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ORIGINAL ARTICLE

The impacts of laser zona thinning on hatching and implantation of vitrified-warmed mouse embryos

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Abstract

Embryo vitrification has advantages in assisted reproduction yet it also induces zona hardening. Laser zona thinning (LZT) is considered as a solution yet its efficacy and security have not been well studied. In this study, we used vitrified-warmed morulae from 2-month-old and 10-month-old ICR female mice as model to investigate the impacts that LZT treatment brings to the in vitro hatching process and implantation by analyzing hatching rate, implantation rate, and blastocyst quality. The results showed that the fully hatched rate was significantly higher after LZT treatment for both young (25.7% vs. 16.2%, $P < 0.05$) and aged (36.6% vs. 13.2%, $P < 0.01$) mice. For zona-thinned morulae in young mice, its onset of hatching occurred earlier (28.6% vs. 8.8%, $P < 0.01$) at D4 and with a greater percentage of U-shaped hatching at D5 (48.3% vs. 33.0%, $P < 0.05$). LZT treatment did not induce expression change of apoptosis-related genes in all groups ($P > 0.05$), but for young mice, the total cell number of day 5 blastocyst in zona-thinned group was significantly less than that of the control group (40.6 ± 5.1 vs. 59.9 ± 14.5, $P < 0.01$). At last, there was an increasing implantation rate in zona-thinned compared to the control group for young (63.8% vs. 52.5%, $P > 0.05$) and aged (55.6% vs. 47.2%; $P > 0.05$) mice after embryos were bilaterally transferred in the same recipient. In conclusion, the significant increase of fully hatched rate after LZT treatment is related to the advanced onset of hatching as well as the enhancement of superior hatching structure, and LZT also lead to a better implantation after embryo transfer.

Keywords Mouse embryo · Vitrification · Laser zona thinning · Hatching · Implantation

Introduction

As one of the milestone for assisted reproductive technology, human embryo was firstly successfully cryopreserved in 1983 [1]. Embryo cryopreservation can long-term preserve those superfluous embryos after in vitro fertilization in vitro culture, and effectively prevent the occurrence of ovarian hyperstimulation syndrome, after ovarian recovery, frozen-thawed embryos are able to transfer to the uterus horn after one or several programmed or natural physiological cycles [2]. However, it has been accepted that zona pellucida (ZP) will undergo problematic hardening during the freeze-thaw procedure [3], which is commonly occurred in ART, exerting adverse effect on embryo hatching and implantation [4]. To be more specific, cryopreservation induces microstructural change on embryo ZP after freezing-thawing [5] and study has demonstrated that the vitrification solution containing DMSO and EG which could induce ZP hardening [6]. Apart from being induced by vitrification, zona hardening is thought to be a phenomenon that is more prevalent among senior women [7]. A study found out that ZP thickness decreased while the density of the ZP increased in two age groups (older, > 38 years; younger, ≤ 38 years) with extended culture; however, the older group had significantly denser ZP compared with the younger group [8], indicating that the microstructure of the ZP is also influenced by age.

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For this situation, Jacques Cohen suggested using assisted hatching to cope with this problem [9]. This is a methodology using various approaches to drill a hole on ZP, which is believed to facilitate the embryo's escape from the ZP, and this strategy has been suggested as a mean of improving pregnancy rates in patients with recurrent implantation failure [10] or in frozen embryo transfer cycles [3]. Later, as a non-invasive technique, laser gradually introduced to assist embryo hatching in early 1990s, which is considered to be safer compared to other assisted hatching techniques [11]. Although laser zona thinning (LZT) is not included as an approach of assisted hatching [12], it is still a commonly used technique in assisted reproduction [13] in view of a functional ZP is critical in protecting the embryos from detrimental factors likes toxins, microorganisms, and antibodies in IVF media [14–16], and zona-opening could lead to the loss of embryonic autocrine growth factors [17], which will bring potential risk to embryo development. However, whether LZT is necessary to improve the implantation ability of frozen-thawed, embryo is still under debate [18]. Some researchers suggested that cryopreservation will induce zona change, so LZT can increase the implantation of frozen-thawed embryos [19–21], while other researchers have opposing views because they could not obtain positive outcome [12, 22–25] and two prospective randomized controlled trials [26, 27] even had controversial opinions about the effectiveness of LZT. The different manipulation condition in different assisted reproductive center account for those controversial comments; therefore, reliable outcomes should be obtained from refined randomized controlled trials.

