EXPLORATION OF THE FEMTOSECOND LASER PULSE THERMAL EFFECTS ON THE MOUSE EMBRYOS DURING THE ASSISTED HATCHING PROCEDURE

Kubekina MV[1,2] ✉, Filatov MA[1,2], Silaeva YuYu[1,2], Sitnikov DS[2]

[1] Institute of Gene Biology of the Russian Academy of Sciences, Moscow, Russia
[2] Joint Institute for High Temperatures of the Russian Academy of Sciences, Moscow, Russia

Impaired hatching of the embryo from the *zona pellucida* (ZP), the specialized protective shell, immediately before implantation is one of the factors of infertility. Hatching impairment is often due to the ZP hardening or thickening. In such cases, the laser assisted hatching procedure is used to overcome infertility. During this procedure a hole is drilled in the ZP facilitating the embryo release. The question of the safe use of laser for assisted hatching remains open, since laser beam can heat the environment and cause thermal shock in embryos. The study was aimed to assess safety of the mouse embryo femtosecond laser exposure during the assisted hatching procedure using the embryo viability and HSP gene expression assessment methods. A new type of pulsed laser was used in the study for the ZP dissection — the femtosecond laser. The energy of such pulses was two orders of magnitude lower than the energy of laser dissectors currently used in the clinics. To assess the femtosecond laser exposure to the embryo, the house mouse (*Mus musculus*) embryos were stained with fluorescence dyes, and expression of the genes encoding heat shock proteins (*Hsp90aa1* and *Hspa5*) was assessed. The embryonic cells remained viable after the laser assisted hatching procedure involving the use of a femtosecond laser, while expression levels of the genes encoding heat shock proteins were slightly increased compared to the negative control group ($p = 0.408$).

Keywords: embryo, mouse, blastocyst, *zona pellucida*, laser assisted hatching, femtosecond laser pulses, heat shock proteins

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✉ **Correspondence should be addressed:** Marina V. Kubekina
Vavilova, 34/5, 119334, Moscow, Russia; marykumy@gmail.com

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ИССЛЕДОВАНИЕ ТЕПЛОВОГО ВОЗДЕЙСТВИЯ ФЕМТОСЕКУНДНЫХ ЛАЗЕРНЫХ ИМПУЛЬСОВ НА ЭМБРИОНЫ МЫШИ В РАМКАХ ПРОЦЕДУРЫ ВСПОМОГАТЕЛЬНОГО ХЕТЧИНГА

М. В. Кубекина[1,2] ✉, М. А. Филатов[1,2], Ю. Ю. Силаева[1,2], Д. С. Ситников[2]

[1] Институт биологии гена Российской академии наук, Москва, Россия
[2] Объединенный институт высоких температур Российской академии наук, Москва, Россия

Одной из причин бесплодия является нарушение хетчинга — процесса вылупления эмбриона из специальной защитной оболочки (ZP, *zona pellucida*,) непосредственно перед имплантацией. Часто нарушение хетчинга обусловлено уплотнением или утолщением ZP. Для преодоления бесплодия в таких случаях прибегают к процедуре вспомогательного лазерного хетчинга, когда с помощью лазера в ZP делают отверстие, облегчая выход эмбриона из нее. Вопрос безопасности применения лазера для проведения процедуры вспомогательного хетчинга остается открытым, поскольку лазерное излучение способно нагревать окружающую среду и потенциально вызывать у эмбрионов термический шок. Целью настоящей работы было оценить безопасность фемтосекундного лазерного воздействия на эмбрионы мыши в рамках процедуры вспомогательного хетчинга, используя методы исследования жизнеспособности эмбрионов и уровней экспрессии генов HSP. Для диссекции ZP в работе использовали новый тип источника лазерных импульсов фемтосекундной длительности. Энергия указанных импульсов на два порядка ниже энергии миллисекундных лазерных диссекторов, применяемых в клиниках в настоящий момент. Для оценки фемтосекундного лазерного воздействия на эмбрион производили окрашивание эмбрионов домовой мыши (*Mus musculus*) флуоресцентными красителями, а также оценивали уровни экспрессии генов, кодирующих белки теплового шока: *Hsp90aa1* и *Hspa5*. После выполнения процедуры вспомогательного лазерного хетчинга с использованием фемтосекундного лазера клетки эмбрионов сохраняли жизнеспособность, а уровни экспрессии генов, кодирующих белки теплового шока, повышались незначительно по сравнению с группой отрицательного контроля ($p = 0{,}408$).

