

Effect of Laser Optoperforation of the Zona Pellucida on Mouse Embryo Development *in vitro*

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Abstract—The effect of laser optical perforation of the zona pellucida on the viability and development of mouse embryos has been studied. Operations of zona pellucida thinning and single or double perforation were carried out on 2-cell embryo, morula, and blastocyst stages with a laser pulse (wavelength 1.48 μm , pulse duration 2 ms). Embryo development up to the blastocyst stage and hatching efficiency were statistically analyzed. It was found that 2-cell or morula stage embryo zona pellucida thinning or single perforation did not affect development to the blastocyst stage and number of hatched embryos, but it accelerated embryo hatching compared to control groups one day earlier *in vitro*. Double optoperforation on 2-cell embryo or morula stage did not significantly affect development to the blastocyst stage, but it strongly decreased the number of hatched embryos. Also, zona pellucida perforation at the blastocyst stage had a negative effect: hatching did not occur after this manipulation. Blastocyst cell number calculation after single zona pellucida perforation at 2-cell and morula stages showed that cell number of hatching or hatched blastocysts did not differ from the same control groups. This fact points out that the laser single optoperforation method is a useful and safe experimental tool that allows further manipulations within the zona pellucida.

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Laser operations on cells and embryos are an important field of current photobiology and biophotonics. The high power density of tightly focused laser irradiation provides an efficient impact on matter of cells or embryos. Precise focusing of the laser spot allows strictly controlled perforation of the membrane. The present work was devoted to studying the influence of optoperforation of mammalian embryonic zona pellucida with a tightly focused laser beam with 1.48- μm wavelength on further development of the embryo. Such a laser operation was proposed for application in *in vitro* fertilization (IVF) practice and intracytoplasmic sperm injection into the oocyte (ICSI). For cultured *in vitro* oocytes and embryos, the process of natural exiting from the zona pellucida (“hatching”) is often impaired, which decreases probability of implantation and pregnancy [1]. Assisted

hatching – an artificial disruption of the embryo zona pellucida integrity during mechanical or chemical action – was proposed for increasing embryo implantation into the endometrium of the uterus [2]. Laser perforation of the zona pellucida is the most recently described method for assisted hatching [3, 4]. The laser provides high precision of action, sterility, and minor invasiveness, which makes the laser a convenient tool for performing micromanipulations. However, there are ambiguous results of using such methods. It was reported that frequency of pregnancy was significantly increased when using assisted hatching [5, 6]. At the same time, negative effect of the method was also reported [7]. Some reports indicate that there is no difference in groups with or without artificial perforation [8, 9].

The goals of the present work were to determine the influence of different manipulations on development of embryos *in vitro* until blastocyst formation and on the

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ability of blastocysts to exit zona pellucida, and the efficiencies of different laser-assisted hatching methods. Cell counting was performed to estimate quality of blastocysts developed *in vitro* after laser perforation.

MATERIALS AND METHODS

Obtaining embryos. Female mice of C57BL/5 strain and male mice of CBA strain were used in the experiments. All the animals were 1.5–2.0-month-old. To obtain sufficient numbers of embryos, females were stimulated by hormones. Hormones used to reach superovulation were pregnant mare serum gonadotropin (Intervet, USA) and human chorionic gonadotropin (Intervet). The hormones (10 U.I.) were injected intraperitoneally (i.p.) with 48-h intervals, usually in the evenings (4–6 p.m.). This time was chosen because ovulation in mice begins in 10–13 h after chorionic gonadotropin injection, this time approximately corresponding to the time of copulation (late night–early morning). Embryos were obtained at the 2-cell stage (second day after fertilization) by washing the oviducts with M2 medium (Sigma, USA). Manipulations with embryos were performed in a drop of M2 medium on the cover slip. Embryos were cultured in an incubator in four-well dishes (Nunc, USA) with M16 medium at 5% CO₂ and 37°C.