To our knowledge, this prospective randomized study was for the first time to investigate what impacts will LZT treatment bring to hatching dynamics of vitrified-warmed mouse embryo. Moreover, vitrified-warmed embryos from laser-thinned and control groups were bilaterally transferred to the same recipient to investigate the impact of LZT treatment on implantation. Furthermore, age is also taken into account as a factor to study whether LZT treatment has distinct effectiveness on different ages. This study can provide valuable information to the enhancement of human-assisted reproductive technology.

Materials and methods

Unless otherwise indicated, all chemicals and media were purchased from Sigma-Aldrich (MO, USA). The protocols for the animal studies were approved by and performed in accordance with the requirements of the Institutional Animal Care and Use Committee of China Agricultural University.

Vitrified-warmed mouse embryos collection

CD-1 (ICR) mice purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) were used to collect oocyte and spermatozoa. Mice were under free feed intake, housing with a 12-h light/dark photoperiod and maintained in an air-conditioned (22–25 °C) and 40% humidity room. Female ICR mice (2 months of age, 31.3 ± 1.8 g; 10 months of age, 42.8 ± 5.0 g) were injected with 7.5 IU pregnant mare serum gonadotrophin intraperitoneally (PMSG; Tianjin Animal Hormone Factory, Tianjin, China) then 7.5 IU human chorionic gonadotrophin (hCG; Ningbo Animal Hormone Factory, Ningbo, China) after 48 h. After being sacrificed by cervical dislocation, sperm from the caudal epididymis of adult male ICR mice (10–14 weeks of age) were suspended in human tubal fluid (HTF) medium (Millipore, MA, USA) and incubated at 37 °C under 5% CO_2 and 95% humidity for 1 h in advance. Oocytes were collected from the oviducts of female mice 13–15 h after hCG injection. The capacitated sperm suspension was gently added to the cumulus-oocyte complexes at a final motile sperm concentration of 10^6 cells/ml. The oocytes and sperm were incubated in HTF medium for fertilization. After 4 h, the fertilized oocytes were washed for three times and transferred to fresh culture medium. Double pronuclei were confirmed with phase-contrast microscopy. All zygotes were cultured in micro drops (80 μL) of medium in culture dishes (Nunc, Roskilde, Denmark) overlaid with mineral oil for 3 days. Embryo culture, capacitation and fertilization dishes were prepared 4 h prior to embryo culture so as to allow gas and temperature equilibration.

Morulae in good quality were collected then vitrified after 3-day culture. The quality of morula on day 3 was assessed according to the previous criteria [28]. For vitrification, pretreatment solution contained 10% dimethyl sulfoxide (DMSO) and 10% ethylene glycol (EG) in PBS medium. Vitrification solution (EDFS35) contained 17.5% DMSO (v: v), 17.5% EG (v: v), 30% Ficoll (w: v) and 0.5 M sucrose in PBS medium. Embryos were vitrified in EDFS35 by the open-pulled straws (OPS) method [29]. First, embryos were rinsed in pretreatment solution for 30 s, then transferred to vitrification solution in the narrow end of the OPS, and held for 25 s. Then the straws were immediately plunged into liquid nitrogen (LN_2). For thawing, the embryos were rinsed in 0.5 M sucrose for 5 min, then rinsed three times in M2 medium.

Laser zona thinning

Laser manipulation were performed according to previous reports [30, 31]. We used the XYClone Laser System (Hamilton Thorne, MA, USA), including an integrated class I laser (1.46 μm, 300 mW) with 20 × objective, and a 640-μm pilot light on an inverted microscope with heated stage (TL3-220P,

Olympus, Japan). The pilot light was calibrated to a target on a video monitor (Hamilton Thorne, MA, USA) which was used as the primary visualization mode for aiming the laser during use. Prior to manipulation, vitrified-warmed morulae were randomly divided into two groups, then the experimental group was positioned on the microscope stage with the laser target located on the outer edge of the ZP, the thinning area should be located at the maximum distance between the blastomere and the target site to avoid blastomere damage. Position the innermost isotherm ring (red) of the laser so that it is centered over the ZP target site using the 20 × objective. Click the "Fire" button to produce a single laser pulse, and a maximum of six ablations were made successively around the ZP to achieve a depth of 50–60% of the ZP thickness for a total length of approximately 80 μm (quarter thinning) (Supplemental Figure 1). All manipulations were performed at room temperature (25–26 °C).

