ASSESSMENT OF THE ZONA PELLUCIDA MICRODISSECTION ON ITS THICKNESS IN MAMMALIAN EMBRYOS

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The zona pellucida (ZP) is a dynamically changing object that plays an important role during the preimplantation stage of embryogenesis. The ZP thickness may affect the implantation success and pregnancy rate, it is considered as a prognostic factor in a number of studies. The study was aimed to assess the dynamic changes in the mouse embryonic ZP thickness after laser assisted hatching (LAH) that involved breaching the ZP integrity at the blastocyst stage. Femtosecond laser pulses were used to perform the zona microsurgery. The zona thickness was measured both at the stage of blastocyst microsurgery (~E3.5, i.e. 3.5 days of embryogenesis) and at the hatching stage (~E5). Significant differences in the ZP thickness were revealed in the control group of embryos: from 6.21 μm (E3.5) to 5.4 μm (E5). The changes in thickness from 6.6 μm (E3.5) to 6.2 μm (E5) observed in the group subjected to LAH were non-significant. Tracing the ZP thickness of a particular embryo from the blastocyst stage to the hatching stage made it possible to estimate the thinning coefficients in the experimental and control groups. The findings that indicate lower tensile strength of the zona in case of LAH can provide the basis for further research on the ZP properties in case of using the embryo cryopreservation protocols.

Keywords: blastocyst, zona pellucida thickness, thinning, laser assisted hatching, femtosecond laser pulses

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Compliance with ethical standards: animals were handled in accordance with the Declaration of Helsinki and the guidelines issued by the Bioethics Commission at the Institute of Gene Biology of the Russian Academy of Sciences.

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ИССЛЕДОВАНИЕ ВЛИЯНИЯ МИКРОДИССЕКЦИИ БЛЕСТЯЩЕЙ ОБОЛОЧКИ ЭМБРИОНОВ МЛЕКОПИТАЮЩИХ НА ЕЕ ТОЛЩИНУ

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Блестящая оболочка (*zona pellucida*, ZP) — динамически меняющийся объект, играющий важную роль на преимплантационной стадии развития эмбриона. Ее толщина может оказывать влияние на успешность имплантации и частоту наступления беременности, в ряде исследований ее рассматривают как прогностический критерий. Целью работы было исследовать динамику толщины блестящей оболочки эмбриона мыши в результате процедуры вспомогательного лазерного хетчинга (ВЛХ), когда нарушение целостности ZP проводят на стадии бластоцисты. Для микрохирургии оболочки использовали импульсы излучения фемтосекундной длительности. Измерение толщины оболочки проводили как на стадии микрохирургии бластоцисты (~E3.5, т. е. 3,5 дня эмбрионального развития), так и на стадии вылупления (~E5). Обнаружены статистически значимые различия толщины ZP эмбрионов в контрольной группе — с 6,21 мкм (E3.5) до 5,4 мкм (E5). В группе, подвергавшейся ВЛХ, изменения толщины с 6,6 мкм (E3.5) до 6,2 мкм (E5) оказались статистически не значимыми. Отслеживание толщины ZP индивидуального эмбриона от стадии бластоцисты до стадии вылупления позволило оценить коэффициент истончения для контрольной и экспериментальной групп. Полученные данные свидетельствуют о снижении предела прочности оболочки при проведении ВЛХ и могут служить базисом для последующих исследований ее свойств в случае применения протоколов криоконсервации эмбрионов.

Ключевые слова: бластоциста, толщина блестящей оболочки, истончение, вспомогательный лазерный хетчинг, фемтосекундные лазерные импульсы

Финансирование: экспериментальные исследования по ВЛХ были выполнены с использованием УНУ «Лазерный тераваттный фемтосекундный комплекс», входящим в состав ЦКП «Лазерный фемтосекундный комплекс» ОИВТ РАН при финансовой поддержке Министерства образования и науки РФ в рамках Государственного задания № 075-01129-23-00. Эмбрионы были получены с использованием Уникальной научной установки «Трансгенбанк» при финансовой поддержке Министерства образования и науки РФ в рамках проекта (Соглашение № 075-15-2021-668 от 29.07.2021).

