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Evaluation of morphokinetic characteristics of zona pellucida free mouse pre-implantation embryos using time-lapse monitoring system

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ABSTRACT The mammalian zygote cleaves and develops to blastocyst within the zona pellucida (ZP) *in vivo*. The presence or absence of ZP may affect the characteristics of the embryo, including blastomere alignment, cell-cell junction, and compaction. This study aimed to compare the morphokinetic characteristics of ZP-intact and ZP-free mouse pre-implantation embryos with a time-lapse monitoring system. Mouse 2-cell embryos were collected 1.5 days post coitum (dpc), and their ZPs were removed by treatment with acid Tyrode's solution. All embryos were cultured *in vitro* up to the outgrowth stage at 7.5 dpc. In this study, ZP did not influence the cumulative times from 2-cell to further stages and blastulation. Interestingly, ZP-free embryos at 4-cell stage have three patterns of blastomere alignment according to the number of contact points between blastomeres. However, blastomere alignment did not lead to any differences in morphokinetic comparisons. Regardless of the presence or absence of ZP, embryos compacted after the 8-cell stage took shorter time to become blastocysts than embryos compacted pre-8-cell stage. Nevertheless, cell-cell junction proteins required for successful compaction were similarly expressed between ZP-intact and ZP-free embryos. ZP-intact embryos compacted post-8-cell stage had a higher rate of reaching blastocysts than compacted ZP-intact embryos before 8-cell stage, while the outgrowth/blastocyst rate was similar. In this study, the presence or absence of ZP did not influence embryonic development and expression of cell surface glycoproteins, whereas compaction timing may be one of the criteria for evaluating embryo quality. ZP-free embryos may become an alternative for overcoming cases with ZP problems in human ART programs.

KEY WORDS: *timing of compaction, zona pellucida, developmental competence, time-lapse monitoring system*

Introduction

The mammalian zygote cleaves and develops to the blastocyst stage within the zona pellucida (ZP) *in vivo*, coinciding with dynamic changes in morphology and gene expression (Govindasamy *et al.*, 2019, Tosenberger *et al.*, 2019). Before the blastocyst stage, blastomeres within the ZP are tightly bound together through a process known as compaction, and become individually indistinguishable (Cockburn and Rossant 2010, White *et al.*, 2016). The compaction process usually occurs around the 8-cell stage in mice and humans. This process involves functional changes with expanding membrane channels and junctional formation serving as the intracellular communication pathway (Ma *et al.*, 2009). E-cadherin/β-catenin cell adhesion complexes appear at the time of

Abbreviations used in this paper: ART, assisted reproductive technologies; dpc, days post-coitum; ET, embryo transfer; hCG, human chorionic gonadotropin; ICM, inner cell mass; IVF, *in vitro* fertilization; OG, outgrowth; PMSG, pregnant mare's serum gonadotropin; QABM, Quinn's SAGE Blastocyst Media; TE, trophectoderm; WOW, well-of-well; ZP, zona pellucida.

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compaction and facilitate the cells adhering to one another more tightly than previously.

The ZP is a thick extracellular elastic coat composed of long interconnected sulfated glycoprotein fibrils (Greve and Wassarman 1985). The ZP ultrastructure is net-like and permeable to large molecules, including some small viruses, through which they can gain entry into the embryo (Michelmann *et al.*, 2007). The ZP has diverse biological functions, including protecting the oocyte from uncertain physical stresses during growth and ovulation, preventing not only polyspermic fertilization but also blastomere separation before compaction, protecting against viral and bacterial infections, creating a microenvironment through selective permeability to soluble factors in oviductal and uterine fluids, and others (Hunter 1976, Zusman *et al.*, 1984, Kapur and Johnson 1986, Suzuki *et al.*, 1995, Van Soom *et al.*, 2010). As aforementioned, physiological functions of ZP are essential for folliculogenesis and fertilization. However, it has been controversial in preimplantation development of mammalian embryos (Wassarman and Litscher 2008, Wassarman and Litscher 2012).