Embryo in vitro culture and hatching dynamics observation

After LZT treatment, zona-thinned and control vitrified-warmed mouse morulae were continue to culture in the same HTF medium at 37 °C in an atmosphere containing 5% CO_2. The embryonic development were observed and photographed respectively at 24 h, 48 h, and 72 h after re-culture. The definition of blastocyst formation and hatching status were based on previous criteria [32]. For further verification, live recording of embryonic development was conducted in confocal scanner box CellVoyager (CV1000, Yokogawa, Japan), and images were captured every 60 s, lasting for 3 days. At 24 h and 48 h, the hatching rate calculated as the number of hatching blastocysts divided by the number of existing blastocysts at 48 h × 100 (%). At 72 h, the fully hatched rate was calculated as the number of hatched blastocyst divided by the number of existing blastocysts at 48 h × 100(%). Blastocyst formation rate calculated as the number of blastocyst at 48 h divided by the number of cultured vitrified-warmed embryos × 100 (%). We took previous study as a reference for the definition of 8-shaped and U-shaped hatching structure of blastocysts [33] (Fig. 1a).

Cell number counting of d5 blastocyst

D5 blastocyst were collected and stained with DAPI to mark all cell of embryos according to previous reports [34]. Briefly, after overnight, 4% paraformaldehyde (with DEPC) fixing, the blastocysts were rinsed with phosphate-buffered saline (PBS) for three times, then placed into 50 μL of 300 nM DAPI (with anti-fade mounting medium) (H1200, Vector, CA, USA) staining solution for 1 min incubation and put onto a slide glass for observation under a fluorescence microscope (BX50, Olympus, Japan) (Fig. 2a).

cDNA preparation and real-time PCR analysis

RNA was extracted from 50 expanded blastocyst using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The first cDNA strand was synthesized using a cDNA reverse transcription kit (Applied Biosystems, MA, USA). Quantitative real-time PCR was carried out using the CFX96TM real-time PCR detection system (Bio-Rad, CAL, USA) under standard conditions. At least triple samples were analyzed for each gene (*Bax*, *Caspase 3*, *Caspase 9*, *Cyto C*) and β-actin was used as a reference gene. The expression levels were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method [35]. The primers are shown in Supplemental Table I.

Embryo transfer and implantation

Female mice were mated with vasectomized males to induce pseudo-pregnancy in the evening. On the next morning, female mice with vaginal plugs were defined as pseudo-pregnant for 0.5 day. At DOP2.5 (day-of-pregnancy), pseudo-pregnant recipient mice were anesthetized with 2%

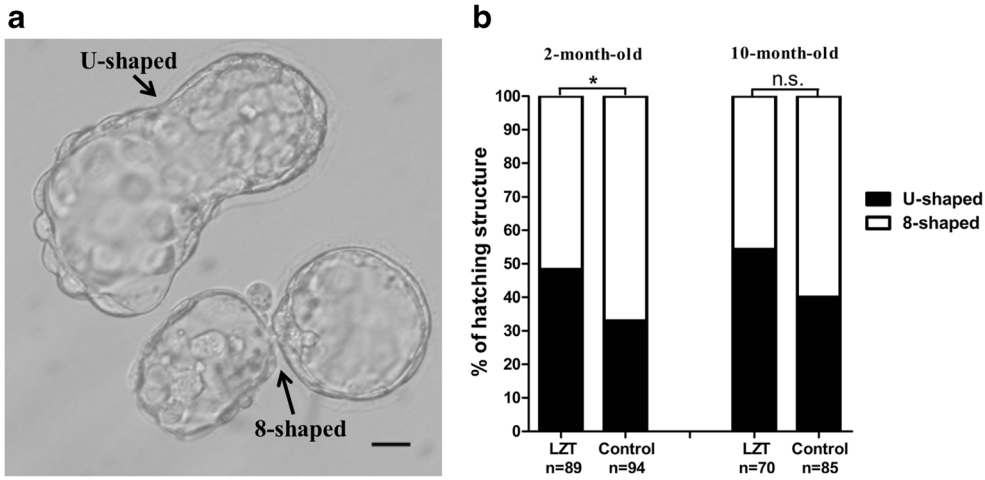

Fig. 1 **a** shows different hatching structures based on the size of opening (scale bar indicates 20 μm). **b** shows a significant difference on percentage of U-shaped hatching for 2-month-old mice after LZT treatment ($P < 0.05$)

