22 (14%) $P = 0.69$	5/22 (23%) $P = 0.0005$	4/22 (18%) $P = 0.11$
9	2 nJ, 60 ms	9.6 mJ	19	7/19 (37%) $P = 0.003$	9/19 (47%) $P = 0.009$	2/19 (11%) $P = 1.7 \cdot 10^{-5}$	1/19 (5%) $P = 0.48$
Control			20	0/20 (0%)	4/20 (20%)	16/20 (80%)	0/20 (0%)

irradiation on the oocyte development was evaluated the next day. The separation of polar bodies, indicating the completion of oocyte maturation, was observed by differential interference-contrast microscopy, by the light field methods and fluorescence microscopy after staining with the chromatin dye Hoechst 33342 (Carl Zeiss Auxion Vision, Germany, lens ×20, numerical aperture NA = 0.4). In total, 209 oocytes were analyzed (Table 1).

The images of NLBs before and after exposure to laser radiation are shown in Fig. 2. The NLB tissue section on the difference image (difference between two images in the numerical form before and after laser irradiation) looks like a light spot localized in the lens focus. The black field on the difference image indicates that the difference between images before and after exposure is close to zero (i.e., changes in the nucleoplasm morphology at the exposure modes used are practically absent).

The analysis of images of the entire oocyte shows the preservation of membranes and zone pellucida. The localization of laser radiation is determined by the fact that the wavelength of 780 nm gets into the transparent region of the biological tissue, and the linear light absorption is negligible. At femtosecond pulse energies of 0.5 to 2 nJ, the laser light intensity ranges from 3.4×10^{11} to 13.6×10^{11} W/cm^2, respectively. At such intensity the oocyte, which is clear at a wavelength of 780 nm, absorbs n quanta of light with the probability $P_n = \sigma_n I^n$, where $n \geq 2$ and σ_n is the absorption cross-section for the n-photon process. The absorption of laser pulse energy, which is most significant in the maximum intensity area (i.e., near the focus of the lens), as well as the n-photon absorption of laser radiation by NLBs lead to multi-photon dissociation and/or photoionization and destruction of NLB material. Photoionization means the formation of low-density plasma [12]. Plasma formation is confirmed by the observation of a broadband structureless luminescence spectrum with a maximum at approximately 500–600 nm from the laser pulse waist area in NLB. The NLB material was incised at a light intensity of 3.4×10^{11} W/cm^2 and above. The threshold of water breakdown by the femtosecond pulse was in the range of 6.6 to 9.0×10^{12} W/cm^2 [13]. The destruction of NLB material at a relatively low-intensity laser radiation was apparently due to the low-lying energy levels of protein and RNA molecules (the edge of absorption bands was close to 300 nm). The light absorption by these protein and RNA molecules is possible due to the photon process even at $n = 3$, whereas the water breakdown is observed at $n = 5$.

The local diameter of an NLB section depends on the pulse energy and pulse train duration (Fig. 3). The intensity is proportional to the single pulse energy, and the dose of the incident energy on the NLB is proportional to the product of the energy of a single pulse and the train duration. Since the damaged area diameter in Fig. 3 is less than 1 μm, the waste diameter $2w_0$ and the Z_R parameter can be used to estimate the upper

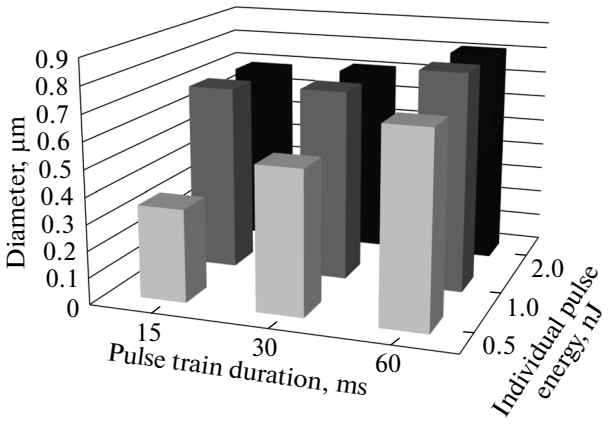

Fig. 3. Dependence of the incision diameter in NLB on the pulse energy and train duration.

volume of the NLB section, which is equal to 2.7×10^{-11} cm^3. At an NLB diameter of 10 μm the NLB volume is 5.24×10^{-10} cm^3. The proportion of the damaged NLB material does not exceed 5.2% by volume.