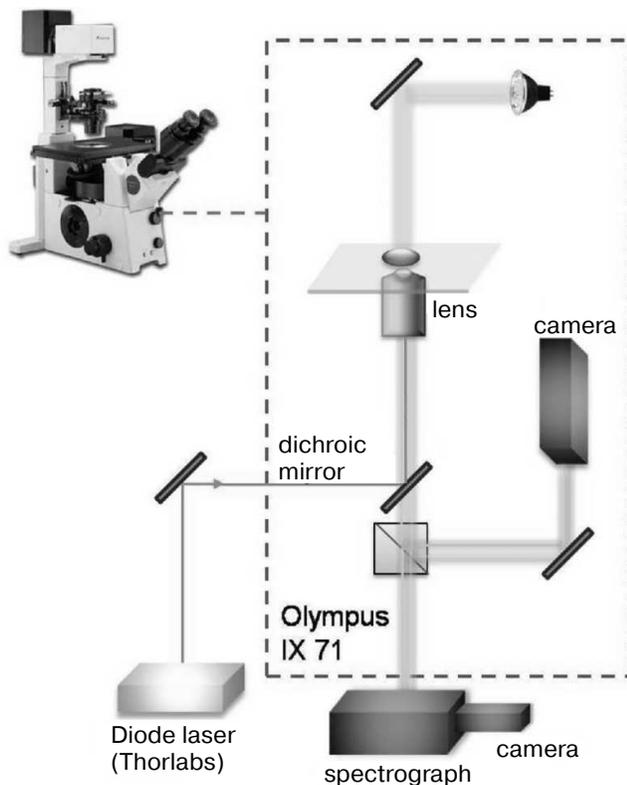


Fig. 1. Schematic diagram of the laser device for perforation. Elements of the device are indicated on the figure.

Laser perforator. Microsurgical operations were performed with a laser manipulator device designed in the Institute of Chemical Physics, Russian Academy of Sciences. The scheme of the optical block used in the experiment is shown in Fig. 1. Diode laser irradiation (Thorlabs, USA; wavelength $\lambda = 1.48 \mu\text{m}$, output power 500 mW, laser irradiation power at the object plane was 110 mW) was supplied via an optical fiber with microlens on the end into the optical path of an inverted microscope OLYMPUS IX71 through a dichroic mirror. Irradiation was supplied as a pulse and/or series of distinct pulses. Pulse duration was varied from 50 ns to 5 s using a controlled laser power supply that was developed based on an impulse generator (Thorlabs). In all the perforation experiments, pulse duration was 2 ms. Laser irradiation was focused with an Olympus 60 \times LUCPLFLN lens with numerical aperture NA = 0.7. The beam diameter was $2w_0 = 1.22 \lambda/\text{NA} = 2.58 \mu\text{m}$; depth of focus was $z_0 = (\pi w_0)^2/\lambda = 3.53 \mu\text{m}$ [10]; energy density at the focus area was $4.2 \cdot 10^3 \text{ J/cm}^2$. Absorption coefficient of water for wavelength 1.48 μm is 0.003 cm^{-1} [11], and the typically used pulse duration of 2 ms led to temperature jump $\sim 75^\circ\text{C}$ at the cell constriction (spot 2.58 μm in diameter). The X,Y-coordinates of the stage of the microscope and duration of laser pulses were computer-controlled. Microsurgery of embryonal zona pellucida was performed at the most distant from blastomeres area to prevent blastomere injury by the heat wave from the cell constriction spot after the laser pulse. The used laser pulse led to formation of round perforation 2 μm in size. According to the program, the laser spot moved over the zona pellucida field, impulse was applied and, thus, the layer perforation occurred sequentially. Laser perforation operations were made on the cover slip where embryos were placed into a 50- μl drop of M2 medium. After laser action, embryos were transferred to four-well dishes with M16 medium and cultured in a CO₂ incubator. The operation did not last more than 2 min, and thus effects related to drop evaporation were insignificant.

Number of cells in blastocysts was estimated by two methods: making air-drying preparations using the Tarkowski method and Hoechst staining with subsequent visualization under a confocal fluorescence microscope. For making air-drying preparations by the Tarkowski method [12], we used hypotonic NaCl solution, methanol–glacial acetic acid mixture (3 : 1), and 7% solution of Giemsa stain (PanEko, Russia). The embryo was placed into a drop of hypotonic NaCl solution on the cover slip for ~ 5 min. When the hypotonic solution was almost completely evaporated, a drop of fixing solution (methanol–glacial acetic acid mixture) was applied over the embryo. After evaporation of the fixing mixture, the cover slip was transferred into 7% solution of Giemsa stain for 15 min. Then, nuclei of blastocysts were counted under the microscope.

