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Femtosecond laser is an effective instrument to remove DNA in pronuclei of mouse zygotes

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Abstract. Applying femtosecond lasers in assisted reproductive technologies is very promising. The removing of a pronucleus (called epronucleation) in three-pronuclear zygotes may help to increase the number of normal diploid embryos. In this work we demonstrated the possibility of epronucleation in mouse zygotes by the femtosecond laser irradiation. We showed that the femtosecond laser could effectively destroy the pronuclei. At the same time, zygotes retained their viability and did not destroy.

Keywords: epronucleation, zygote, femtosecond laser, nanosurgery, non-invasive

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Фемтосекундный лазер – эффективный инструмент энуклеации зигот

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Аннотация. В работе мы показали возможность удаления пронуклеусов в зиготе с помощью фемтосекундного лазерного облучения. Мы полагаем, что этот метод более эффективен и менее инвазивен, чем механическое удаление микроманипулятором. Цитопласты, подготовленные с помощью лазера, более жизнеспособны и пригодны для дальнейших биотехнологических манипуляций.

Ключевые слова: энуклеация, зиготы, фемтосекундный лазер, пронуклеусы зигот, нанохирургия, малая инвазивность

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Introduction

Triploidy is the most common [1] abnormality of the chromosome set. Triploid embryos usually abort the development at early post-implantation stages, which results in miscarriages. In artificial human technologies triploid zygotes can be easily identified by the presence of extra pronucleus (normal diploid zygotes have two pronuclei). These zygotes are always considered as abortive material and does not used for transfer. The removing of extra pronucleus could help to obtain diploid, transferable embryos for couples with exclusively abnormally or few normally fertilized oocytes [2].

Another application of epronucleation is preparation of recipient cytoplast for mitochondrial replacement therapy (MRT) [3]. MRT is applied to avoid mitochondrial diseases and it realized by two different approaches: spindle transfer and pronuclear transfer. The quality of the recipient cytoplast may be the key factor of MRT efficiency.

Epronucleation by a microneedle is only existed technique to perform this manipulation. It implies the cell puncturing and using cytoskeleton relaxants. The survival rate after epronucleation by a microneedle is able to reach 100%, but an ability of blastocyst formation in not very high [4]. In this work we suggest a novel approach of epronucleation, which does not require the use of cytoskeleton relaxants and the cell puncturing. We used mouse eggs as a model object and we showed that the destruction of a pronucleus by a near-infrared femtosecond laser does not disturb the whole oocyte and does not accompany with the loss of cytoplasm. Although the pronuclei are clearly visible without any staining, we applied Hoechst 33342 dye to reduce the threshold of DNA breakdown.

Materials and Methods

Oocyte collection and staining. C57Bl/6 female mice aged eight- to ten weeks were mated with males of the same strain. Mice were sacrificed and the oviducts were removed. Fertilized oocytes with two pronuclei were washed and then cultured in KSOM culture medium (MR-101-D, EmbryoMax). The zygotes were stained with 5 μg/ml Hoechst 33342 dye (B2261, Sigma-Aldrich) for 15 minutes in KSOM medium in the incubator and then washed twice. During the experiment the zygotes were placed onto a cover glass in a 50 μl drop of M2 (M7167, Sigma) medium.

Laser parameters and enucleation process. Femtosecond radiation (λ=790 nm) was generated by a reformed laser (Chameleon Discovery, Coherent) and coupled to an inverted microscope Olympus IX71. In our experiments we used following parameters for the irradiation of the pronuclei: λ = 795 nm, υ = 80 MHz, pulse energy 0,5 nJ (40 mW power); 100 fs pulse duration, pulse train duration 60 ms. Laser radiation was focused by 60× objective lens (NA = 0.7). Pronuclei are easily visualized, we exposed laser on one of them. We repeated the exposure multiple times, until the DNA luminescence had stopped.

Confocal imaging. Fluorescence imaging was performed using a laser scanning confocal microscope Zeiss LSM 980 (Carl Zeiss Microscopy, Jena, Germany), 63x Plan-Apochromat objective (NA = 1.4, oil immersion). The oocytes were placed in M2 medium drop on a 0.17 mm cover glass (Zeiss). Hoechst 33342 luminescence excitation were performed by 405 nm laser, detection range 410–479 nm.

Results and Discussion

Femtosecond laser was successfully applied for elimination of one or two pronucleus in the mouse oocytes. At the first moment, two pronuclei were clearly visible (Fig. 1,*a*). After the laser irradiation contour and nucleoli of the pronucleus disappeared (Fig. 1, *b*, *c*). The oocytes retained their viability after the irradiation at least for 24 hours.