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Соблюдение этических стандартов: все манипуляции с животными производили в соответствии с требованиями Хельсинкской декларации и рекомендациями комиссии по биоэтике Института биологии гена РАН.

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The zona pellucida (ZP) is an extracellular matrix that surrounds the oocyte at the early stages of development and performs a number of important functions during oogenesis, fertilization, and preimplantation development. The ZP plays a role of physical barrier protecting the embryo from microorganisms, viruses, and immune cells, which could be present in the oviduct [1]. It allows the oocyte to move freely in the fallopian tube after ovulation, prevents embryo implantation in the oviduct wall [2], enables species-specific fertilization, induction of acrosome reaction in sperm, and blocks polyspermic fertilization [3]. Limitation of volume of the embryo surrounded by the ZP ensures a close contact between blastomeres that is essential for compaction at the stage of cleavage [4] and later maintains integrity of the inner cell mass (ICM) [5]. The ZP thickness of the embryo is crucial: it affects the embryo's implantation in the endometrium.

There are certain factors, the effects of which make it difficult for the embryo to hatch from its shell on its own and then to implant: for example, in case of thicker (more than 17 μm thick) [6, 7] or dense ZP, or in case of impaired production of enzymes responsible for the ZP lysis [8]. In the last decade, laser assisted hatching (LAH) has become increasingly used in the clinics. This procedure is based on the ZP exposure to laser pulses aimed at the zona thinning/dissecting and facilitation of subsequent embryo hatching. Millisecond infrared laser dissectors are usually used for this purpose. Laser pulse absorption by the shell results in the local ZP protein disruption due to heating to several hundred degrees [9]. The size of the dissection site of 5–20 μm is determined by the laser pulse energy and thermophysical properties of the object. There is a possibility of thermal damage to embryonic cells adjacent to the ZP, that is why it is recommended to perform laser microdissection in strict compliance with the regulations at the early stages of preimplantation development characterized by wider perivitelline space (i.e. the space between the ZP and the cell membrane of oocyte or zygote) compared to the later stages of preimplantation development.

The laser microsurgery technique involving the use of femtosecond laser pulses can be an alternative to the applied commercial solutions. The physics of the femtosecond pulse interaction with the biological objects is based on the multiphoton absorption processes, and the local breaching of the ZP integrity is of non-thermal nature [10]. This fact makes it possible to precisely define the area of laser exposure confined to the focal spot size of ~1.5–2 μm. The benefits of using femtosecond laser pulses that include minimum ZP dissection width and minimization of the radiation thermal effects on adjacent embryonic cells were earlier implemented in the technologies for controlled laser assisted hatching [11] and embryo tagging during preimplantation development [12, 13].

The interest in studying the ZP properties is increased due to the desire to discover reliable indicators of oocyte quality. It is oocyte quality that is considered one of the major factors of female fertility, since it determines the original potential of embryogenesis [14]. Thus, there is a number of studies of the correlation of the ZP thickness (as one of the indicators) [15, 16] and its variability in embryos with the pregnancy and implantation rates [6, 15, 17, 18]. The ZP mechanical characteristics were also considered as predictors of successful oocyte development after fertilization [19], embryo quality [20], and successful implantation [21].

Despite the fact that LAH has been used in clinical practice for several decades, there are just a few studies focused on the impact of this procedure on the ZP structure and morphology. The relevance of such studies is beyond doubt, since comparison of the ZP dynamic changes associated with experimental breaching of its integrity with the natural cycle makes it possible to acquire additional data on the mechanisms underlying the ZP disruption/thinning and the factors that determine successful hatching and subsequent embryo implantation. Furthermore, effective approaches to embryo cryopreservation by vitrification at any stage of preimplantation development have been introduced into clinical practice. At the same time, there are data on alteration of the ZP physical and chemical properties in such embryos, as well as on the ZP thickening (the so called zona hardening) [22]. In this regard it is essential to study the effects of LAH on alterations of the ZP properties, since the findings can provide the basis for comparative analysis of hatching peculiarities in the embryos subjected to vitrification.