For successful implantation, the blastocyst must hatch from the ZP to invade the uterine endometrium. ZP hardening and thickening may occur as a consequence of *in vitro* fertilization-embryo transfer (IVF-ET), which may lead to low implantation and pregnancy outcomes (De Vos and Van Steirteghem 2000). Several studies have suggested ZP removal as an option to overcome fertilization and hatching failure due to an abnormal ZP structure in human embryos (Ueno *et al.*, 2016, Nishio *et al.*, 2006). However, procedures of ZP removal may damage the oocyte and affect the resulting rat embryo's developmental potential (Okuyama and Funahashi 2012, Li *et al.*, 2013). Despite this risk, Urman *et al.,* reported that the absence of ZP on day 5 human embryos improved pregnancy rates in patients with poor IVF/ICSI (*in vitro* fertilization/intracytoplasmic sperm injection) prognosis (Urman *et al.*, 2002). The roles of ZP in embryo fertilization, implantation, and development are still controversial within the field of human IVF-ET.

Embryo selection based on morphology remains the most common and generally accepted method in ART (Balaban *et al.*, 2011). However, the spatial arrangement of the blastomeres within the embryos is not currently considered as a potential criterion for embryo selection (2011, Ebner *et al.*, 2003). In human embryos, the arrangement of blastomeres at the 4-cell stage is associated with their developmental potential (Cauffman *et al.*, 2014). Interestingly, the arrangement of blastomeres at the 4-cell stage may be changed by ZP removal. Unfortunately, the exact correlation between blastomere arrangement and embryonic development in ZP-intact and ZP-free embryos has not yet been fully investigated.

It has been found that compaction timing is a control of a major developmental transition in early mouse embryogenesis (Levy *et al.*, 1986). Moreover, despite the development of a time-lapse monitoring system, few research groups have attempted to demonstrate the correlation between compaction timing and development (Iwata *et al.*, 2014, Milewski *et al.*, 2018). In addition, no study has yet to demonstrate the impact of compaction timing on murine embryo-