Ключевые слова: эмбрион, мышь, бластоциста, блестящая оболочка, *zona pellucida*, вспомогательный лазерный хетчинг, фемтосекундные лазерные импульсы, белки теплового шока

Финансирование: работы по проведению манипуляций с эмбрионами при помощи лазера и оценка уровней экспрессии генов, ответственных за синтез белков теплового шока, выполнены при финансовой поддержке РНФ в рамках научного проекта 23-19-00424 на оборудовании УНУ «Лазерный тераваттный фемтосекундный комплекс», входящей в состав ЦКП «Лазерный фемтосекундный комплекс» ОИВТ РАН. Работы по получению эмбрионов были выполнены при финансовой поддержке гранта номер 075-15-2021-668 (от 29.07.2021) УНУ Трансгенбанк.

Вклад авторов: М. В. Кубекина — проведение иммунофлуоресцентного окрашивания и определение уровня экспрессии белков теплового шока, написание статьи; М. А. Филатов — работа с эмбрионами, статистические методы обработки, написание статьи; Ю. Ю. Силаева — общее руководство экспериментом; Д. С. Ситников — проведение лазерной микрохирургии, обработка результатов, написание статьи; все авторы — обсуждение и редактирование статьи.

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✉ **Для корреспонденции:** Марина Владиславовна Кубекина
ул. Вавилова, д. 34/5, 119334, г. Москва, Россия; marykumy@gmail.com

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The problem of infertility affects millions of people [1]. Its causes are extremely diverse; assisted reproductive technologies, such as *in vitro* fertilization (IVF) and in vitro (extracorporeal) embryonic culture are used for treatment. Mammalian preimplantation embryos (including human) are surrounded by the specialized protective shell: *zona pellucida* (ZP). It is composed of glycoproteins forming a porous structure [2, 3]. Due to the presence of very small pores (several dozen nanometers in diameter only), ZP provides access to the embryo for nutrients from the environment, but prevents the entry of viruses and bacteria, i.e. has a protective function. The embryo goes through several stages in its development: cleavage, formation of morula and blastocyst. When the blastocyst stage is reached, the embryo has to get off the ZP. The process of the embryo release from the ZP is called hatching. Hatching is normally followed by implantation, i.e. by the embryo attachment to the uterine wall. When hatching is impaired, the embryo cannot get off from the ZP and get implanted. Infertility due to impaired implantation is a very common phenomenon. The causes of hatching impairment are diverse: too thick or too hard ZP, insufficient levels of enzymes needed for local ZP breaching. In such cases the laser assisted hatching procedure, during which a hole is drilled in the ZP facilitating the embryo release, is used.

There are various assisted hatching methods: chemical, mechanical and laser. When using a chemical method, the ZP is treated with specific chemicals causing the ZP disruption, such as acidic Tyrode's solution [4], or enzymes, such as pronase [5]. However, such treatment can have a direct effect on the embryonic cells, which can result in the decrease of its capacity of further normal development. When using mechanical hatching, the hole in the ZP is made with a specialized microneedle [6, 7], however, embryonic cells are likely to be damaged during this procedure, which adversely affects embryogenesis. Laser assisted hatching (LAH) is most popular in many IVF clinics [8, 9]. During LAH, a cut-out in the ZP is made with millisecond laser dissectors. These allow one to make a 5–10 μm hole in the ZP, which is enough for both zona thinning and dissection to the full depth. The ZP disruption is due to heating the medium to a temperature of several hundred degrees [10]. The adverse effects of using millisecond (ms) dissectors include heating the environment surrounding the exposed area, which can result in embryonic cell damage. Thus, when using the Zylos-tk dissector (Hamilton Thorne; USA) with the pulse duration of 0.5 ms and power of 300 mW, heat spreads to the distance of about 16 μm from the laser beam center and leads to the temperature increase to 60 °C within the pulse duration [11]. That is why it is recommended to perform dissection of the embryo's ZP during the early stage of development, when the perivitelline space is large enough (more than 15 μm).

The use of femtosecond (fs) lasers allowing one to achieve high beam intensity with low laser pulse energy is considered to be promising. Femtosecond laser exposure results in medium heating of the area of beam focus, however, heat does not have time to spread sidewise due to short pulse duration. Theoretical estimations have shown that the size of the heated area is comparable with the spot size of a focused laser beam [12], while maximum heating of aqueous medium with the laser pulse intensity of 1.8–5 TW/cm^2 at a wavelength of 514 nm matches the range of 5–30 °C [13]. In the earlier studies, irradiation at a specified wavelength with the intensity of ~2.5 TW/cm^2 was used for embryo microsurgery [14]. In this study, the intensity in laser beam focus was 3 TW/cm^2. To assess safety of using a femtosecond laser beam for assisted hatching of mouse embryos, we conducted experiments focused on exploring embryo viability after the ZP laser microsurgery. Furthermore, the experiments focused on assessing the expression of genes encoding heat shock proteins (HSPs), the synthesis of which in the cells starts when the temperature increases, were conducted. HSPs are essential to maintain native spatial protein structure, which becomes especially important under heat stress. In eukaryotes, heat shock proteins of the families Hsp70 and Hsp90 are most strongly activated by heat stress; that is why members of these families were selected for the study [15].