The study was aimed to assess the effects of LAH performed using femtosecond laser on the ZP characteristics of the house mouse (*Mus musculus*) embryos at the blastocyst stage. This stage of embryogenesis represents the beginning of embryo hatching, during which the ZP is subjected to substantial strain. The fundamental possibility of the ZP microsurgery at the blastocyst stage (i.e. on the later stage of preimplantation development) is ensured by using femtosecond laser pulses. As shown earlier [11, 23], the ZP microsurgery involving such pulses has no adverse effect on embryogenesis.

METHODS

Experimental apparatus

Microsurgical experiments involving embryos were performed using the femtosecond laser scalpel set-up (Fig. 1) created by the Joint Institute for High Temperatures of the Russian Academy of Sciences [24]. The beam generated by the TETA femtosecond laser source (Avesta; Troitsk) was used as a laser scalpel. After conversion to the second harmonic in the DKDP crystal, the laser pulse parameters were as follows: pulse duration 280 fs, energy 50 μJ, wavelength 514 nm, pulse repetition rate 2.5 kHz. The laser beam was fed through the right side port of the Olympus IX-71 inverted microscope and focused into the spot with a diameter of about 2 μm (FWHM) with the 20× UPlanFL objective lens (Olympus; Japan) having a 0.5 numerical aperture (NA). Attenuator was mounted on the laser beam path before entering the microscope for the laser pulse energy adjustment, and the telescope was used to align the laser beam diameter with the objective lens aperture. When performing microsurgical procedures, the laser pulse energy was 20 nJ, which corresponded to the intensity of 2.5 TW/cm^2. The embryos were distributed into droplets of growth medium in Petri dishes with the glass bottom thickness of 170 μm. A Petri dish was placed on the motorized microscope stage (Märzhäuser Wetzlar; Germany) to move the embryo relative to the stationary laser beam. The beam was focused in the plane of maximum embryo cross-section, i.e. in the equatorial plane. Imaging of the embryos was performed using the DFK 72AUC02 camera (The Imaging Source; Germany). Software allowing the operator to set the trajectory of the laser beam motion over the image of the embryo was used to automate the microsurgical procedure.

Animals

Embryos were collected from the C57Bl/6JxCBA F1 hybrid mice purchased from the animal house of the Federal Medical Biological Agency of Russia (Stolbovaya branch; Russia).

Animals were kept in the controlled environment (22–24 °C, 14 : 10 h light/dark cycle (day : night)) with *ad libitum* access to food (special extruded diet for mouse breeding) and water.

Hormonal stimulation of ovulation

The widely accepted protocol for ovulation induction was used to collect a large number of embryos at once. Immature females about 3 weeks of age with body weight of 10–12 g were used as donors. Hormonal stimulation was performed in accordance with the two-step protocol: the pregnant mare serum gonadotropin (PMSG) was administered intraperitoneally at 13:00 of day one, 5 IU per animal. HCG (Chorulon, Merck Animal Health, USA), 10 IU per animal were administered 48 h later. After that the females were housed with males for mating.

Obtaining embryos

Animals were euthanized by cervical dislocation. The zygote stage embryos were collected on the day of the copulation plug detection. Oviducts were cut out from the animal's body with scissors and placed in the HEPES-containing Ooclean medium (PanEco; Russia) heated to 37 °C. Then the oviduct ampullae were dissected with the syringe needles under stereomicroscope to extract the cumulus-oocyte complexes. Then about 0.03 g of hyaluronidase (Lydase; Microgen, Russia) were added to this droplet to provide zygote purification from the cumulus cells. The zygotes obtained were serially washed in four droplets of Ooclean medium and transferred to the culture medium.

Transportation of embryos

To be transferred from the Institute of Gene Biology of the Russian Academy of Sciences to the Joint Institute for High Temperatures of the Russian Academy of Sciences for LAH, the embryos were placed in the HEPES-containing Ooclean medium (PanEco; Russia) previously heated to 37 °C. The embryos were transported in the 2 ml eppendorf tubes. The temperature of +37 °C was maintained during transfer of the tube containing embryos. After transportation the embryos were washed in the culture medium. For that these were transferred from one droplet to another three times.