Confocal microscopy. Hoechst 33342 (Sigma, USA) staining was performed in M16 medium in a CO₂ incuba-

tor in 5 $\mu\text{g/ml}$ dye solution for 10–20 min. Then the embryos were washed with dye-free medium, and fluorescence was registered. Fluorescence in the hatching blastocysts was localized using a Carl Zeiss LSM-710-NLO confocal laser-scanning microscope (Jena, Germany). Samples from the *in vitro* culturing dishes were transferred to sterile Petri dishes with thin glass bottom (0.16 mm thick) in the M2 manipulation medium. Images were obtained using a Plan_Apochromat lens with 20 \times magnification (aperture 0.8). Two-photon excitation of Hoechst 33342 fluorescence was executed by a Chameleon Ultra II femtosecond laser (Coherent, USA) with wavelength 770 nm and maximum power density 25 mW. Fluorescence was registered at 400–550 nm with confocal diaphragm 600 μm in diameter. To obtain images in transmitted light, a CW laser at 561 nm was used. Image dimensions were 512 \times 512 pixels (0.58 nm/pixel), and scanning speed was 1.58 $\mu\text{s/pixel}$. For 3D reconstruction of blastocysts, series of images every 5.3 μm in the vertical direction were taken.

The following operations for laser optoperforation of zona pellucida were performed:

– *thinning of 0.4-rad of zona pellucida arch*. In this case, the zona pellucida of embryos at 2-cell stage was thinned from outside with laser with arch length of 20 μm to depth of half-layer thickness. Both experimental and control groups included 30 embryos;

– *thinning of π -rad of zona pellucida arch*. In this experiment, we thinned half of the zona pellucida circle (π rad, or 180°). Operations were made on 2-cell embryos and at the morula stage. Perforation result is shown in Fig. 2. There were 30 embryos in both experimental groups and 26 embryos in the control group;

– *single laser perforation of zona pellucida*. We made one perforation 20 μm in diameter through the zona pellucida depth. Operations were made on 2-cell embryos and at the morula stage. The results are presented in Fig. 2. The experimental group with perforation at the stage of two blastomeres included 33 embryos, the experimental group with perforation at the morula stage – 37 embryos, and the control group – 30 embryos;

– *double laser perforation of zona pellucida*. In this experiment, we made two perforations at the opposing sides of the embryo. Operations were made on 2-cell embryos and at the morula stage. In the first experimental subgroup, 2-cell embryos were perforated with perforation area size of 10 μm . In the second subgroup, embryos at the morula stage were perforated likewise. In the third subgroup, two perforations 20 μm in diameter were made in the morula stage embryos. The perforation result is shown in Fig. 2. There were 30 embryos in each group;

– *single laser perforation of zona pellucida of embryo at the blastocyst stage*. In this experiment, the effect of zona pellucida laser perforation on the fifth day of embryonal development at the blastocyst stage was studied. Embryos

were extracted from murine oviducts on day 2 of embryonal development at the 2-cell stage, and they were cultured in the M16 medium until day 5. On day 5, blastocysts were subjected to laser irradiation (perforation diameter was about 20 μm). The control group was cultured until day 6. There were 30 embryos in each group. The control group embryos were not subjected to laser action.

Statistical analysis of the results was performed using STATISTICA 6.0 software. To validate statistical reliability, the z -test was used with significance level $p = 0.05$.

RESULTS AND DISCUSSION

Embryos at different developmental stages – hatching or hatched blastocysts – are depicted in Fig. 3. During normal development, blastocyst formation *in vivo* occurs on day 4 (here, the day that follows fertilization occurring overnight or early in the morning is considered day 1). Hatching of blastocyst from the zona pellucida and its implantation into the uterus happens on day 5. *In vitro* developments proceeds with about 12-h delay, and on day 5 we see expanded blastocysts and on day 6 – hatching from zona pellucida [13]. The table includes the results of counting embryos that reached blastocyst stage (5th day of development), the embryos hatching on day 5, and the embryos that hatched on day 6 after several types of optoperforation: (i) thinning of 0.4 rad of zona pellucida arch; (ii) thinning of π rad of zona pellucida arch; (iii) single laser perforation of zona pellucida; (iv) double laser perforation of zona pellucida.

