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Fusion of blastomeres in mouse embryos under the action of femtosecond laser radiation. Efficiency of blastocyst formation and embryo development

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Abstract. **Using the method of femtosecond laser surgery we study the fusion of two-cell mouse embryos under the action of tightly focused femtosecond laser radiation with the fusion efficiency reaching 60%. The detailed statistical analysis of the efficiency of blastomere fusion and development of the embryo up to the blastocyst stage after exposure of the embryos from different mice to a femtosecond pulse is presented. It is shown that the efficiency of blastocyst formation essentially depends on the biological characteristics of the embryo, namely, the strain and age of the donor mouse. The possibility of obtaining hexaploid embryonal cells using the methods of femtosecond laser surgery is demonstrated.**

Keywords: *laser nanosurgery, preimplantation development.*

1. Introduction

Nanosurgery, i.e., operations within cells and embryos using pico- and femtosecond lasers, is a front line of research in present-day biophotonics. The high light power density in the IR range, where biotissues are transparent, combined with the small total energy of the femtosecond pulse provide highly efficient nonlinear optical interaction of light with the cell substance. High localisation of the femtosecond pulse action in a nanolitre volume of the laser beam waist region allows one to perform operations in individual organelles inside a cell/embryo, without affecting the cell environment and damaging the membrane of the cell, whereas the low pulse energy ensures the absence of thermal stress. Precise focusing of the laser beam spot provides precisely controlled perforation of membranes, which is important for performing optotransfection or cell fusion operations. Femtosecond laser technologies allow high-efficiency operations aimed at selective fusion of a few blastomeres within the embryo, which is hardly possible using the traditional methods of chemical fusion or electrofusion. The fusion of cells makes it possible to solve important problems in biotechnology, in particular, to prepare polyploid cells and to develop new cloning technologies [1–10].

Up to date the papers devoted to laser fusion of somatic cells are published and the protocols for working with different cell lineages are proposed [8, 11–14]. Successful fusion of two, three and even four (by two pairs) blastomeres inside a tetrachoric embryo was reported [7]. For two-cell mouse embryos the successful fusion achieved 61.5%, the development to the blastocyst stage occurred in 78.1% of fused embryos. The analogous operation in parthenogenetic two-cell pig embryos was successful in 54% of the cases, and about 95% of operated embryos developed to blastocysts [8]. In Russia the studies in the field of femtosecond laser micro- and nanosurgery are also carried out. In particular, high efficiency of femtosecond laser fusion of blastomeres in two-cell mouse embryos was reported [15–18]. These achievements confirm the high potentialities of laser fusion of cells and embryos. However, as shown by our practical experience, the fusion efficiency in particular samples of embryos can vary from batch to batch of biological material. It remains unclear, what factors, besides the precisely controlled parameters of laser setup, affect the efficiency of embryonal cells (blastomeres) fusion and the further development of operated germs.

The present paper is devoted to the study of influence of such factors as the mouse strain and age, the time from the moment of hormonal stimulation of the female mouse to the beginning of laser fusion operation, on the success of cell fusion inside the embryo. Besides the analysis of the blastomere fusion efficiency, we analyse such biological indicators of the embryo condition as the probability of its development to the blastocyst stage and the probability of realisation of the next development stage, blastocyst hatching from the *zona pellucida*.

The fusion of two cells under the action of a laser pulse is a random event. Four outcomes are possible: the formation of a single fused cell, the absence of any effect, the death of one cell and the survival of the other, and the destruction of both cells. The goal of our work is to study the efficiency of fusing two large cells – blastomeres in the embryo and to assess the probability of further embryo development in the case of the first three outcomes being implemented.