Embryo culture

The embryos were cultured to the morula stage (about 2.5 days of embryogenesis, E2.5) in the four-well plates (Thermo Fisher Scientific Nunc; USA) using the CSCM-C 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 droplets of the CSCM-C medium (Fujifilm Irvine Scientific; USA) covered with mineral oil, 2–3 embryos per droplet.

Experiment

Three droplets containing culture medium were previously formed in each glass bottom Petri dish (catalogue number № 200350; SPL Lifesciences, Korea), then the droplets were covered with mineral oil. A day before the experiment, the morula stage embryos were distributed into well-prepared Petri dishes and transferred to the incubator. Three embryos were placed in each droplet. Microsurgery of the embryos' ZP was performed at the blastocyst stage (~E3.5).

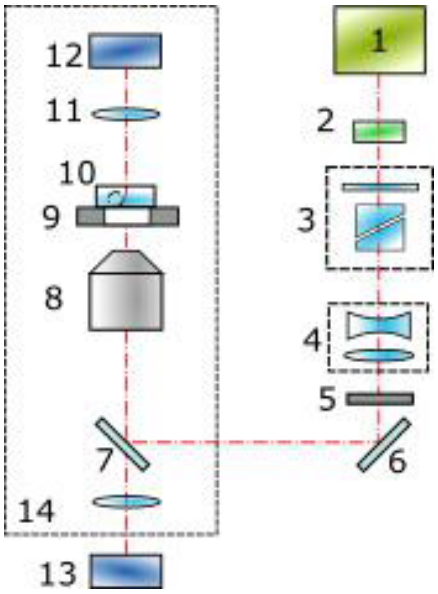

Fig. 1. Scheme of femtosecond laser scalpel. 1 — femtosecond laser; 2 — converter to second harmonic; 3 — attenuator; 4 — telescope; 5 — mechanical laser interrupter; 6, 7 — mirrors for the laser beam wavelength; 8 — objective lens; 9 — motorized microscope stage; 10 — Petri dish containing embryo; 11 — substage condenser; 12 — substage lamp; 13 — CMOS camera; 14 — inverted microscope

A total of 99 embryos were divided into two groups: experimental group (EG) of 63 embryos and concurrent control group (CC group) of 36 embryos. The operator took a picture of the embryo in its initial state, set the trajectory of the laser beam motion for the embryo's ZP microdissection, performed LAH, and took a final picture of the embryo after the procedure. The total time spent on one embryo did not exceed 1.5 min. The embryos usually hatched at the E5 stage, during which they were photographed again.

Statistical data processing

Statistical processing of the results was performed using the Statistica 7.0 software package (Dell; USA) and Microsoft Excel 2013 (Microsoft Corporation; USA). The hypotheses were tested for normality using the Kolmogorov–Smirnov and the Shapiro–Wilk tests. Since the distributions of the data provided in the study were non-normal, nonparametric tests were used for analysis. In this case, median, quartiles (25–75%), and variation (minimum and maximum values of the studied parameters) were considered as significant indicators. The Mann–Whitney U test was used to compare two independent groups; two dependent groups were compared using the Wilcoxon signed-rank test for dependent samples. Significance levels of all statistical parameters were set to 0.05.

RESULTS

Fig. 2A shows a fragment of the embryo's ZP before exposure to laser beam. The primitives (green lines 1–3) set the trajectory of the laser beam motion. Laser exposure of the area along the polyline 1 was essential for breaching of the embryo's ZP integrity during the LAH procedure. About 80–90% of the ZP thickness were cut. Lines 2 and 3 were auxiliary ones (to confirm that the embryo hatched through the drilled hole (Fig. 2B)). The ZP thickness of each embryo of the experimental group at the time of the procedure was measured. The ZP thickness Δ was measured in three different areas with the angular increment of ~90–120°. Similar measurements of the embryo's

ZP thickness were performed at the stage of embryo hatching and after hatching in both experimental (Fig. 2C) and CC (Fig. 2D) groups.