Analysis of these data reveals that optoperforation does not affect development of embryos until the blastocyst stage (5th day of development), significantly affecting the number of hatching blastocysts on day 5. Unlike the control groups where hatching of blastocysts from zona pellucida was not observed, hatching from zona pellucida occurred in groups with optoperforations. On day 6, number of embryos completely hatched from the zona pellucida was equal for control group and groups with single perforation and layer thinning.

Double perforation significantly reduces the number of hatching embryos. Hatching of embryo from the zona pellucida, occurring on day 5, stops, and the number of completely hatched blastocysts was small. This negative effect emerged more clearly in the case of double 10- μm perforation. In the case of double perforation at the 2-cell stage, only 3.3% of embryos hatched (1/30), and 0% of embryos hatched in the case of perforation at the morula stage (0/30). In the control group, 30% of blastocysts were completely hatched (9/30). In the group where 20- μm perforations were made at the morula stage, 10% (3/30) of blastocysts were completely hatched. For hatching, embryos predominantly used only one of the two perforations. Injurious effect of double perforation observed in our work corresponds to the data of work [14], where it

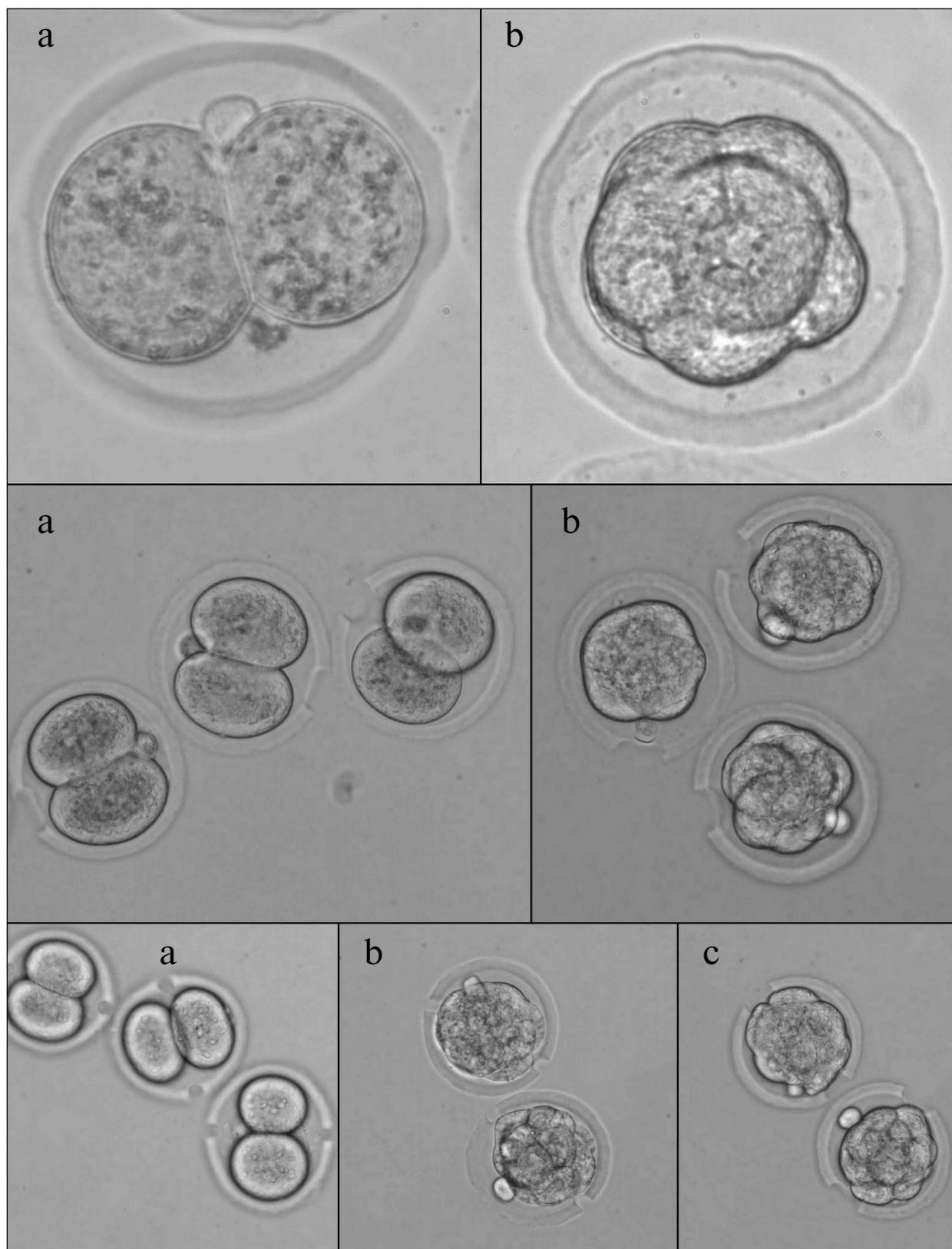


Fig. 2. Image of murine embryos subjected to optoperforation. Upper row: operation of thinning of π radians segment of zona pellucida arch; second row: operation of single perforation; third row: operation of double perforation. a) 2-cell embryo; b) morula stage embryo; c) hatched embryos.

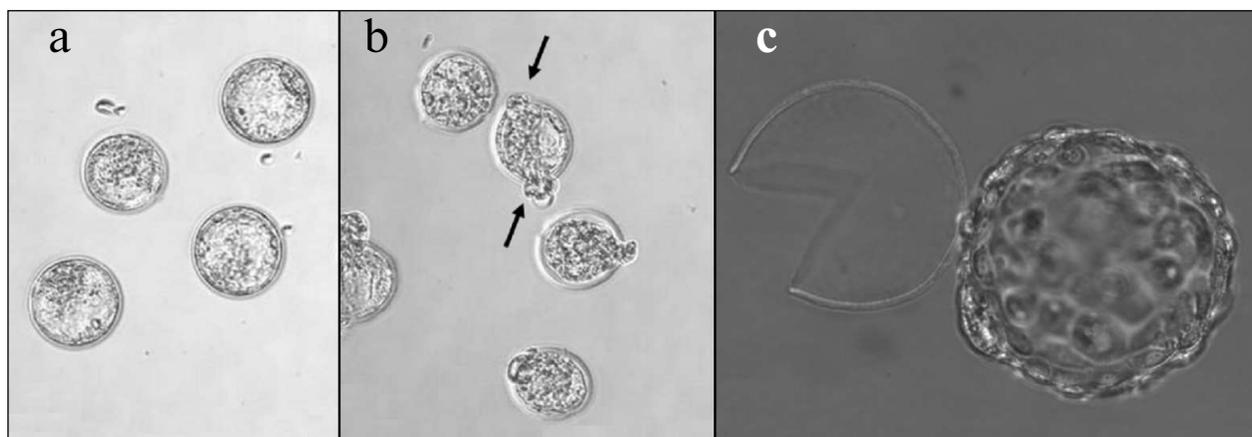


Fig. 3. Image of embryos at different developmental stages. a) Blastocyst in control group (5th day of development); b) hatching blastocyst (5th day of development). Exiting of blastocyst from two perforations is indicated with arrows; c) hatched embryo.