2. Experimental part

2.1. Materials and methods

In the experiments we used female mice of different age belonging to the pure-bred (C57BL/6) and hybrid (CDF/

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C57Bl/6) strains. The coupling was implemented with males of the CBA strain. To get sufficient number of embryos, the females were subjected to hormonal stimulation with gonadotropin of the pregnant mare serum (A036A02, Intervet) and human chorionic gonadotropin (HCG) (A038A01, Intervet). The dry hormones were diluted with 0.9% physiological solution of sodium chloride to the concentration of 10 IU per 100 μL. The intraperitoneal introduction of hormones was performed with the interval 48 hours in the amount of 10 IU. The embryos were extracted on the second day of development by washing the oviducts with the PBS medium (P3813, Sigma). For manipulating with embryos in air, the medium M2 (M7167, Sigma) was used. The embryos were cultivated in the CO_2 incubator with 5% concentration of the carbon dioxide at the temperature 37 °C in the medium M16 (M7292, Sigma) in four-well dishes (179830, Nunc). The development of the embryos was observed from the stage of extraction (the second day of development, the stage of two blastomeres) to the blastocyst stage (the fifth day of development); embryo hatching from the *zona pellucida* was also fixed (the sixth day of development).

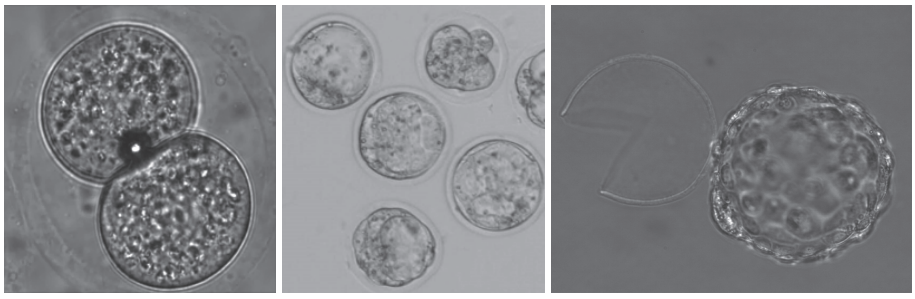
2.2. Experimental setup

In the experiments we used the inverted optical Olympus IX71 microscope with the objective 60× and NA = 0.7. The embryo membrane optoperforation was carried out using a femtosecond Mai Tai Ti : sapphire laser (Spectra Physics) generating the radiation at the wavelength 780 nm. The diameter of the beam waist was estimated using the formula $2w_0 = 1.22\lambda/\mathrm{NA} = 1.36$ μm. In the object plane of the microscope the pulse duration amounted to 100 fs, the pulse energy was 1 nJ and the pulse repetition rate was 80 MHz. The estimated power density in the beam waist was equal to 6.9×10^{11} W cm^{-2}. The length of the pulse train, controlled with a chopper, amounted to 30 ms. Both the duration of the pulse train and the laser pulse energy were chosen close to the threshold of the steam-to-gas cavitation bubble formation. The visual control was implemented using the Sony ExwaveHAD camera. The setup scheme is thoroughly described in Ref. [19].

To perform the laser micromanipulations the embryos were transferred to 24×24 mm cover glasses into a drop of the embryonal medium M2 with the volume 50 μL. For fusing two blastomeres the pulse was directed to the zone of maximally dense contact between them. The impact was considered as successful if it lead to the formation of a cavitation steam-to-gas bubble (Fig. 1a). With the parameters of the laser pulse being the same, the dimension of the bubble randomly varied from 1 to 4 μm. The complete fusion of two cells-blastomeres occurred during 1 h. On the fifth day of development the number of expanded blastocysts was counted (Fig. 1b), and on the sixth day the embryo hatching from the *zona pellucida* occurred (Fig. 1c).