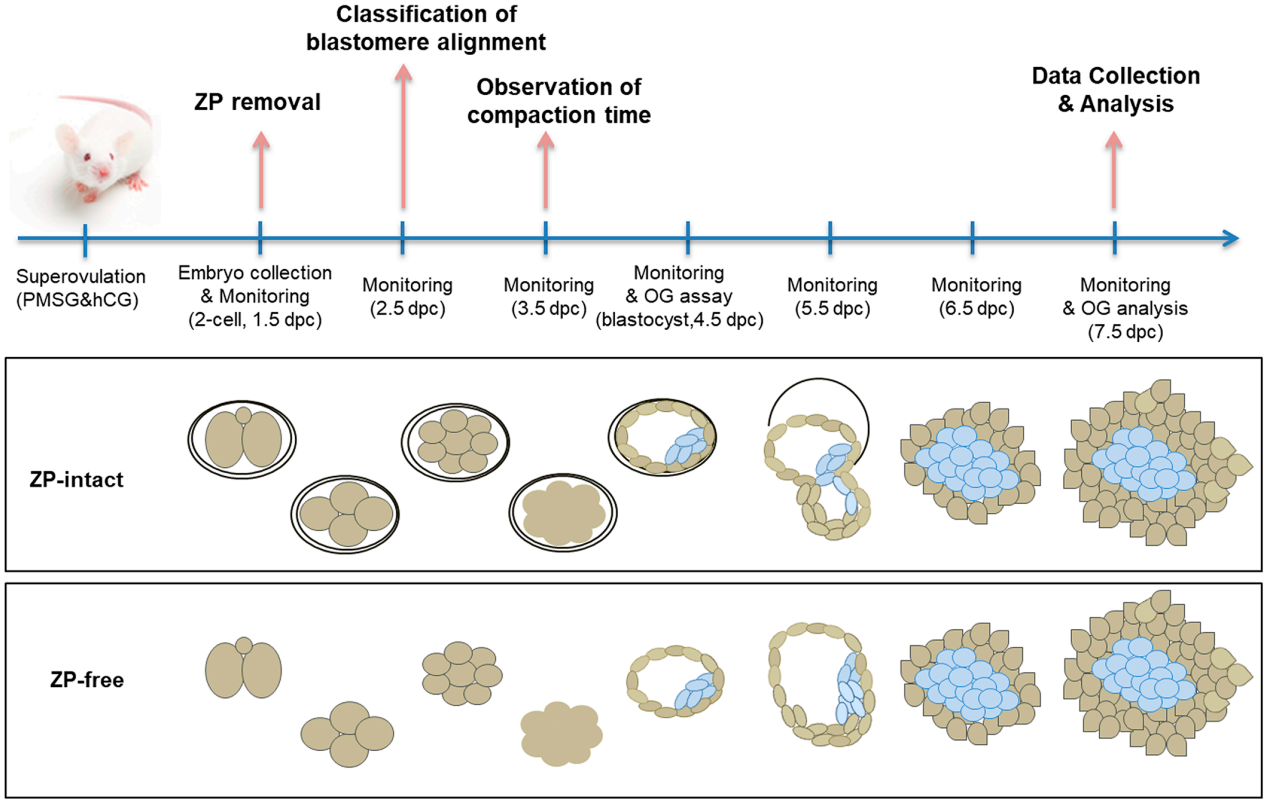

Fig. 1. Schematic illustration of this experiment. *Mouse 2-cell embryos were collected and their zona pellucida (ZP) was removed on 1.5 dpc. The patterns of blastomere alignment and stages of the compaction process were evaluated in both ZP-free and ZP-intact embryos.* In vitro *embryonic development was assessed by monitoring blastocyst formation and outgrowth from 1.5 to 7.5 dpc. Data were collected at the end of monitoring. Abbreviations: dpc, days post coitum; hCG, human chorionic gonadotropin; OG, outgrowth; PMSG, pregnant mare's serum gonadotropin; ZP, zona pellucida.*

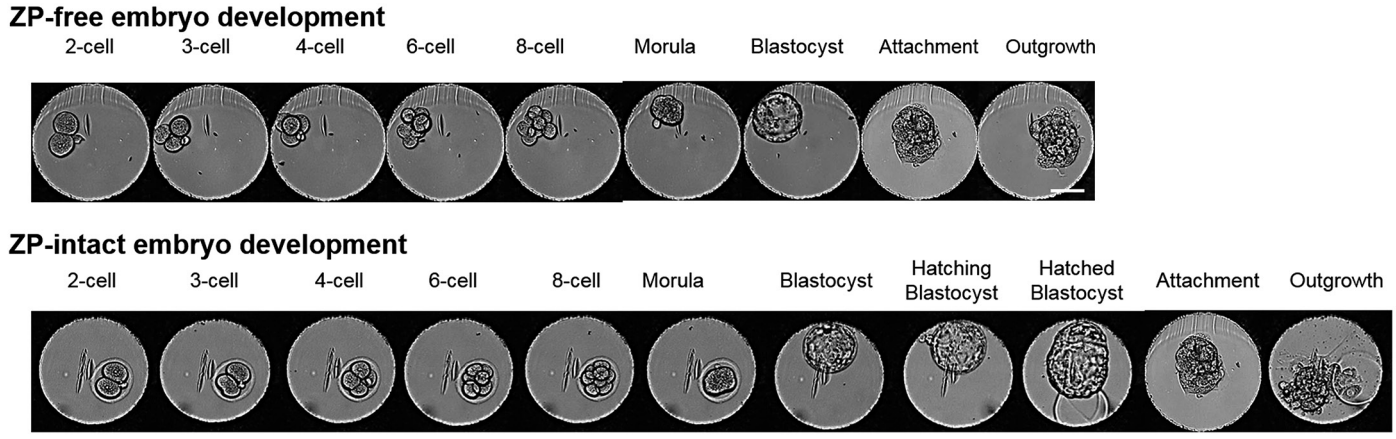

Fig. 2. Time-lapse monitoring of embryos during preimplantation development and outgrowth *in vitro*. *Zona pellucida (ZP) free or intact two-cell embryos on 1.5 dpc were cultured to the outgrowth (OG) stage for 6 days with Primo vision monitoring system. Images were captured once every 30 min during culture periods. The developmental stages were 2-cell, 4-cell, 6-cell, 8-cell, morula, early blastocyst, blastocyst, hatching blastocyst, hatched blastocyst, attachment, and OG. ZP-free embryos did not show hatching process in this study. ZP, zona pellucida; dpc, days post coitum. Scale bar, 100 μ.*

genesis, regardless of the presence or absence of ZP.