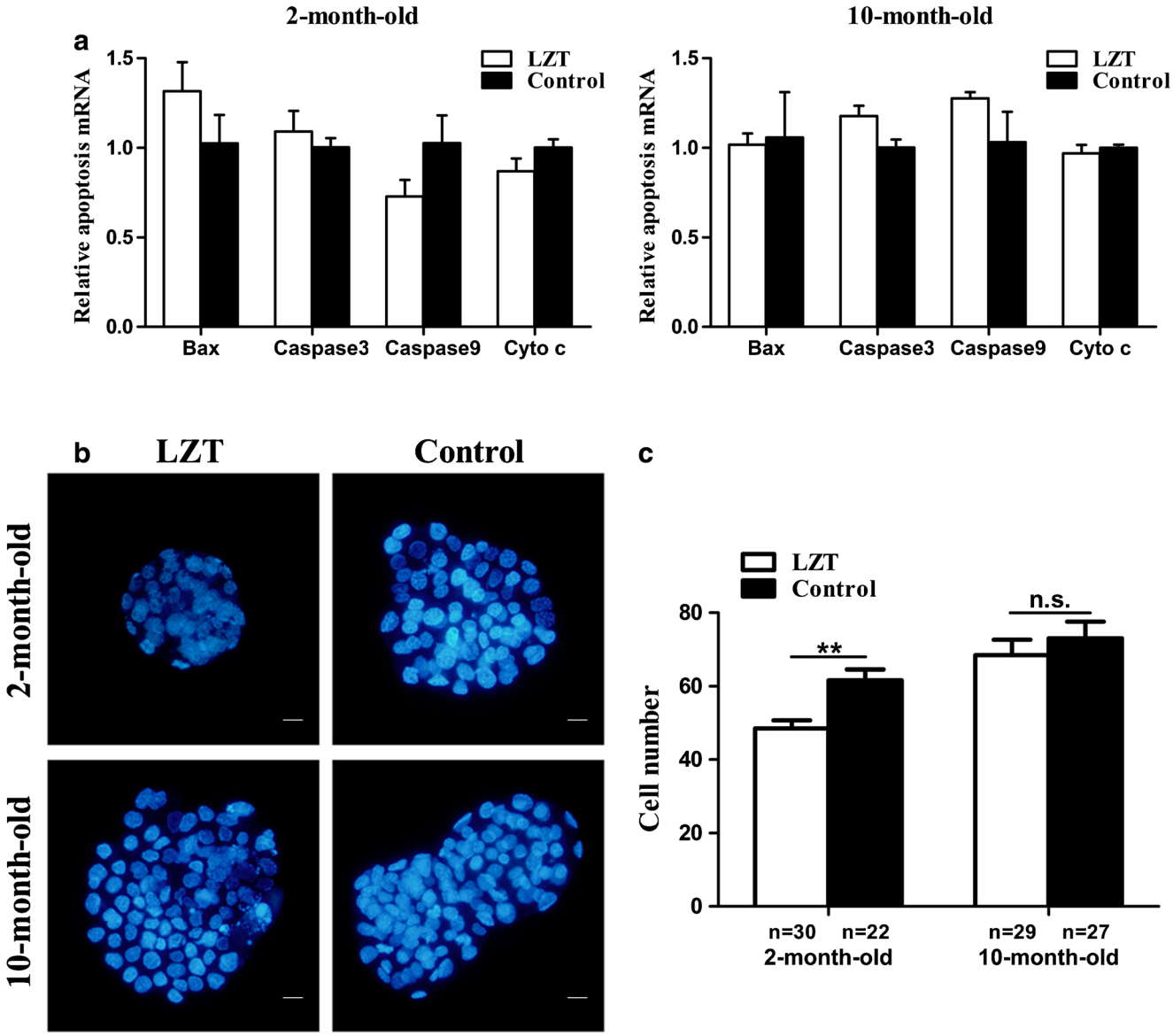

Fig. 2 **a** shows there was no significant difference on apoptosis gene mRNA expression after LZT treatment for both age groups. **b** shows the D5 blastocyst after DAPI dyeing (scale bar indicates 10 μm). **c** shows in 2-month-old mice, the total cell number of D5 blastocyst in zona-thinned group was significantly lower than that of control group ($P < 0.01$)

Avertin (0.1 mL/5 g body weight), then eight laser-thinned and eight control vitrified-warmed morulae were transferred bilaterally to each uterine horn according to previous reports [36]. At DOP7.5, implantation sites were visualized by intravenous injection of 1% solution of Chicago sky blue dye, and the implantation rate was calculated as the number of implantation sites divided by the number of transferred embryos × 100 (%).