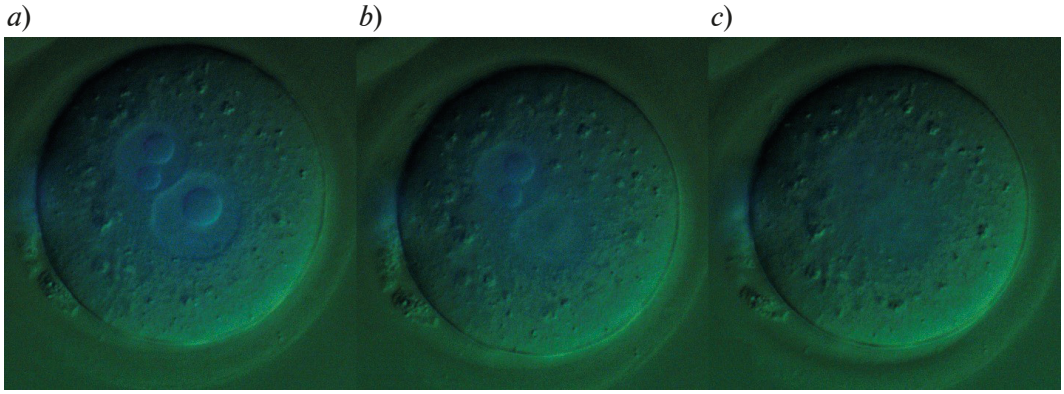

Fig. 1. Femtosecond laser destruction of the pronuclei in the mouse zygote: before the irradiation (*a*); after irradiation of one pronucleus (*b*); after irradiation of two pronuclei (*c*)

To control enucleation, we observed a decrease in the level of DNA luminescence. We measured the initial level of luminescence, then destroyed one of the pronuclei and compared the levels of luminescence after the exposure. Fig. 2 shows the pronuclei, they are in different focal planes. In this experiment, the pronucleus shown in the images on the left (*a*) was irradiated. Fig. 2,*b* shows the second, right pronucleus, without laser irradiation. The average luminescence intensity of the region in which the irradiated pronucleus is located decreased by more than half (73.5 before, 31.6 after). The intensity of the luminescence of the non-irradiated pronucleus practically did not change (27.7 before, 27.6 after).

A decrease in DNA luminescence by two times can be observed for the left protonucleus, and no significant changes for the right one

We examined an ability of epronucleated zygotes to develop in vitro. Oocytes with one irradiated pronucleus completely stopped the development (cleavaged 0 of 13) as well as oocytes with two irradiated pronucleus (cleavaged 0 of 10), whereas control zygotes successfully formed blastocysts (12 of 13). However, fragmentation or degradation of epronucleated zygotes was not observed at least for 24 hours. We admit that these results are preliminary and require further investigation.

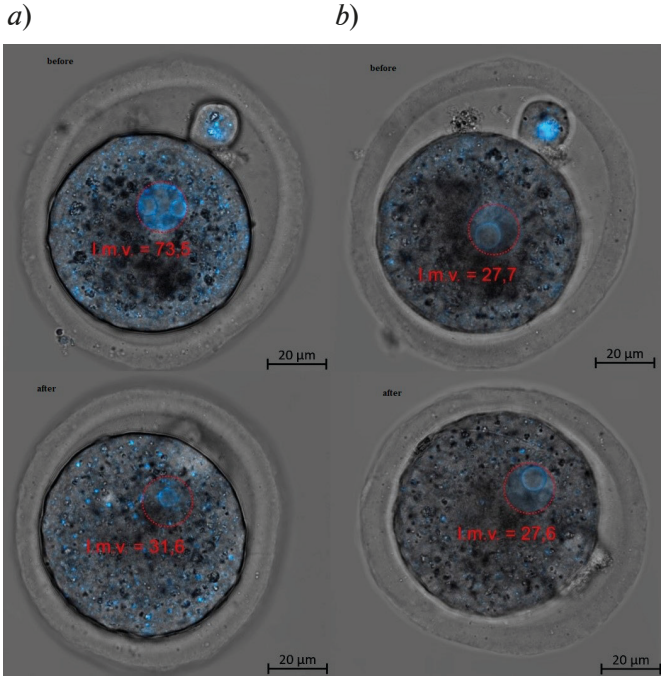

Fig. 2. Pronucleus after irradiation by a femtosecond laser (*a*); non-irradiated protonucleus (*b*)

Discussion

The use of femtosecond lasers for the oocyte and embryo manipulation can significantly improve some important techniques in artificial human technologies, but it requires considerable effort and fundamental research. We have already studied the range of non-invasive near-infrared laser impact [5] and examined viability of mouse germinal vesicle oocytes [6] and two-cell embryos development [7] after the laser exposure within this range. Moreover, we have successfully performed enucleation of metaphase II oocyte [8] and we demonstrated the efficiency and safety of this method. Thereby we assume that epronucleation within the range of non-invasive diapason could be effective and low-invasive as well. Nonetheless, the proper study of viability after the epronucleation ought to be performed. Another important thing that must be studied is the process of DNA destruction by the femtosecond laser: it is not yet clear, is there any remains of DNA after the exposure.

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