Results of embryo development after optoperforation

Embryo group/ <i>n</i> sampling	Blastocysts on day 5	Hatching on day 5	Hatched on day 6
Thinning of 0.4-rad segment of zona pellucida arch			
2-cell stage/ <i>n</i> = 30	93.3% (28/30)	6.6% (2/30)	33.3% (10/30)
Control/ <i>n</i> = 30	90.0% (27/30)	0% (0/30)	30.0% (9/30)
Thinning of π -rad segment of zona pellucida arch			
2-cell stage/ <i>n</i> = 30	90.0% (27/30)	0.00% (0/30)	30.0% (9/30)
Morula stage/ <i>n</i> = 30	96.6% (29/30)	26.6% (8/30)*	46.7% (14/30)
Control/ <i>n</i> = 26	92.3% (24/26)	0.00% (0/26)	26.9% (7/26)
Single laser perforation of zona pellucida			
2-cell stage/ <i>n</i> = 33	90.9% (30/33)	84.8% (28/33)*	30.3% (10/33)
Morula stage/ <i>n</i> = 37	91.8% (34/37)	67.5% (25/37)*	32.4% (12/37)
Control/ <i>n</i> = 30	93.3% (28/30)	0.00% (0/30)	30.0% (9/30)
Double laser perforation of zona pellucida			
Two 10- μ m perforations at the 2-cell stage/ <i>n</i> = 30	80.0% (24/30)	46.6% (14/30) from one perforation	3.3% (1/30)
Two 10- μ m perforations at the morula stage/ <i>n</i> = 30	90.0% (27/30)	63.3% (19/30): 60% (18/30) from one perforation; 3% (1/30) from two perforations	0.00% (0/30)
Two 20- μ m perforations at the morula stage/ <i>n</i> = 30	93.3% (28/30)	58% (18/30): 56% (17/30) from one perforation; 3.3% (1/30) from two perforations	10.0% (3/30)
Control/ <i>n</i> = 30	93.3% (28/30)	0.00%	30.0% (9/30)
Laser perforation of blastocyst zona pellucida			
Blastocyst/ <i>n</i> = 30			0.00% (0/30)
Control/ <i>n</i> = 30			36.6% (11/30)

* Significant difference.

was reported that two 12-15- μm perforations in the zona pellucida at the zygote stage hinder complete hatching of the blastocyst. All the embryos used both perforations for hatching and then degraded.

After the zona pellucida perforation at the blastocyst stage, hatching of the operated embryos was not observed, while embryos of the control group hatched from the

zona pellucida much more successfully: 36.6% (11/30), $p < 0.05$.

Cells in blastocysts after a single perforation were counted. Mean numbers of cells after perforation in different experimental groups are shown in Fig. 4. Statistical analysis revealed significant decrease in cell number in blastocysts that have not hatched from the zona pellucida