Statistics analysis

For comparison of the continuous variables, the Student's *t* test was used, and for comparison of non-continuous variables, the standard chi-squared test was used. Data were analyzed by SPSS (IBM, Version 22.0, CA, USA). Results were considered statistically significant if $P < 0.05$.

Results

In this study, for both ages, the in vitro-fertilized embryos shared similar cleavage rate and morula rate during 3 days in vitro culture ($P > 0.05$) (Supplemental Figure 2). Table 1 summarizes the embryonic development from D4 to D6. Firstly, the blastocyst formation rates (D5) between mice in different ages had no significant difference ($P > 0.05$). At 24 h, the hatching rate was significant higher in zona-thinned group of young mice (28.6% vs. 8.8%, $P < 0.01$), indicating the

Table 1 Hatching rates in the zona-thinned and control groups

Groups	Rate, % (*n*) 2-month-old	*P* value	Rate, % (*n*) 10-month-old	*P* value
Blastocyst formation				
LZT	73.7 (140/190)		77.7 (101/130)	
Control	81.8 (148/181)	0.062	84.1 (106/126)	0.191
Hatching (24 h)				
LZT	28.6 (40/140)		27.7 (28/101)	
Control	8.8 (13/148)	0.000**	23.6 (25/106)	0.495
Hatching (48 h)				
LZT	77.1 (108/140)		74.3 (75/101)	
Control	39.9 (59/148)	0.000**	58.5 (62/106)	0.017*
Hatched (72 h)				
LZT	25.7 (36/140)		36.6 (37/101)	
Control	16.2 (24/148)	0.047*	13.2 (14/106)	0.000**

*Indicates $P < 0.05$ and **indicates $P < 0.01$. Blastocyst formation rate = total blastocyst after 48 h/cultured morula after warming; 24 h hatching rate = hatching blastocyst after 24 h/total blastocyst after 48 h; 48 h hatching rate = hatching blastocyst after 48 h/total blastocyst after 48 h; hatched rate = hatched blastocyst after 72 h/total blastocyst after 48 h

onset of hatching occurred earlier after LZT treatment (Supplemental Figure 3). This trend became more obvious at 48 h (Supplemental Figure 4) for both groups (77.1% vs. 39.9%, $P < 0.01$; 74.3% vs. 58.5%, $P < 0.05$). When it comes to 72 h, zona-thinned group yielded a significantly higher fully hatched rate compared with control group for both ages (25.7% vs. 16.2%, $P < 0.05$; 36.6% vs. 13.2%, $P < 0.01$). Two distinct kinds of hatching structure were recorded during in vitro culture (Fig. 1a), and it was found out that LZT treatment could significantly increase the percentage of U-shaped hatching structure in young mice group (48.3% vs. 33.0%, $P < 0.05$; Fig. 1b).

The total cell number of D5 blastocyst in the zona-thinned group of young mice was significantly lower than that of the control group (40.6 ± 5.1 vs. 59.9 ± 14.5, $P < 0.01$; Fig. 2b). However, LZT treatment did not induce significant mRNA expression change of apoptosis related genes in different groups ($P > 0.05$; Fig. 2c).

There was a trend that implantation rate increased when transferring zona-thinned embryos for both ages, yet there still had no statistic difference in implantation rate between the zona-thinned and control groups for both ages (63.8% vs. 52.5%, $P > 0.05$; 55.6% vs. 47.2%, $P > 0.05$; Table 2).

Table 2 Implantation rates in the zona-thinned and control groups

Groups		Rate, % (*n*)	*P* value
2-month-old	LZT	63.8 (51/80)	
(*n* = 10)	Control	52.5 (42/80)	0.149
10-month-old	LZT	55.6 (46/80)	
(*n* = 10)	Control	47.2 (39/80)	0.267

Discussion

Previous studies seldom focused on how LZT treatment influence the embryo's pre-implantation development and the effectiveness of LZT was mostly assessed by analyzing the ultimate clinical pregnancy or live birth rate after embryo transfer, hence we took vitrified-warmed mouse embryos from different ages as model to investigate the impacts of LZT on hatching dynamics and implantation, so as to offer more objective and thorough information about its effectiveness and security.