The time-lapse monitoring system for the embryo provides interesting topic of study, including intercellular contacts at the four-cell stage and the cumulative time to reach the blastocyst stage (Milewski, Szpila and Ajduk 2018, Kim *et al*., 2017, Kelley and Gardner 2017). Therefore, we investigated the impacts of intercellular contact points and the cumulative time needed to reach blastocyst via a time-lapse monitoring system.

Therefore, this study was performed to compare the morphokinetic characteristics and developmental competence of ZP-intact and ZP-free mouse pre-implantation embryos using a time-lapse monitoring system. We investigated whether the lack of ZP affects preimplantation embryonic development and trophoblastic outgrowth potential. Furthermore, we evaluated whether the blastomere alignment pattern of ZP-free embryos is associated with developmental competence and outgrowth potential *in vitro.*

Results

Comparison of mean cumulative times and developmental competence according to the presence or absence of ZP

Mouse 2-cell embryos were cultured *in vitro* to the OG stage for six days (from 1.5 to 7.5 dpc), and the development rates of ZP-free and ZP-intact embryos were compared. There were no differences between the mean cumulative times for each developmental stage between the ZP-free and ZP-intact embryos (Table 1). The blastulation of ZP-free (89.2%, 91/102) and ZP-intact (85.2%, 92/108) embryos on 4.5 dpc was similar. However, the OG rate was significantly higher in ZP-free embryos at 88.2% (90/102), than in the ZP-intact embryos at 38.0% (41/108). The trophoblastic OG area was not significantly different between the ZP-free (11.8 ± 1.0 mm^2) and ZP-intact (11.3 ± 0.5 mm^2) embryos.

Patterns of blastomere alignment in ZP-free embryos at the 4-cell stage

ZP-free embryos have three (Fig. 3A, 22/102, 21.6%), four (Fig. 3B, 40/102, 39.2%), or ≥5 intercellular contact points at the 4-cell stage (Fig. 3C, 40/102 39.2%). Most embryos have more than three points of intercellular contact at the ZP-free 4-cell stage (Fig. 3D, 78.4%)

Comparison of mean cumulative times and developmental competence according to the patterns of blastomere alignment at 4-cell stage embryos in ZP-free embryos

There were no statistical differences in the mean cumulative times from the 2-cell to OG stage among the three types of blastomere alignments at the 4-cell stage in ZP-free embryos (Table 2). The blastulation rates were similar between ZP-free embryos with four and more than four points of contact (P=0.15).

Comparison of mean cumulative times and developmental competence according to the timing of compaction in ZP-free and ZP-intact embryos

ZP-free and ZP-intact embryos were retrospectively assigned to two different groups based on the compaction timings (pre-8C; compaction before 8-cell *versus* post-8C; compaction after 8-cell). In ZP-free embryos, compaction before the 8-cell stage showed significantly longer mean cumulative times from the start of blastulation (tB) to the OG stage than embryos compacted after the 8-cell stage (Table 3, $P < 0.01$) in each developmental stage. Embryos compacted before the 8-cell stage also showed increased mean cumulative times from 2-cell to the morula, blastocyst, and

TABLE 1

COMPARISON OF MEAN CUMULATIVE TIMES (H) FOR EACH DEVELOPMENTAL STAGE BETWEEN ZP -FREE AND -INTACT EMBRYOS

Developmental stage	ZP-free embryos (n=102) Post-hCG(46h)	ZP-intact embryos (n=108) Post-hCG(46h)	*P* value
t2 (from hCG)	46.0 ± 0.0 (102)	46.0 ± 0.0 (108)	0.319
t3 (cc2)	53.4 ± 4.3 (102)	52.6 ± 3.4 (108)	0.135
t4	54.3 ± 4.5 (102)	53.8 ± 4.4 (104)	0.326
t6	67.2 ± 5.9 (94)	66.6 ± 5.0 (95)	0.517
t8	68.7 ± 6.0 (94)	68.4 ± 5.4 (95)	0.375
tMo	72.2 ± 5.8 (94)	78.0 ± 5.9 (94)	0.162
tSB	78.7 ± 7.5 (91)	93.7 ± 7.6 (92)	0.286
tB	98.1 ± 7.3 (91)	99.0 ± 8.5 (92)	0.628
tOG	160.2± 14.2 (90)	164.7 ± 14.2 (41)	0.090