Thus, the study was aimed to assess safety of the mouse embryo femtosecond laser exposure during the laser assisted hatching procedure. For this embryo viability and HSP gene expression in embryos were investigated.

METHODS

Experimental apparatus

Microsurgical manipulations on embryos were performed using the "Femtosecond Laser Scalpel" system (Joint Institute for High Temperatures RAS; Russia) [16]. The beam generated by the TETA femtosecond laser (Avesta; Troitsk, Russia) was used to produce a laser scalpel. Laser pulses emitted by the source had the following parameters: pulse duration τ — 280 fs, energy — 330 μJ, wavelength λ — 1028 nm, pulse repetition rate — 2.5 kHz. There was a KDP (potassium dihydrogen phosphate, KH_2PO_4) crystal installed on the beam path to convert infrared radiation into visible light; radiation leaving the crystal had a wavelength (λ) of 514 nm. Then it was fed through the right side port of the Olympus IX-71 inverted microscope and focused into a spot with a radius r = 1.4 μm at the 1/e level with the 20xUPlanFL microlens (Olympus; Japan) having a 0.5 numerical aperture (NA). Attenuator installed next to the laser source (Fig. 1) was used to adjust pulse energy. A telescope was used to align the laser beam diameter with the objective lens aperture, as well as to avoid energy loss associated with

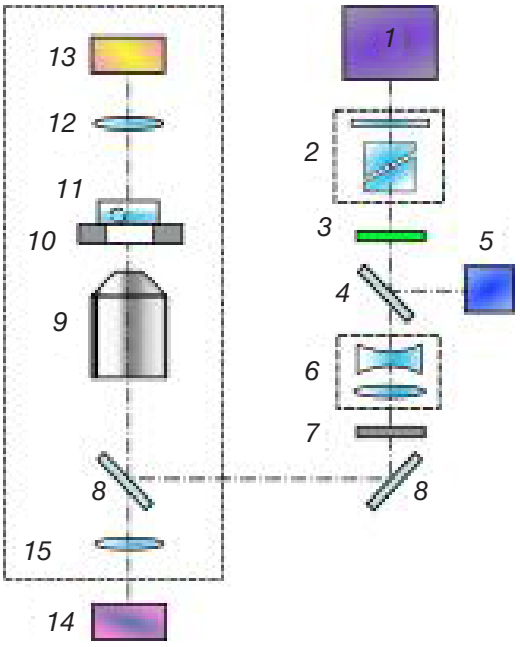

Fig. 1. Femtosecond laser scalpel scheme. 1 — fs laser, 2 — attenuator, 3 — KDP crystal, 4 — glass plate, 5 — photodiode, 6 — telescope, 7 — laser beam shutter, 8 — mirrors for the laser beam wavelength, 9 — microlens, 10 — motorized microscope stage, 11 — Petri dish with embryos, 12 — substage condenser, 13 — substage lamp, 14 — video camera, 15 — inverted microscope

the microlens frame beam limiting. The DET36A2 photodiode (Thorlabs Inc.; USA), to which part of the laser beam was branched using a thin glass plate (Fresnel reflection) was used to control the laser pulse energy. Photodiode output signal was digitized with the Tektronix TDS 5054 oscilloscope. The photodiode signal amplitude was calibrated using a power meter consisting of the S120VC photodiode detector and the PM100D console (Thorlabs Inc.; USA). The detector was temporarily installed on the SCAN IM 120×80 microscope stage (Märzhäuser Wetzlar; Germany) to record the power of laser beam leaving the microlens.

To perform microsurgery, embryos were placed in Petri dishes with the glass bottom. The thickness of the glass bottom was 170 μm (beam was focused through the dish bottom). The Petri dish was placed on the motorized microscope stage (Märzhäuser Wetzlar; Germany) to move the embryo relative to the stationary laser beam. Embryo imaging was performed with the DFK 72AUC02 camera (The Imaging Source; Germany), and the image was displayed on the personal computer screen. Software written in LabView (National Instruments, USA) was used for automatization of microsurgical procedure, which allowed the operator to control such parameters, as energy, pulse repetition rate and laser beam divergence, and set the laser beam trajectory drawing it directly on the image of the embryo. The ZP dissection was performed using a sequence of laser pulses with the speed of embryo movement relative to the beam of 0.01 mm/s.

Animal handling

The study involved CBA x C57Bl/6J F1 hybrid mice. Animals were kept at the 14 : 10 h light/dark cycle at a temperature within the range of 22–24 °C. They had *ad libitum* access to water and food (special extruded feed for mouse breeding). All the procedures involving animals were conducted in accordance with the guidelines of the Bioethics Commission, Institute of Gene Biology RAS.