In this study, we found out that LZT treatment could significantly improve the hatching outcomes of vitrified-warmed embryos for both age groups during in vitro culture (Table 1), probably because the tension from expanded blastocyst released easier when the ZP is thinned. ZP with a distinct bi-layered structure, composing of a less dense, easily digestible thick outer layer and a more compact but resilient inner layer [37, 38], once the outer layer, which is used to maintain the integrity of ZP, is ablated by laser, then expanded blastocysts can easily come out. On the basis of that, the change of ZP integrity further influence hatching structure, and we found out that the percentage of U-shaped hatching in zona-thinned groups were both higher than that of the control group for both age groups, especially, there was a significant difference in young mice (Fig. 1b). Previous study already demonstrated that the hatched rate of U-shaped hatching is significantly higher than that of 8-shaped hatching [33], indicating that U-shaped is conducive to hatching. Although previous study has demonstrated that zona-opening could lead a higher fully hatched rate compared to zona-thinning for fresh mouse embryo [12], its thinned area adopted a width of 35 μm, which was different from generally used protocol (quarter or half thinning) [27, 30, 39, 40]; moreover, it has been proved that

the larger size of ZP thinning area, the higher rate of clinical pregnancy and implantation in vitrified-warmed embryo transfers at the cleavage-stage [31].

The onset of hatching is based on the size and cell number of embryo at blastocyst stage [41]. Due to the tension from expansion, ZP gets thinner along with the embryonic development from cleavage to blastocyst under natural condition [14], in the meantime, lots of secretory vesicles contained trypsin-like protease are clustering around the opening as well as digesting the glycoprotein form ZP [42], and ultimately blastocyst escapes from the breached ZP. Unlike normal condition, in this study, LZT treatment made the onset of hatching advance, likewise, previous study used acidic Tyrode's solution to perform cruciate-thinning on fresh day 3 mouse morulae and results implied that cruciate-thinning of the zona acts optimally to promote both early and complete hatching [43]. We assumed that this advancement could influence the embryonic development and it was found out that total cell number of D5 blastocyst in zona-thinned group of young mice was significantly less than that of the control group (Fig. 2b), which was probably caused by inadequate embryonic development and expansion due to the advanced timing of hatching (Table 1), and a previous reports on fresh mouse embryo also demonstrated that the ICM/TE was significantly lower after LZT treatment, meanwhile the hatched rate was significantly higher [44]. However, the RT-qPCR results showed that the mRNA expression of four apoptosis related genes were barely change with or without LZT treatment (Fig. 2c), indicating that the LZT would not induce the embryo apoptosis. Consequently, LZT is a comparatively safe technology, at least there was no evidence of an increase in chromosomal aberrations or congenital malformations for children born after laser-assisted hatching [45]. But considering the total cell number of blastocyst can be a reflection of developmental competence [46] the long-term impact from LZT treatment still need further investigation.

As far as is known, this is the first study comparing the implantation rates of vitrified-warmed embryo transfer cycles between zona-thinned and zona-intact embryo under the same uterine microenvironment, aiming at giving a more objective evaluation of LZT, because the separation of the uterine body and the pre-vaginal portion of the uterine cervix into two canals by a septum guarantee the absence of embryo transuterine migration in mice [36]. Although there was no significant difference between the LZT and control group on implantation rate for both ages, the results indicate a tendency that LZT treatment increased implantation rate (Table 2). Therefore, it can be concluded that LZT is beneficial to implantation in vitrified-warmed embryo transfer cycle, and those random controlled trials of LZT remains controversial and elusive, to a large extent, might influenced by maternal factors and the different clinical operation, since it has been reported that mechanisms of recurrent implantation failure are zona hardening, asynchrony between the embryo and the endometrial implantation window after ovarian stimulation, or a deficiency in the cellular energy required for hatching [47].

In conclusion, as for vitrified-warmed embryo, the significant increase of fully hatched rate after LZT treatment is related to the advanced onset of hatching as well as the enhancement of superior hatching structure. LZT treatment is also associated with an increased chance of implantation after embryo transfer.

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Compliance with ethical standards

Ethical approval The use of the animals and the experimental procedures were approved by the Animal Care Committee at China Agricultural University. The experiment was conducted at China Agricultural University. All methods were carried out in accordance with the approved guidelines.

Conflict of interest The authors declare that they have no conflict of interest.

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