The data on the ZP thickness for the embryos of the experimental and control groups are provided in Fig. 3. As is commonly known, the ZP is stretched during hatching in the natural conditions. Thus, we have shown that the ZP thickness demonstrates a significant decrease in Δ_m (from 6.21 μm (E3.5) to 5.4 μm (E5)) in the control group of animals not exposed to laser beam. No such ZP thinning has been revealed in the experimental group, and the measured Δ_m values are 6.6 μm (E3.5) and 6.2 μm (E5). Statistical processing of the experimental data using the Mann–Whitney U test has confirmed significant differences between the ZP thickness (E5) values observed during the natural hatching cycle and after the LAH procedure.

The ZP thinning coefficient $K = \Delta_{E5}/\Delta_{E3.5}$, where $\Delta_{E3.5}$ and Δ_{E5} are the ZP thickness values of specific embryo obtained 3.5 and 5 days after fertilization, respectively, is one more interesting parameter. The median value of this parameter in the experimental group ($K_{med.e}$) is 0.95. The bars of the bar chart (Fig. 4A) to the right of the $K = 1$ line show that the ZP thickness measured at the stage of hatching is greater than that measured at the time of microsurgery. In certain cases the differences reported reach 23%. This may be due to the fact that the blastocyst demonstrates cyclic volume changes (expansion and collapse) during hatching, thereby stretching the ZP [25]. The ZP thickness measured at the microsurgery stage (E3.5), therefore, corresponded to the stretching phase. In the control group, the ZP thickness was also assessed in the embryos that had reached the blastocyst stage (E3.5). According to Fig. 4B, the ZP of the vast majority of embryos becomes thinner during hatching. The share of embryos with $K > 1$ is less than 20%. Similar to the experimental group, this can be due to the changing thickness of the blastocyst ZP (E3.5) during expansion. The median coefficient values ($K_{med.cc} = 0.84$) also indicate greater ZP thinning in non-treated embryos.

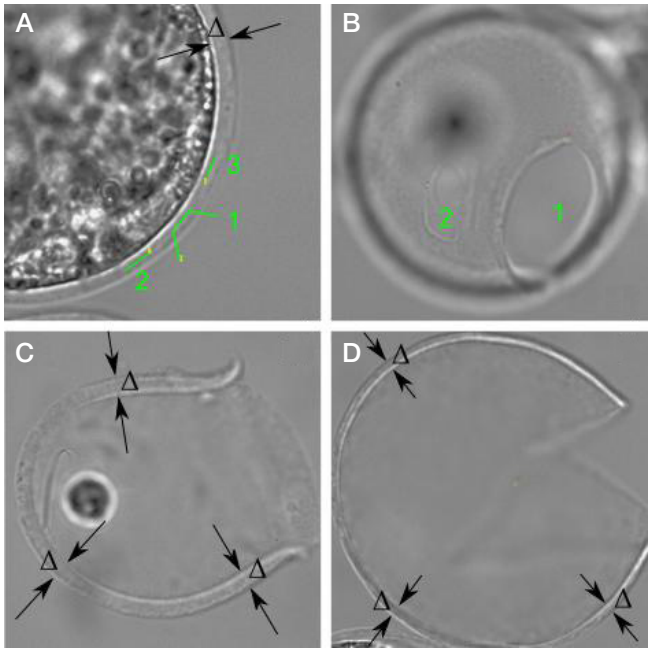

Fig. 2. Embryos before zona microsurgery (**A**); zona pellucida after hatching (**B**). 1 — hole in the ZP after blastocyst hatching resulting from incision along the trajectory 1 (on the fragment a); 2 — results of incision along the trajectory 2 (on the fragment a). The zone after hatching of the embryo in the experimental group (**C**) and CC group (**D**)

DISCUSSION

The embryo hatching from the ZP is an important step that is essential for further embryo implantation in the uterus. Hatching is preceded by cyclic blastocyst expansion and collapse. The changes in blastocyst volume result in physical impact on the ZP, since these cause ZP stretching and thinning. Exposure to tensile stress and proteases (such as trypsin) synthesized by the uterus and/or blastocyst results in the ZP breaching that makes blastocyst hatching possible.