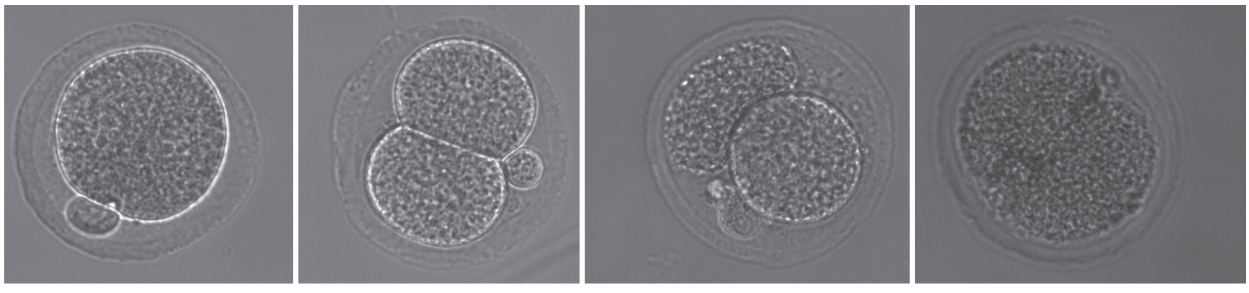
3. Results and discussion

The two-cell embryos after the laser pulse action are shown in Fig. 2. Figure 2a presents a single large cell produced from two fused blastomeres. Figure 2b shows the case when two blastomeres did not fuse after the formation of the cavitation bubble, induced by the laser pulse. Further figures show the case of one of the blastomeres destructed after the laser pulse (Fig. 2c) and the laser-induced destruction of both blastomeres (Fig. 2d). In all cases the diameter of the cavitation



a b c

Figure 1. (a) Two-cell mouse embryo at the moment of vapour-to-gas cavitation bubble formation under the action of a train of femtosecond laser pulses; (b) expanded blastocysts inside the *zona pellucida*; and (c) the blastocysts, hatching from the *zona pellucida*.



a b c d

Figure 2. Embryos after the impact of laser radiation: (a) fused embryo, (b) non-fused embryo, (c) embryo with one of the blastomeres destructed and (d) completely destructed embryo.

Table 1. The efficiency of fusion under the impact of femtosecond laser radiation and the development of fused embryos in pure and hybrid line mice.

Experiment No.	Strain	Time after HCG introduction/h	Total number of embryos	Number of fused embryos	Number of non-fused embryos	Number of embryos with one of destructed blastomeres	Number of completely destructed embryos	Fused embryos that attained the blastocyst stage (%)	Tetraploid blastocysts from the operated embryos (%)
1	C57BL/6	43.5	30	3 (10%)	18 (60%)	7 (23.3%)	2 (6.7%)	100 (3/3)	10 (3/30)
2		44.5	20	3 (15%)	10 (50%)	6 (30%)	1 (5%)	100 (3/3)	15 (3/20)
3		44–45	32	8 (25%)	13 (41%)	8 (25%)	3 (9.3%)	87 (7/8)	22 (7/32)
4		44–45	30	8 (27%)	14 (47%)	2 (6.7%)	6 (20%)	37 (3/8)	10 (3/30)
5		45	35	5 (14%)	15 (43%)	0 (0%)	15 (42.8%)	40 (2/5)	6 (2/35)
6		45–46.5	50	10 (20%)	17 (34%)	14 (28%)	9 (18%)	70 (7/10)	14 (7/50)
7		47	12	1 (8%)	6 (50%)	2 (16.7%)	3 (25%)	100 (1/1)	8 (1/12)
8		47	24	6 (25%)	11 (46%)	2 (8.3%)	5 (20.8%)	66 (4/6)	16 (4/24)
Summary data:			233	44 (19%)	104 (45%)	41 (17%)	44 (19%)	75 (30/44)	13 (30/233)
9	CBA/C57Bl/6	44–45	40	13 (33%)	17 (43%)	5 (12.5%)	5 (12.5%)	46 (6/13)	15 (6/40)
10		45	20	4 (20%)	10 (50%)	1 (5%)	5 (25%)	75 (3/4)	15 (3/20)
11		45	20	12 (60%)	4 (20%)	0 (0%)	4 (20%)	41 (5/12)	25 (5/20)
12		45	50	20 (40%)	17 (34%)	3 (6%)	10 (20%)	45 (9/20)	18 (9/50)
13		47	20	10 (50%)	3 (15%)	0 (0%)	7 (35%)	0 (0/20)	0 (0/20)
14		47	20	5 (25%)	8 (40%)	0 (0%)	7 (35%)	0 (0/20)	0 (0/20)
Summary data:			170	64 (38%)	59 (35%)	9 (5%)	38 (22%)	35 (23/64)	12 (23/170)

bubble amounted to 1–4 μm, the spread of its values being random. The outcome of the laser fusion operation can be observed nearly after 30–60 minutes; this time is sufficient for the fusion of both blastomeres into one cell.