Early embryo manipulation

A large number of zygotes were obtained using a widely used protocol for ovulation induction involving the use of the follicle-stimulating hormone and chorionic gonadotropin [17]. Hormonal ovulation induction was performed in accordance to the two-step protocol: PMSG was administered intraperitoneally at 13:00 of day 1 (Follimag; Mosagrogen, Russia), 5 IU per animal; hCG was administered intraperitoneally 48 h later (Chorulon, Merck Animal Health; USA), 10 IU per animal. After that these females were housed with males for mating. The fact of copulation was determined next morning based on the presence of the copulatory plug.

Obtaining embryos

The zygote stage embryos were collected (0.5 days of embryogenesis, E0.5) and cultured *in vitro*. Female mice were euthanized by cervical dislocation to obtain embryos. Collected oviducts were placed in the Ooclean medium (PanEco; Russia), where the adipose tissue was separated by the syringe needles and tweezers. Then oviducts were transferred to the 100 μL pure drop of the Ooclean medium, where the oviduct ampullae were dissected to obtain zygotes surrounded by the cumulus cells. Then about 0.03 g of hyaluronidase (Lydase; Microgen, Russia) were added to this drop to separate zygotes from the cumulus cells. The resulting zygotes were serially washed in four droplets of Ooclean medium and transferred to the culture medium.

Embryo culture

The embryos were cultured to the morula stage (E2.5) in the four-well plates (Termo Scientific Nunc, USA) using the culture medium for gametes and embryos (Fujifilm Irvine Scientific; USA). During the morula and blastocyst stages (E3.5) the embryos were cultured in the 20 μL medium drops covered with mineral oil (Origio; Denmark), 2–3 embryos per drop.

Transportation of embryos

The embryos were obtained and cultured to the morula stage in the Institute of Gene Biology RAS, while embryo culture to the blastocyst stage and LAH were performed in the Joint Institute for High Temperatures RAS. The embryos were placed in the 0.5 mL eppendorf tube filled with the HEPES-containing medium (Ooclean; PanEco, Russia) for transportation. The eppendorf tube with embryos was placed in a thermos flask with water heated to 37 °C in order to ensure constant temperature during the embryo transportation. Embryos were transported within 90 min.

Groups of embryos

The embryos used in the study were divided into four groups: negative (1) and positive (2) controls, non-viable embryos (3), experimental group (4). There were four embryos in the group of non-viable embryos and 30 embryos per group in other groups.

1. The embryos cultured under standard conditions (5% CO_2/air 37 °C) in an incubator (Binder; Germany) were used as negative controls.
2. Blastocysts exposed to the temperature of 42 °C for 30 min were used as positive controls. Thermal exposure was also provided in a CO_2 incubator (5% CO_2/air 42 °C). During exposure, the embryos were in the same culture medium and in the same culture dishes (four-well plates) as intact embryos. Then blastocysts of this group were cultured under standard conditions for 2 h to produce response to thermal exposure.
3. To obtain non-viable embryos, the embryos were treated with a fixative solution (4% paraformaldehyde in phosphate buffered saline (PBS)) and permeabilized in the 0.003% PBS-Triton solution.
4. The experimental group included embryos exposed to laser beam during the LAH procedure. Microsurgery of the embryo's ZP was performed at the blastocyst stage (~E3.5). A day before the experiment, the morula stage embryos were distributed across well-prepared glass bottom Petri dishes (catalogue number 200350, SPL Lifesciences; Korea) (three embryos per droplet) and transferred to the incubator. Three ~20 μL droplets of culture medium were previously formed in each Petri dish and then covered with liquid paraffin oil for IVF 1 media (catalogue number E0350-160; PanEco, Russia). On the day of experiment, the Petri dish was taken out of the incubator and placed on the microscope stage. The embryos' total time out of the incubator did not exceed 10 min.

Experiment

To perform LAH, the operator applied the laser beam trajectory over the embryo image, thereby shaping the embryo's ZP incision (Fig. 2A, green polyline). The incision was U-shaped, as