In terms of mechanics, the observed differences between the experimental and control groups can be interpreted in the following way. The breaking strength is a threshold value of mechanical stress applied to the sample; exceeding the threshold results in destruction of the material. As applied to the hatching process, the embryo's ZP is permanently exposed to tensile stress during the blastocyst expansion stage. Exceeding the tensile strength threshold leads to the ZP rupture and initiation of hatching. There are numerous studies aimed at developing models describing the ZP mechanical properties. The approaches used include describing the ZP as a material having linear elastic, hyperelastic, and viscoelastic properties (the detailed review of the present state of the art in analytical and numerical approaches can be found in the recently published report [26]).

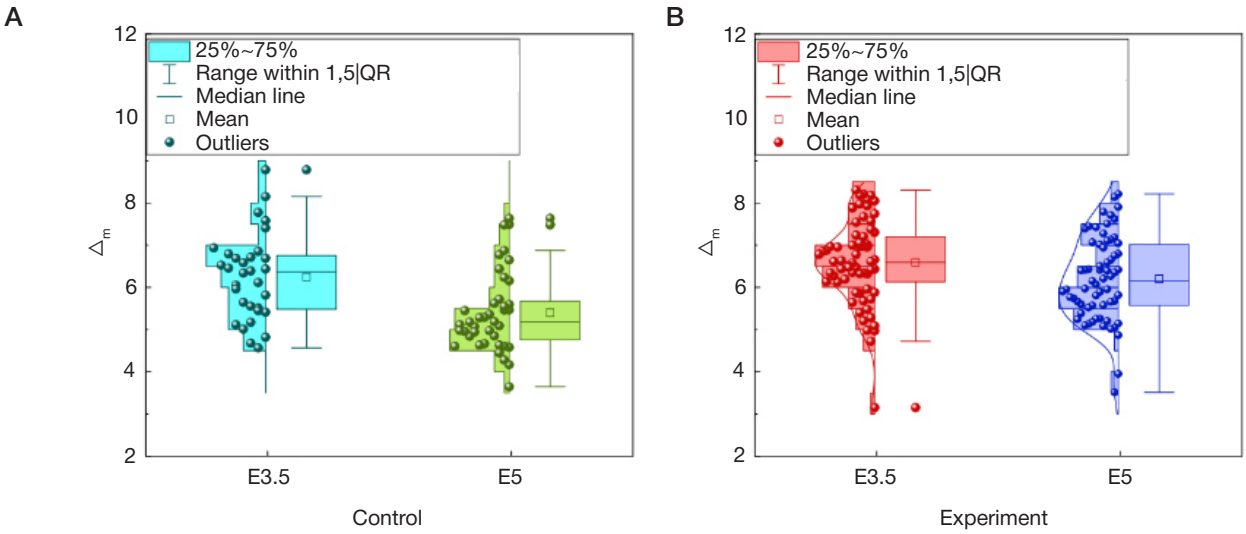

Fig. 3. Changes in the ZP thickness during embryogenesis in the control (**A**) and experimental (**B**) groups