Table 1 summarises the results of all experiments on the fusion of embryos for the two abovementioned strains of mice, and also contains the data on the development of fused embryos. We used the embryos at the stage of development from 43.5 to 47 h (the time was measured from the injection of HCG to the mouse in the process of hormonal preparation of animals). During this period the embryo was at the cell development stage G2. The experiments were carried out on different days, but under similar conditions of laser operation. As seen from the Table 1, the best result for the fusion efficiency (60%) practically coincides with the earlier one (61.5%) [7]. However, the whole set of data presented in Table 1 demonstrates essential spread of the successful fusion in different experiments. First, we can conclude that the fusion efficiency of a two-cell embryo is independent of its development time within the limits of 43.5–47 h. Second, the comparison of probabilities of all possible outcomes (fusion/non-fusion/single-cell embryo/destructed embryo) in mice from the strains C57BL/6 and CBA/C57Bl/6 shows that on average the fusion efficiency in hybrid strain mice is higher (38%±12%) than in pure-bred strain mice (19%±7%). The probabilities of the non-fusion outcome for hybrid and pure strain mice amount to 46%±7% and 33%±13%, respectively. Within the statistical dispersion, the probability of non-fusion is the same for both strains. The ratio of probabilities of destructing one of the blastomeres for hybrid and pure-bred strain is 6.8%±1.9% to 19%±9%, and that of destructing both blastomeres is 4%±3% to 25%±9%. These data show that the hybrid strain provides higher two-cell fusion efficiency, but, on the other hand, the complete destruction of two cells in the cells of this strain is more probable, too.

Experimental series of fusion of blastomeres within the embryos, obtained from the mice of the age from 4 to 9 weeks was carried out. Figure 3 shows the dependence of the fusion efficiency of two blastomeres on the mouse age. The probability was calculated using the samples including from 12 to 50 embryos, the most part of the points were obtained using the samples of 20 embryos. From the figure a correlation can be seen between the donor age and the fusion efficiency, namely, the embryos from younger specimens fuse with greater efficiency.

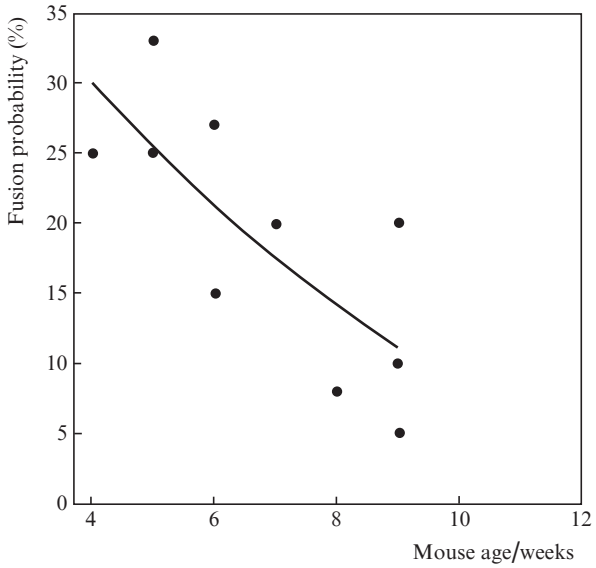


Figure 3. Two-cell embryo fusion efficiency vs. the mouse age. For each point the fusion efficiency was determined using a sample of 12–50 embryos.

The fused embryos and the embryos that were affected by the laser but did not undergo the blastomere fusion, as well as the embryos with one of the blastomeres destructed, are capable of further development *in vitro*. The development of these groups to the stage of blastocyst was observed separately (Table 2). As seen from the data presented in this table, the effect of the femtosecond laser essentially decreases the percentage of appearing blastocysts and even more strongly

Table 2. Development of embryos to the blastocyst stage after the impact of the femtosecond laser.

Embryos	Number of embryos	Number of blastocysts (%)	Number of hatched blastocysts (%)
Control group	156	84 (131/156)	13 (21/156)
Fused embryos	108	49 (53/108)	0.9 (1/108)
Non-fused embryos	136	59 (80/136)	3 (4/136)
Embryos with one of blastomeres destructed	33	39 (13/33)	0 (0/33)

reduces the percentage of hatched ones. Here we should specially mention the embryos in which the fusion did not occur. In these embryos after the action of the pulse no noticeable morphological changes were observed, but the development to the blastocyst stage differed from that in the control group of embryos.