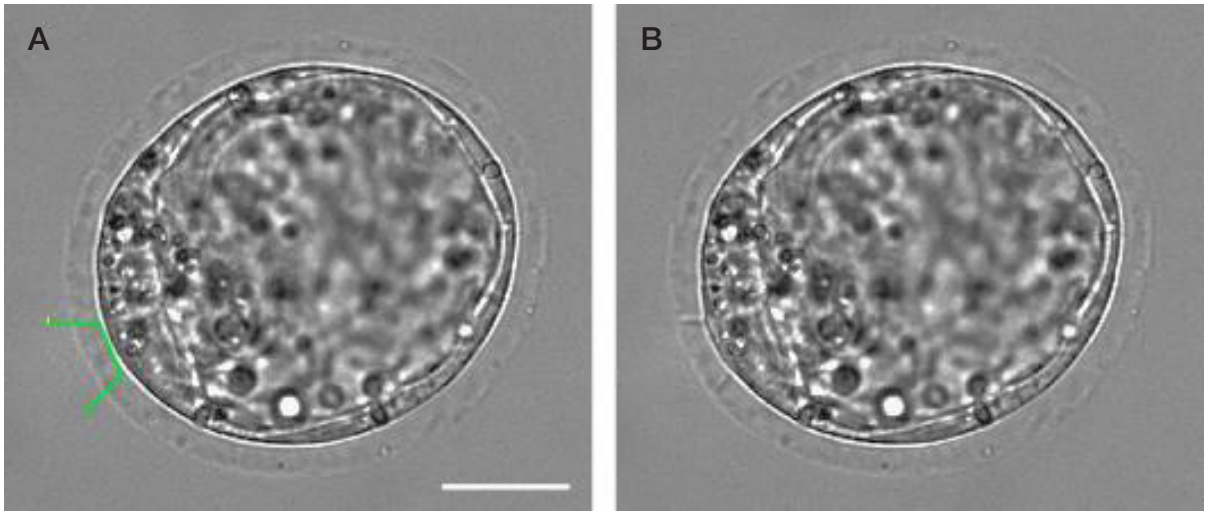

Fig. 2. Images of the embryos obtained before (**A**) and after (**B**) the zona pellucida microsurgery. The scale bar is 20 μm

in the earlier study [16]: laser beam cut the ZP to 80–90% of its thickness. The software performed microsurgery automatically, moving the embryo along the specified trajectory relative to the stationary beam and controlling the moment of the laser emission switch on/off. The laser pulse energy used in the experiment was 30 nJ. Microsurgery outcome is provided in Fig. 2B.

Fluorescence staining

The groups of embryos were stained simultaneously with three dyes:

1) Hoechst 33342 (Thermo Scientific; USA) — control stain, excitation/emission 350/461, working concentration 1 μg/mL;

2) Propidium Iodide (Thermo Scientific; USA) — non-viable cell stain, excitation/emission 493/636, working concentration 500 nmol;

3) Calcein-AM (Sigma Aldrich; USA) — living cell stain, excitation/emission 494/517, working concentration 1 μmol.

Fluorescent dyes were brought to the working concentration in the culture medium for gametes and embryos. This medium was used to culture the embryos for 30 min at 37 °C in the incubator. Then the embryos were washed in three droplets of fresh Ooclean medium (PanEco; Russia) to remove the dyes, and their viability was assessed using the Eclipse Ti-E fluorescence inverted microscope (Nikon; Japan).

Determining gene expression

RNA was extracted using the TRIzol reagent (Thermo Scientific; USA) in accordance with the manufacturer's protocol. To obtain complementary DNA (cDNA), reverse transcription was performed using the MMLV RT kit (Evrogen; Russia) according to the manufacturer's protocol. Gene expression was determined by real-time PCR in the CFX96 amplifier (Bio-Rad; USA) using the qPCRmix-HS SYBR ready-made mixture (Evrogen; Russia). The cDNA samples from blastocysts were amplified in three iterations using primers for the reference *Hprt* and *Hsp90aa1* and *Hspa5* heat shock protein genes. The nucleotide sequences of primers used in the study are provided in Table 1. The *Hsp90aa1* and *Hspa5* expression was calculated using the well-known ΔCt method [18].

RESULTS

Determining embryo viability by fluorescence staining

To determine the effects of laser microsurgery on the mouse embryo viability, all groups of embryos were subjected to fluorescence staining (Fig. 3).

After laser microsurgery the embryos were stained with the Calcein-AM dye, the same as positive and negative control groups; the Propidium Iodide dye was detected in embryos of non-viable group only. Such results make it possible to draw a conclusion that the experimental group embryos remain viable after laser microsurgery. Minor differences in the Calcein-AM and Hoechst 33342 staining intensity can be explained by bias in the settings of the microscope camera, which should be manually adjusted when assessing each group of embryos.

Determining expression of the *Hsp90aa1* and *Hspa5* heat shock genes

It is well-known that exposure of biological object to laser pulse is associated with local heating [19, 20]. The embryo culture at unphysiologically high temperatures (≥ 39 °C) promotes the increase in the expression of genes encoding heat shock proteins [21]. Upregulation of these genes is part of the cellular response to thermal shock. To determine the laser microsurgery effects on the expression of genes Hsp90aa1 (Hsp90 family) and Hspa5 (Hsp70 family) encoding heat shock proteins in embryos, expression of these genes was assessed in the negative and positive control groups, as well as in the experimental group of embryos exposed to laser beam (Fig. 4).