Data represent as a mean ± SD. t2, t3, t4, t6, t8, time to 2, 3, 4, 6,8 cells divisions, respectively; tMo, morula formation; tSB, starting blastulation; tB, expanded blastocyst stage; tOG, blastocyst outgrowth.

hatching blastocyst (HB) stage ($P < 0.05$) compared with embryos compacted after the 8-cell stage in the ZP-intact embryo.

In Table 4, ZP-intact embryos that compacted before the 8-cell stage showed significant decreased blastulation rate ($P < 0.05$), while there was no significance in before or after 8-cell stage compacted ZP-free embryos. However, the compaction timings did not affect the outgrowth/blastocyst rate in either ZP-free or ZP-intact embryos.

Furthermore, we observed the compaction process in both ZP-free and intact embryos according to their compaction timing shown before or after 8-cell stage. As shown in Table 5, compacted before the 8-cell stage of ZP-free embryos have showed the more incidence of incomplete compaction process than embryos compacted after the 8-cell stage ($P<0.05$). It is suggested that timing of compaction process is important for completion in ZP-free embryos. Interestingly, the proportion of appearance compaction process was similar in ZP-intact embryos. The more tightly aligned blastomeres might affect the compaction process which affect the implantation potential.

Localization of cell-cell contact molecules, β-catenin, and E-cadherin, in compacting embryos

Fig. 4 shows immunofluorescence images for the β-catenin and E-cadherin of compacting embryos. β-catenin and E-cadherin were expressed and localized on the surface of each blastomere in ZP-intact and ZP-free compacting embryos. In both ZP-intact and ZP-free embryos, β-catenin was expressed around the nucleus and margin of the blastomeres, while the expression of E-cadherin

TABLE 2

COMPARISON OF MEAN CUMULATIVE TIMES FOR EACH DEVELOPMENTAL STAGE BETWEEN THREE TYPES OF BLASTOMERES ALIGNMENT IN ZP-FREE EMBRYOS AT 4-CELL STAGE

Developmental stage	Pattern of blastomeres alignment (n=102)			*P* value
	3-point of contact	4-points of contact	≥ 5-points of contact	
	Post-hCG (n)	Post-hCG	Post-hCG	
t2 (from hCG)	46.0 ± 0.0 (22)	46.0 ± 0.0 (40)	46.0 ± 0.0 (40)	
t3	53.6 ± 3.5 (22)	54.1 ± 5.1 (40)	52.5 ± 3.7 (40)	
t4	54.8 ± 3.7 (22)	54.9 ± 5.3 (40)	53.4 ± 3.8 (40)	
t6	67.5 ± 6.0 (21)	68.0 ± 6.2 (40)	66.3 ± 5.6 (35)	
t8	70.2 ± 5.9 (21)	69.2 ± 6.3 (39)	67.4 ± 5.7 (35)	No significant
Comp	73.9 ± 6.8 (21)	72.7 ± 5.7 (39)	71.0 ± 5.3 (35)	
tMo	80.2 ± 6.4 (20)	80.0 ± 8.9 (39)	76.3 ± 5.6 (34)	
tSB	93.5 ± 7.1 (20)	94.5 ± 7.7 (38)	90.9 ± 5.3 (33)	
tB	98.6 ± 1.6 (20)	100.0 ± 8.2 (38)	95.7 ± 5.6 (33)	
tOG	158.8 ± 14.8 (20)	159.0 ± 23.6 (38)	158.9 ± 13.5 (33)	

Data represent as a mean ± SD. t2, t3, t4, t6, t8, time to 2, 3, 4, 6,8 cells divisions, respectively; Comp; starting compaction; tMo, morula formation; tSB, starting blastulation; tB, expanded blastocyst stage; tOG, blastocyst outgrowth.