After exposure to the laser pulse, the size of the NLB section changes in time. The video images recorded a decrease in the size of the NLB damage area after laser irradiation as compared to the residual equilibrium size. The characteristic time of this process is close to 2 s. The dynamics of changes in the size of the laser incision in the NLB material at a nanosecond time resolution can be estimated by the time dependence of the intensity of the probing light with a wavelength of 445 nm, which is dispersed in the femtosecond laser focus area after a single pulse (Fig. 4). The optical heterogeneity, generated by a laser pulse in the NLB material, modulates the scattered light intensity at 445 nm. As can be seen from Fig. 4, the probing light scatter level is characterized by an increase for less than 100 ns and a scattering signal decrease after 500 ns; the signal drops to zero to a delay time of 2 μs. Oscillation damped by 1.5 μs can be observed on the kinetic curve of the probing pulse signal. Fourier analysis of the oscillatory component in Fig. 4 revealed a set of frequencies in the range from 15 to 100 MHz. By the value order, this range is close to the frequency of acoustic oscillations of a water bead with a diameter $d = 10$ mm, which is equal to the value $v/d = 150$ MHz, where v is the speed of sound in water (~1.5×10^5 cm/s).

Thus, the formation of a section in NLB under exposure to a femtosecond laser pulse is accompanied by mechanical stress waves, which are manifested in the form of damping acoustic oscillations.

Data on the oocyte development after the laser irradiation are presented in table as a function of pulse intensity and the pulse train duration. After the end of culturing, there are four possible scenarios: (1) NLBs were retained; (2) NLBs were dissolved but the polar bodies were not separated; (3) the polar body was separated, and the MII stage was reached; and (4) the oocyte degraded. Data summarized in Table 1 show that the probability of an oocyte to develop to the MII stage significantly decreases and the probability of

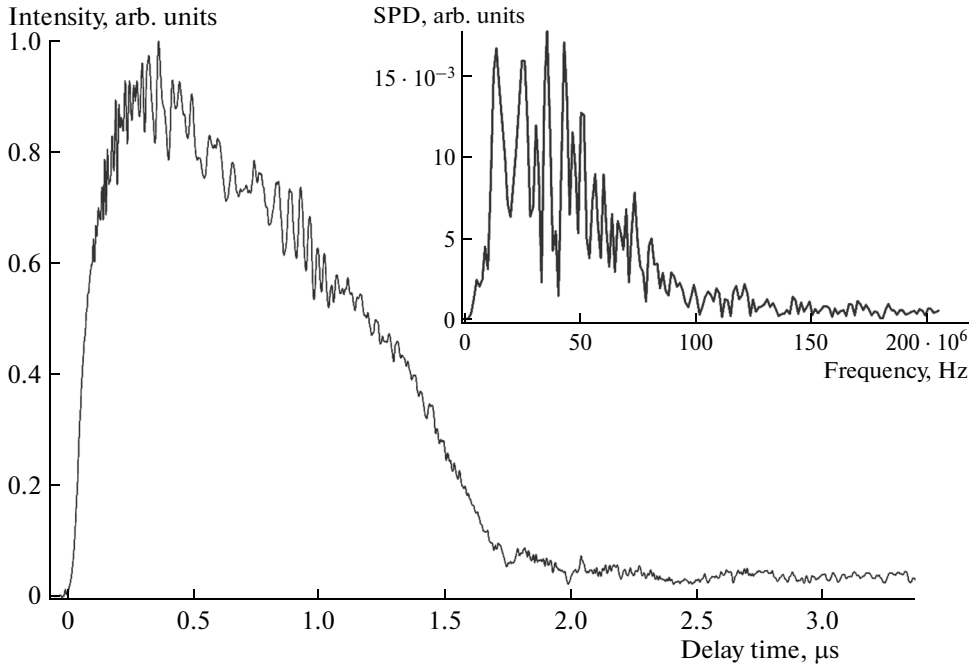

Fig. 4. Time dependence of the scattering signal of the probing light beam passing through the NLB in the femtosecond laser pulse zone. The inset shows the spectral power density (SPD) of the Fourier transform of the oscillating component.

development cessation increases (NLB does not dissolve) if NLB is exposed to laser radiation. The significance of differences between the experimental and control groups in the realization of different scenarios was estimated using exact Fisher test. This method of estimating differences between groups is best suited for small samples. The *P* value characterizes the significant interval of deviations from the null hypothesis; for estimating biological effects, difference is taken significant at a significance level $p \leq 0.05$. The probability of irradiated oocytes to develop to the MII stage significantly decreased compared to the control cells (3 to 7 times at a laser injury level not more than 5.2% of the NLB volume). This fact indicates that NLBs of oocytes that reached the normal size play an important role in mouse oogenesis if the transcription activity in them is low or absent.

To summarize the study, the following conclusions can be made. The local section of NLB material is due to the nonlinear optical absorption of the laser radiation, which is accompanied by multiphoton ionization and dissociation of NLB molecules in the focus area of femtosecond radiation with a wavelength of 780 nm. After the NLB material incision, femtosecond laser pulse initiates damping acoustic oscillations in the NLB. The incision in the NLB reduces the probability of development of oocyte GVs to the MII stage, which indicates an important role of the NLB material organization in mammalian oogenesis, despite the low transcriptional activity of NLB in oocytes prior to ovulation.

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