Distribution of the data obtained was non-normal, therefore, a nonparametric test was used for analysis. The data were

Table. Primers used in the study (sequences are shown in the 5' to 3' direction)

Gene	Genbank No	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product size
Hprt	NM_013556.2	CAGCGTCGTGATTAGCGATGA	GCCACAATGTGATGGCCTCC	174
Hsp90aa1	NM_010480.5	TGAGCAGTATGCCTGGGAGT	CGACCCATTGGTTCACCTGT	75
Hspa5	NM_001163434.1	GGAATGACCCTTCGGTGCAG	GTCTTGGTTTGCCCACCTCC	109

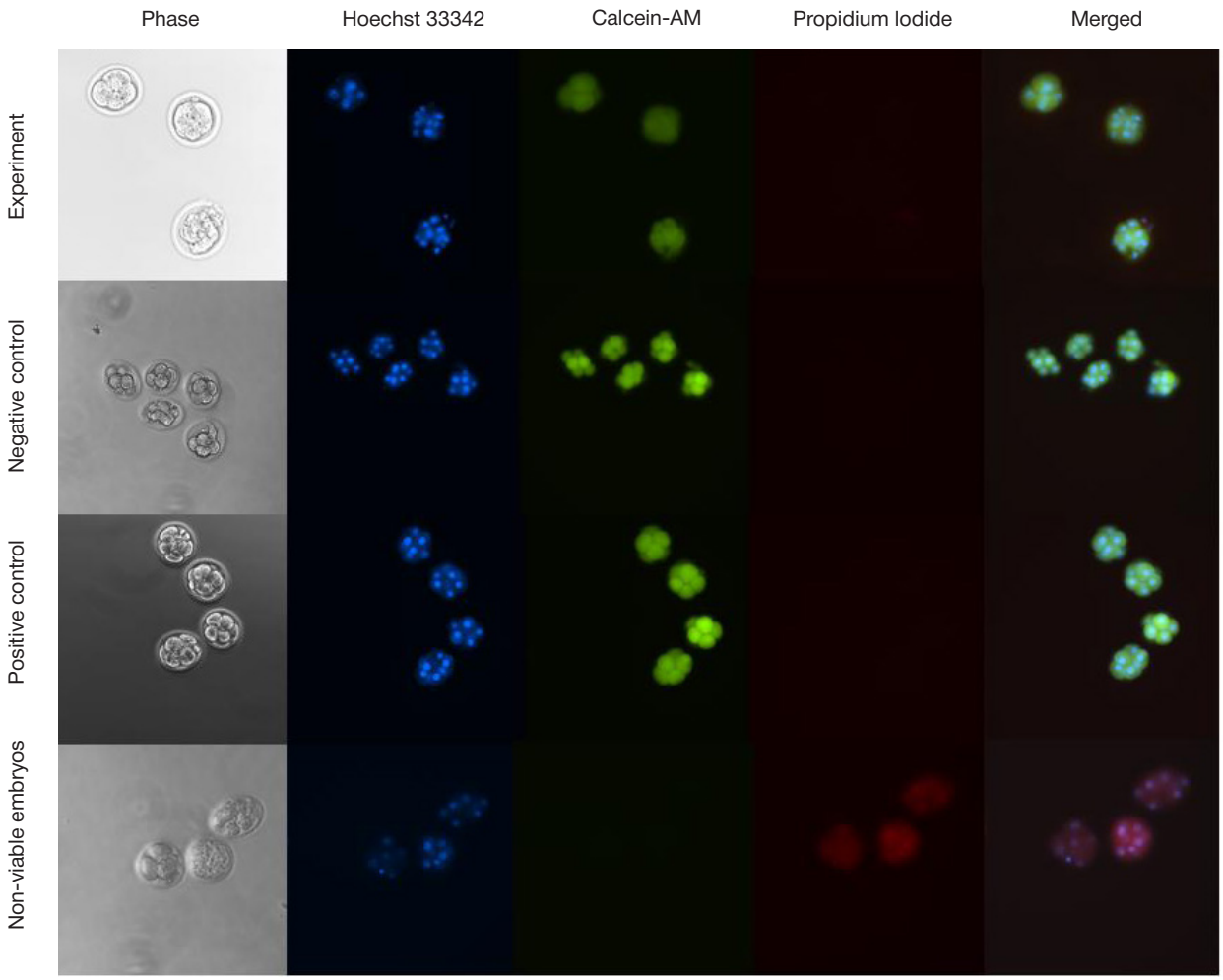

Fig. 3. Fluorescence staining of different groups of embryos. Viable embryos were stained with Calcein-AM, non-viable embryos with Propidium Iodide, nuclei of viable and non-viable embryos with Hoechst 33342

compared using the nonparametric Kruskal–Wallis test. The differences were considered significant at $p < 0.05$. Significant differences between the negative and positive control groups ($p = 0.033$) were reported for both studied genes. The differences between other groups were non-significant: $p = 0.408$ when comparing the experimental group with the positive control group, $p = 0.890$ when comparing the experimental group with the negative control group during assessment of the expression of both genes, *Hsp90aa1* and *Hspa5*.