TABLE 3

COMPARISON OF MEAN CUMULATIVE TIMES FOR EACH DEVELOPMENTAL STAGE ACCORDING TO APPEARANCE OF COMPACTION PROCESS

	ZP-free embryos		ZP-intact embryos	
	Compaction before 8C stage (n=18)	Compaction after 8C stage (n=76)	Compaction before 8C stage (n=17)	Compaction after 8C stage (n=77)
tMo	84.6 ± 7.4	77.6 ± 6.9	80.7 ± 7.4	77.4 ± 5.3 *
tSB	99.5 ± 8.2	91.4 ± 5.7 *	97.2 ± 8.3	92.7 ± 7.0 *
tB	104.9 ± 8.2	96.6 ± 6.2 *	104.3 ± 8.5	97.5 ± 7.5 *
tHB	-	-	116.6 ± 14.7	107.8 ± 13.0 *
tOG	171.3 ± 15.5	157.7 ± 12.6 *	172.1 ± 20.1	163.4 ± 12.3

Data represent as a mean ± SD. 8C, 8-cell; tMo, morula formation; tSB, starting blastulation; tB, expanded blastocyst stage; tHB, hatching blastocyst; tOG, blastocyst outgrowth. Asterisk (*) indicates a statistical significant difference by one-way ANOVA and Student's t- test ($P < 0.05$).

TABLE 4

COMPARISON OF DEVELOPMENTAL RATES ACCORDING TO APPEARANCE OF COMPACTION PROCESS

	ZP-free embryos		ZP-intact embryos	
	Compaction before 8C stage (n=18)	Compaction after 8C stage (n=76)	Compaction before 8C stage (n=17)	Compaction after 8C stage (n=77)
BL rate (%)	17/18 (94.4)	74/76 (97.4)	15/17 (88.2)*	77/77 (100.0)*
OG/BL rate (%)	17/17 (100.0)	73/74 (98.6)	7/15 (46.7)	34/77 (44.2)

8C, 8-cell; BL, blastocyst; OG, outgrowth; OG/BL, proportion of embryos developing to the outgrowth stage per blastocysts. Asterisk (*) indicates a statistical significant difference by Chi-square test or Fisher's exact test ($P < 0.05$).

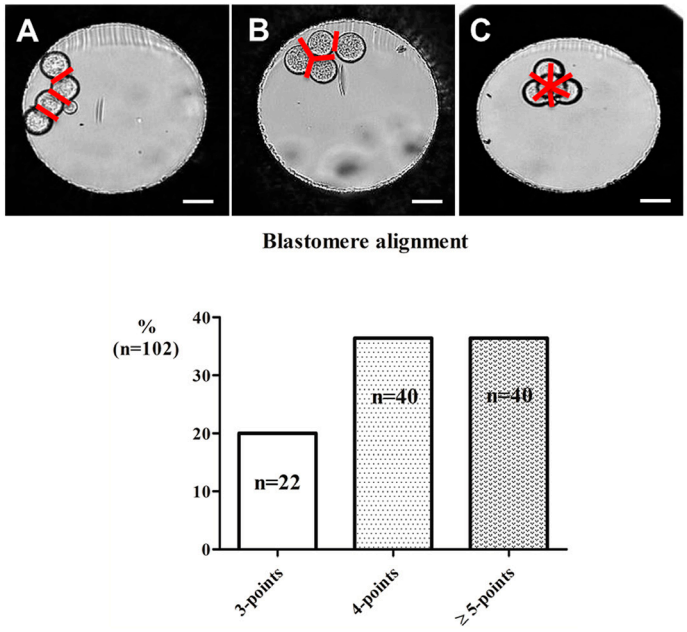

Fig. 3 (above). Pattern and distribution of blastomere alignment in zona pellucida (ZP) free 4-cell embryos on 2.5 dpc. *ZP-free embryos were classified by a diverse pattern of blastomere alignment* **(A)** *3-points,* **(B)** *4-points and more than* **(C)** *5-points of contact in ZP-free 4-cell embryos. Red lines indicate the contact between blastomeres of ZP-free 4-cell embryos.* **(D)** *shows a distribution of blastomere alignment (Y axis) in ZP-free 4-cell embryos. Scale bars, 50 μ.*

was observed only in the margin of the blastomeres. However, there was no differential finding between these two groups. These results demonstrate that the presence or absence of ZP does not have any detrimental effect on the embryo compaction process.