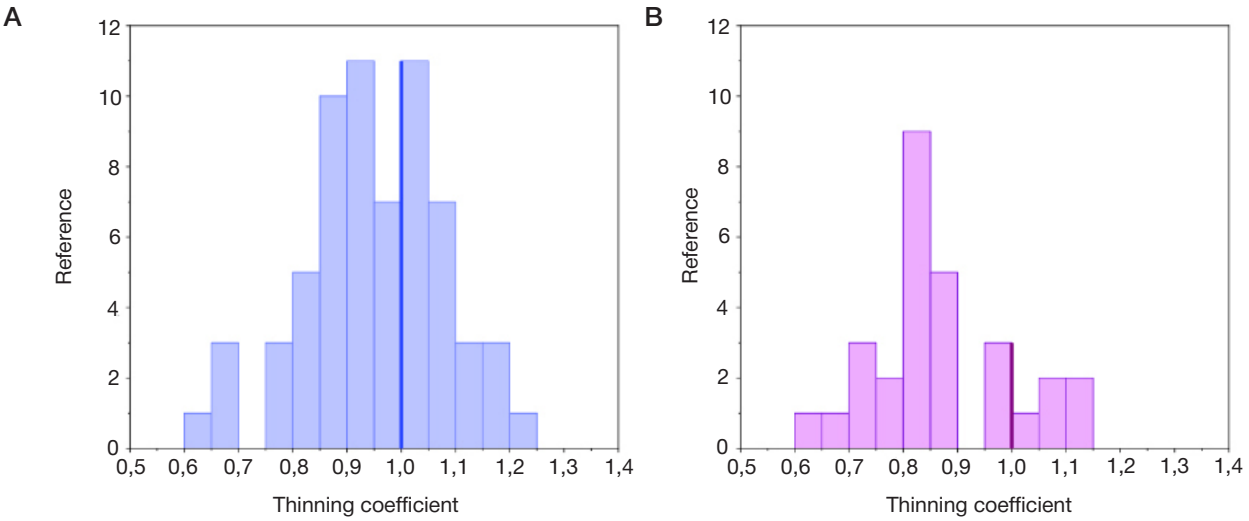

Fig. 4. Distribution of the ZP thinning coefficient in the experimental (**A**) and control (**B**) groups

Constructing the model of the mouse embryo ZP mechanical properties lies beyond the scope of this study. However, the data obtained can be analyzed taking into account the current knowledge about the ZP properties. Analysis of the bar charts provided in Fig. 4 appears to be in qualitative agreement with the available models. With an increase in the blastocyst diameter, the tensile stress at the ZP is increased. The ZP radius is increased, while its thickness is reduced. Currently, there are literary data on the Young's modulus (the ratio of the applied stress to the deformation response of the material) values of the mammalian ZP; these values of the mouse oocyte and embryo are 17.9 and 42.2 kPa, respectively [27]. The Young's modulus characterizes the material properties in the linear section of the stress-strain diagram, right up to the yield strength, at which the elastically loaded sample elongates when stretched, but returns to its original form and size when unloaded. When this limit is exceeded, the material ceases to be elastic and starts to deform plastically (does not fully return to its original state). The thinning coefficient values $K < 1$ provided in Fig. 4B indicate plastic deformation after the tensile stress termination. When the stress threshold value (tensile strength) is reached, the zona breaches and the embryo hatches. Breaching of the ZP integrity during LAH results in significantly lower threshold, as evidenced by the ZP thickness mean values Δ_{E5} and the values of the K thinning coefficient that are greater than that of the control group. The paper [25] reports the hypothesis of reduced implantation capability, possibly due to adverse effects of thick ZP on the blastocyst expansion. This hypothesis is consistent with the theory of strength of materials and the need to apply more force per surface area unit to deform a thicker layer of the zona.

The impact of cryopreservation procedures on the state of the ZP of the oocyte/embryo has been studied fairly extensively. There is abundant evidence that cryopreservation makes their ZP harder [22, 28]. However, the question of how cryopreservation affects the ZP state and properties during the later stages of embryogenesis requires further research. The logical next step of the experiment will be replacing the research object with the embryo subjected to cryopreservation. The data obtained in this study will be used as reference data when studying the ZP thinning in cryopreserved embryos subjected to LAH performed using femtosecond laser pulses at the blastocyst stage.

CONCLUSIONS

This study was focused on assessing the mouse embryo zona pellucida (ZP) thickness both in natural hatching cycle (control group, non-treated embryos) and after the LAH procedure (experimental group). Minimal thermal effects of femtosecond laser beam made it possible to perform microsurgery at the late stage of preimplantation development, at the blastocyst stage before hatching. Measuring the ZP thickness at the blastocyst stage (E3.5) and hatching stage (E5) allowed us to estimate the thinning coefficient of each particular shell. Statistical analysis revealed significant differences in the zona thickness between the experimental and control groups at the blastocyst hatching stage: 5.4 and 6.2 μm, respectively. It was also noted that LAH resulted in almost no stretching of the embryo's ZP: the ZP thickness was 6.6 μm (E3.5) and 6.2 μm (E5), as evidenced by the thinning coefficient median value. We believe that this fact is due to lower tensile stress threshold. The experimental data obtained will provide the basis for comparison during further assessment of the ZP thickness in cryopreserved embryos and embryos grown in media of various compositions.

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