Expression of genes *Hsp90aa1* and *Hspa5* in the negative and positive control groups shows significant differences, however, the differences in expression of both genes between the experimental and negative control group, as well as between the experimental and positive control group are non- significant. Consequently, it can be assumed that laser microsurgery can cause a slight increase in the HSP gene expression in mouse blastocysts.

DISCUSSION

Temperature is a key factor of embryogenesis, and its effects in the context of simulating physiological and biochemical environment of the genital tract have been assessed in a number of studies [22–24]. The earlier research confirms the impact of thermal stress on reproductive efficiency of both domestic animals and humans. The temperature increase affects oocyte maturation and embryogenesis, especially at early stages of development, before the zygotic genome activation. This makes oocytes and embryos sensitive to thermal stress, which can disturb their development. Similar effects were reported as early as several decades ago [22–24].

There are data indicating that laser irradiation can exert adverse effects on the living objects, for example, effects related to thermal exposure [25, 26]. To date, the question of the embryos' millisecond laser exposure safety remains open. The research results show that this procedure has some risks and adverse effects [27]. In particular, the use of laser exposure for assisted hatching at the 6–8 cell stage resulted in significant reduction of the number of cells in the embryos. Similarly, at the two cell stage, elevation of DNA fragmentation at the blastocyst stage was detected. The authors expressed concern toward the fact that, despite the increased hatching rate, DNA damage could affect the implantation potential and further embryogenesis. Similar conclusions about the adverse effects of breaching the ZP integrity at the early stages of pre-implantation development were reported [28].

The study conducted as early as in 1999 showed that blastocyst usually responds to the millisecond laser pulses, which results in the blastocyst collapse [29]. Blastocyst recovery takes time, which makes it difficult to predict the terms of hatching and hampers trophectoderm biopsy. In this regard, the use of femtosecond laser pulses can be more effective, since the ZP microsurgery, even that performed during late embryogenesis, does not result in the blastocyst collapse and delayed hatching. However, available data show that the use

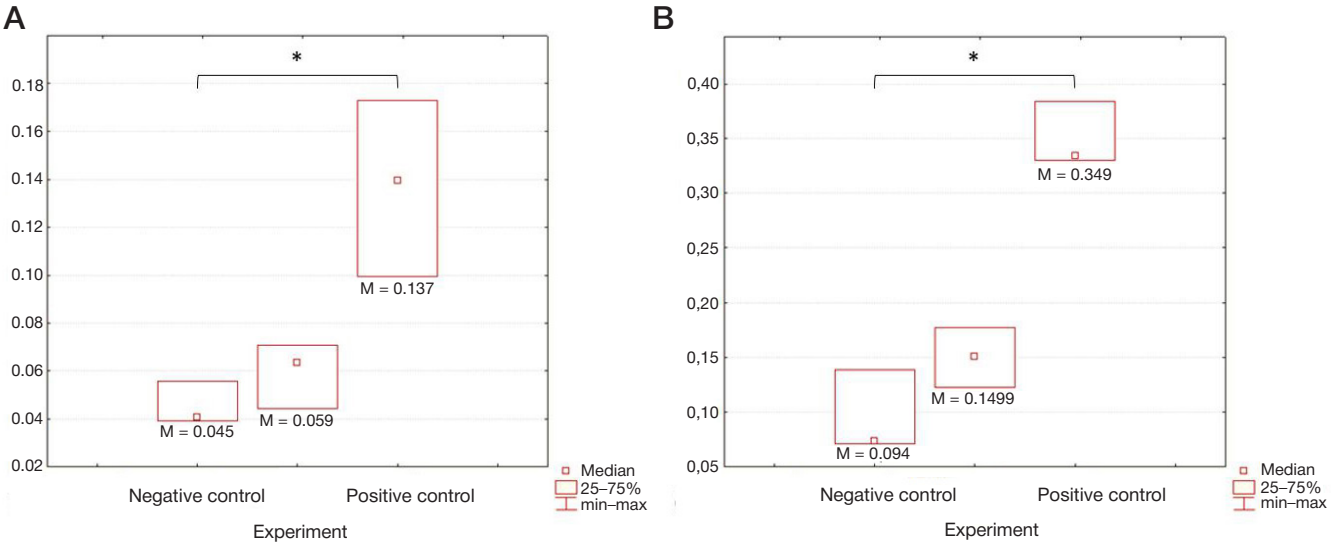

Fig. 4. Graphic representation of heat shock protein gene expression in the negative and positive control groups, as well as in the experimental group of embryos. Expression of genes *Hsp90aa1* (**A**) and *Hspa5* (**B**) encoding heat shock proteins

of femtosecond lasers can be also dangerous for biological objects. Their effects were shown to lead to the rabbit corneal stromal cells' death [30].