Comparison of implantation potential after ZP-free or -intact embryos transferred

Table 6 shows *in utero* implantation rate of ZP-free or intact embryos transferred. Even though no significant difference was observed between ZP-free and ZP-intact embryos, the implantation ability in the ZP-free embryos was slightly increased compared with the presence of ZP in the mouse embryo ($P > 0.05$). ZP removal might not affect the implantation potential *in utero*.

Discussion

Zona pellucida has important functions during sperm-egg recognition, binding, *in vivo* fertilization, and preimplantation embryonic development. For successful implantation, embryos need to be hatched-out from the ZP and implanted on the maternal uterine endometrium (Tu *et al*., 2014). Even though some of these functions are not essential during *in vitro* culture, a suboptimal *in vitro* culture condition may also affect ZP flexibility and elasticity, and consequently decrease *in vitro* hatching rates. Removal of ZP is an alternative method to prevent side effects derived from changes in ZP by using chemical reagents and enzymes (acid Tyrode's solution or pronase), laser, or other techniques. (Trounson and Moore 1974, Ji and Bavister 2000, Wu *et al*., 2004, Park *et al*., 2014) Several studies have shown that ZP-free embryos can be cultured without compromising blastocyst formation rates, quality, and even epigenetic alterations. (Ribas *et al*., 2006, Lagutina *et al*., 2007, Ueno *et al*., 2014, Bodri *et al*., 2015) Moreover, a few cases have reported the successful pregnancy and delivery of ZP-free embryos in the human-assisted reproductive techniques (ART) program (Shu *et al*., 2010, Stanger *et al*., 2001, Ueno *et al*., 2014). In this study, we found that ZP removal from mouse embryos at the 2-cell stage did not decrease the developmental competence to the blastocyst and outgrowth stage embryos. The *in vivo* implantation rate was comparable between ZP-free and intact embryos (Table 6, p=0.115).

The ZP protects the integrity of the pre-compaction embryo during embryonic development. Suzuki *et al.,* reported that the contact pattern in ZP-free embryos might affect their