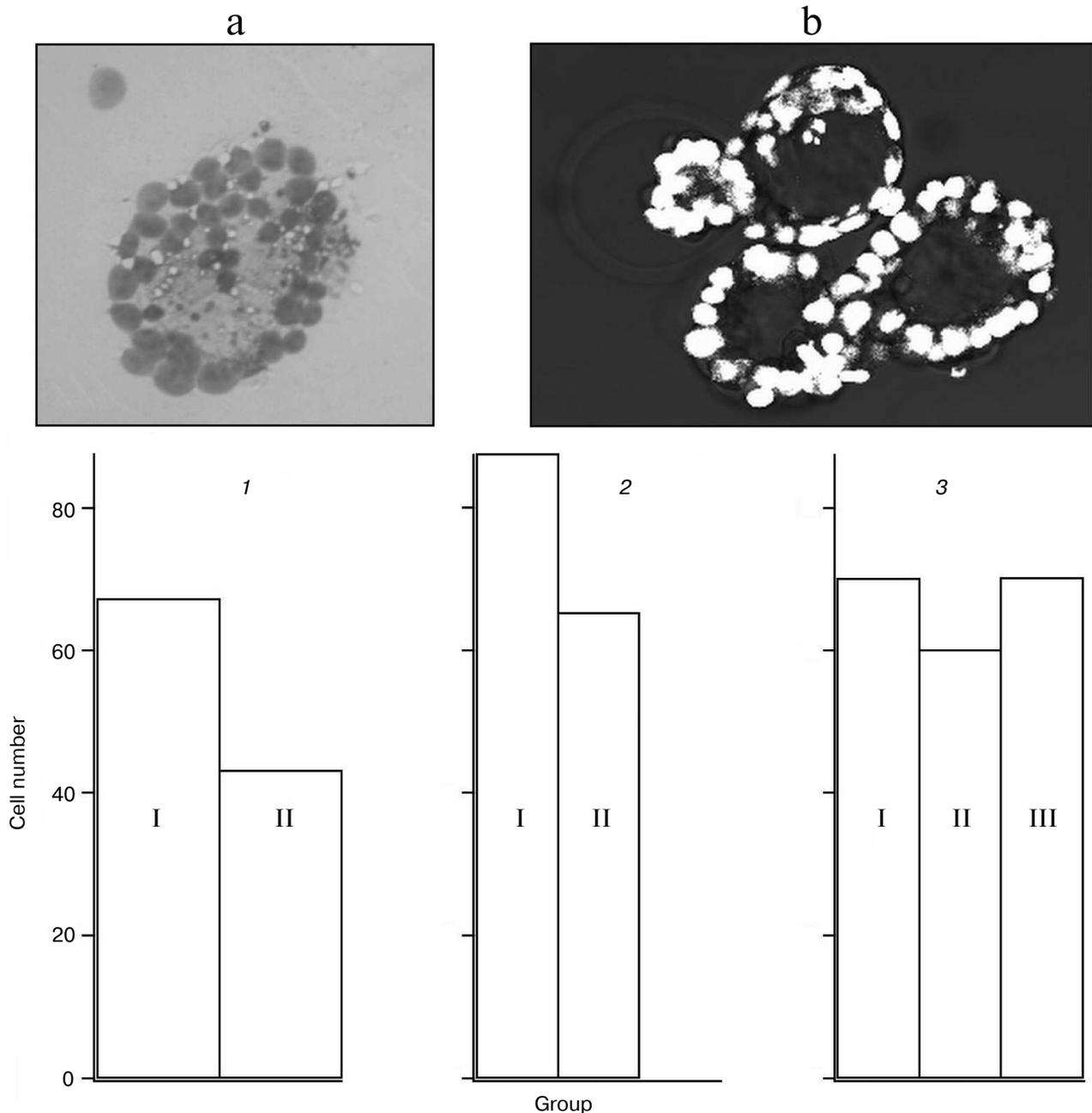


Fig. 4. Results of counting cells in blastocysts. Insert: a) nuclei of embryo cells stained with the Tarkowski method; b) images of nuclei obtained on confocal microscope. Cell number in blastocyst: 1) not hatched from zona pellucida (I – control group, $n = 7$, mean value 67.3; II – group after single perforation at the morula stage, $n = 6$, mean value 43.2); 2) hatched from the zona pellucida (I – control group, $n = 4$, mean value 87.5; II – group after single perforation at the 2-cell stage, $n = 13$, mean value 65.3); 3) hatching from the zona pellucida (I – control group, $n = 9$, mean value 70.1; II – group after single perforation at the 2-cell stage, $n = 7$, mean value 60.1; III – group after single perforation at the morula stage, $n = 9$, mean value 70.2).

after optoperforation at the morula stage. This observation partially corresponds to results of recently published work [15] where cell number decrease in blastocysts after zona pellucida perforation was reported. In our work, this decrease is typical for non-hatched blastocysts, while single perforation of hatching or completely hatched blastocysts had no appreciably affect.

Utilization of assisted hatching including laser action for extracorporeal fertilization remains questionable [1, 16, 17]. In the presented work, we show experimentally that laser action on the zona pellucida at different embryo development stages did not significantly affect ability of embryos to develop until the blastocyst stage. Half circle thinning of the zona pellucida at the morula stage and single laser perforation accelerated hatching. For hatching, embryos always used the auxiliary perforation introduced by the laser and did not make new perforations. Thinning of a small area of zona pellucida as well as thinning of half-perimeter and single perforation did not affect the number of blastocysts that are completely hatched on the 6th day of development. However, multiple laser perforation decreases the fraction of hatched blastocysts. Perforation of the zona pellucida at the blastocyst stage has a negative effect on embryo development.

Analysis of cell counting revealed that after perforation, cell number is only decreased in the blastocysts that did not hatch out from the zona pellucida. After single optoperforation of the zona pellucida, cell number in hatching or completely hatched blastocysts did not differ from the control group.

This shows that single laser perforation of the zona pellucida can be widely used when it is required to penetrate this layer for additional manipulations with the embryo. From this point of view, the method of single laser optoperforation is a convenient and safe experimental approach for manipulating the embryo inside the zona pellucida.

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