To assess thermal effects of laser pulses, modeling of the femtosecond pulse absorption vs. millisecond pulse absorption was performed (Fig. 5) [31]. Spatial distributions of temperatures for the ms (λ = 1480 nm, τ = 0.6 ms, P = 180 mW) and fs (λ = 514 nm, τ = 280 fs, intensity I0 = 2.5 × 1012 W/cm^2) pulses both at the time of reaching maximum temperature (solid curve) and at the stage of medium cooling (dotted curves) are provided. The exposure parameters are considered to be optimal for the ZP dissection [10, 31]. In case of femtosecond laser exposure, heat is concentrated primarily within the beam focus area (r < 1.4 μm), which paves the way for safe embryo microsurgery within the framework of LAH. At the same time, a millisecond pulse is capable of heating a significantly larger area of the medium; one should be careful when performing the ZP dissection to minimize the risk of thermal damage to the embryonic cells adjacent to the ZP.

To assess safety of using femtosecond lasers for LAH, we conducted experiments involving staining of embryos with fluorescence dyes and determining the HSP gene expression. It was found that the embryos remained viable even after the ZP microsurgery (staining of embryos with the Calcein-AM vital dye was observed).

To determine thermal shock experienced by the embryos due to laser beam exposure, we measured the expression of genes encoding two key heat shock proteins: Hsp90aa1 and Hspa5. We found significant differences in expression of these genes between the positive and negative control groups, while expression of these genes in the experimental groups showed no significant differences from the control groups. The findings are consistent with theoretical estimates.

CONCLUSIONS

The findings of this pilot study suggest that the effects observed can result from local laser beam exposure, and the embryo generally remains viable. Thus, comprehensive assessment of the efficacy and safety of laser assisted hatching involving the use of the fs laser requires further research. It is necessary to perform experimental comparison of the femtosecond laser and millisecond lasers widely used in clinical practice in the context of their impact on the heat shock protein expression, as well as to assess the femtosecond laser effects on the mouse embryos *in vivo* by transferring the embryos to gestational surrogates.

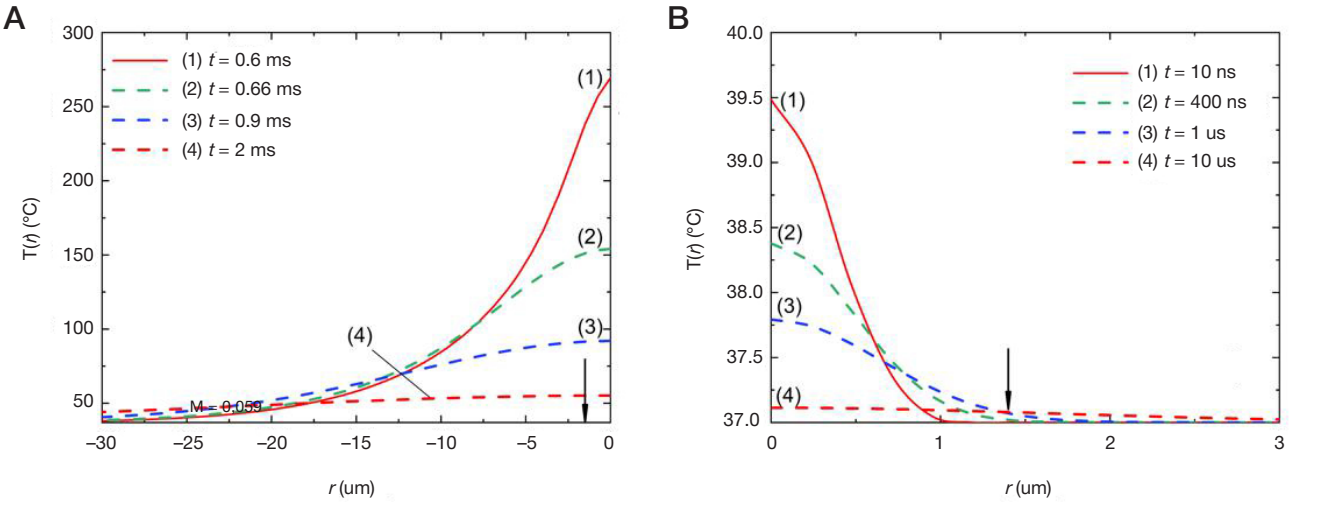

Fig. 5. Radial temperature profiles at different times for the millisecond (**A**) and femtosecond (**B**) pulse. Laser beam size at 1/e level is marked with arrows. Time (t) is counted down from the beginning of the pulse

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