Since the probability of blastocyst formation is a significant parameter that characterises the viability of the embryo after the laser treatment, we carried out the comparative analysis of the embryos development to the blastocyst stage in the control group and in the three groups with different outcomes of the operation, namely, in which no fusion occurred, two blastomeres successfully fused, and one of the blastomeres was destructed. To find out the significance of the difference between the groups we used Fisher's exact test [20], which is suitable for the statistical analysis of small samples of data and is widely used in biology [20]. The results of the analysis have shown that in the control group the embryos developed significantly better than the embryos after the impact of femtosecond laser radiation, the significance level of the statistical estimates was $p = 2\times10^{-6}$ for the control group and the group without fusion (in biology the limit significance level is $p < 0.05$ [20]). The development of embryos after the destruction of one of the blastomeres and the embryos with fused blastomeres appeared to be statistically insignificant ($p = 0.42$). Also statistically insignificant was the development of embryos with fused blastomeres and the embryos were the fusion did not occur ($p = 0.15$). Thus, in all three groups with different outcomes of operation the probability of the embryo development to the blastocyst stage decreased by nearly the same value as compared to the control group. The result of the operation (fusion, non-fusion, or destruction of one of the blastomeres) appeared to be statistically insignificant for the probability of the embryo development to blastocyst.

The comparison of the fusion efficiency and further development of embryos, obtained from the mice of C57BL/6 and CBA/C57Bl/6 strains, has shown that the pure-bred strain embryos fuse with low efficiency (19%, 44/233), but develop rather well to the blastocyst stage (75%, 30/44), while the embryos of the hybrid strain, on the contrary, fuse with higher efficiency (38%, 46/170), but more rarely achieve the blastocyst stage (35%, 23/64). However, the relative number of tetraploid blastocysts obtained from the operated embryos of both strains appeared to be the same, namely, 13% (30/226) for C57Bl/6 and 12% (23/170) for CBA/C57Bl/6. The data, describing the development of fused embryos in each experiment, are presented in Table 1. The dispersion analysis (ANOVA) [21] with subsequent comparison of the groups revealed significant differences between the fusion efficiency (0.0046, the significance level $p < 0.05$) and the development of fused embryos to the blastocyst stage (0.018). However, it did not reveal significant differences in the total efficiency of producing tetraploid blastocysts (the fraction of blastocysts, obtained from the fused embryos, with respect to the total number of operated embryos) in the pure-bred and the hybrid strains (0.9).

The statistical analysis of the fusion efficiency and further development of embryos allowed the conclusion that the biological characteristics of an embryo essentially affect the efficiency of producing blastocysts after the operation using a femtosecond laser.

Above we presented the data on the fusion of two-cell embryos. It is worth special noting that in comparison with the methods of chemical and electric fusion the method of femtosecond laser fusion offers a unique possibility, namely, it allows the fusion of several chosen cells inside a multicellular structure [7]. The possibility of cell fusion using the femtosecond laser at the stage of three blastomeres is also demonstrated in the present paper (Fig. 4). The presence of three nuclei in one cell is demonstrated by means of staining with the Hoechst 33342 dye followed by visualisation using a confocal microscope.

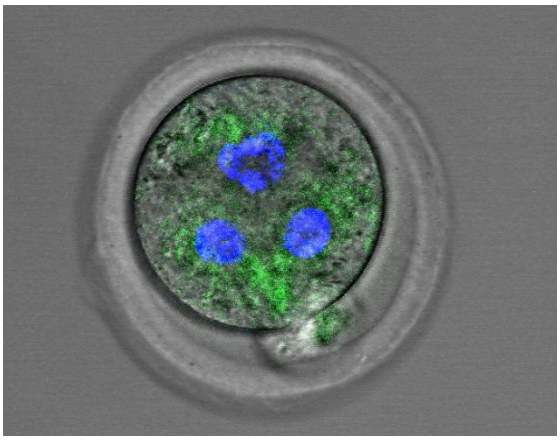


Figure 4. (Colour online) Embryo obtained by fusion of three blastomeres.

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