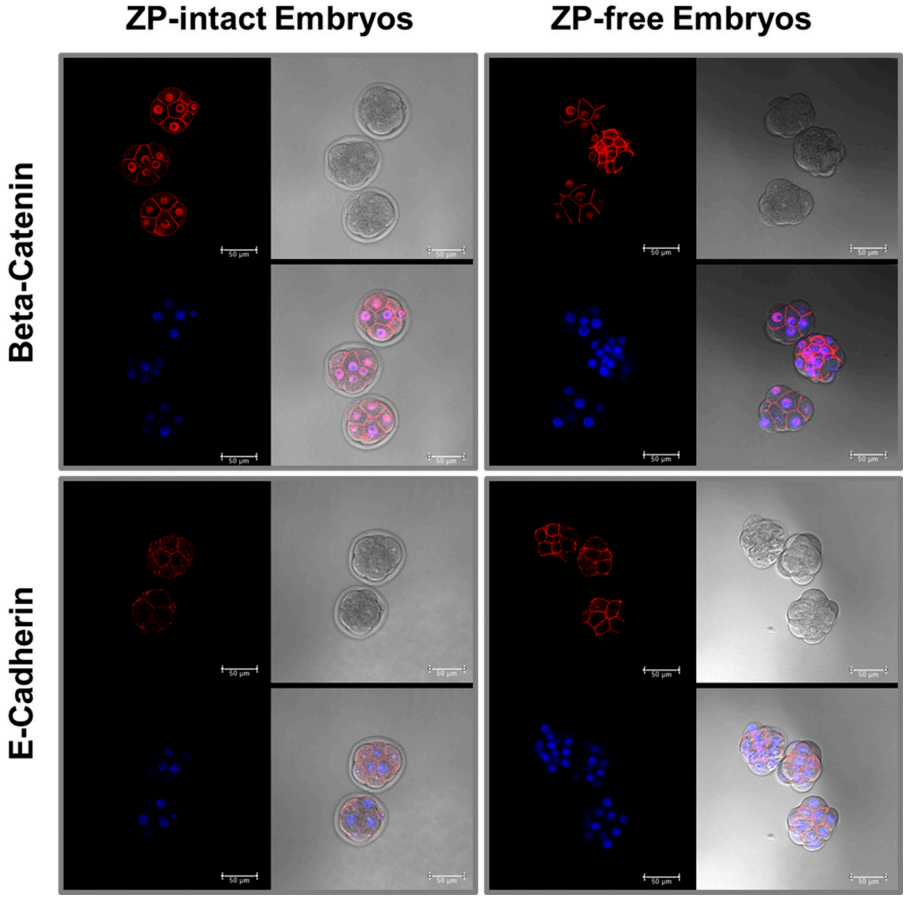

Fig. 4. Localization of β-catenin and E-cadherin on zona pellucida (ZP)-intact and ZP-free compacting morula. *The presence or absence of ZP does not affect cell-cell contacts molecules, β-catenin and E-cadherin, during compacting process. In the upper panels, red and blue signals respectively indicate the expression of β-catenin and nucleus while red and blue signals in lower panels respectively mean the expression of E-cadherin and nucleus in compacting embryos. Magnification, x400; scale bars, 50 μm.*

TABLE 5

PATTERNS OF COMPACTION PROCESS IN ZP-FREE AND INTACT EMBRYOS

	ZP-free embryos		ZP-intact embryos	
	Before 8C stage (n=18)	**After 8C stage (n=76)**	**Before 8C stage (n=17)**	**After 8C stage (n=77)**
Fully compaction (normal)	16.7% (n=3)	60.5% (n=46)	35.3% (n=6)	42.9% (n=33)
Fully compaction (normal)	16.7% (n=3)	26.3% (n=20)	29.4% (n=5)	35.4% (n=27)
Incomplete compaction	66.7%* (n=12)	13.2%* (n=10)	35.3% (n=6)	22.1% (n=17)

8C, 8-cell. Asterisk (*) indicates a statistical significant difference by Chi-square test or Fisher's exact test ($P < 0.05$).

developmental competence (Suzuki *et al*., 1995). This conclusion was drawn from observing the blastomeres of ZP-intact and ZP-free embryos, where the former appeared to be more tightly packed than the latter (Katayama *et al*., 2010). Interestingly, Liu *et al.,* demonstrated that embryos with fewer than six intercellular contact points at the end of the four-cell stage showed compromised subsequent development and reduced implantation potential in human IVF (Liu *et al*., 2015). The most of ZP-intact embryos have a tetrahedral shape with more than four intercellular contact points (Cauffman *et al*., 2014), but the number of intercellular contact points varied in ZP-free embryos. About 20% of ZP-free embryos have decreased contact points of blastomeres. ZP removal interrupts the increase in cell-cell contact resulting in the loss of the tetrahedral structure in ZP-free embryos. However, losing their tetrahedral shape in ZP-free conditions did not alter their developmental competence with unchanged localization of E-cadherin and β-catenin in morula which are known to be involved in the cell-junctional organization during embryonic development as shown in Fig. 4. (De Vries *et al*., 2004)

The compaction process is an essential step during pre-implantation embryo development. When embryos are compacted before reaching the 8-cell stage, they might fail to develop into further stages (Alikani *et al*., 2000). Iwata *et al.,* reported that early compaction before the 8-cell stages with cytokinetic defects might be a cause of aberrant pre-implantation embryonic development in humans (Iwata, Yumoto, Sugishima, Mizoguchi, Kai, Iba and Mio 2014). During the compaction process, the cell boundaries progressively disappear until the embryo is fully compacted. However, in the fair quality of morula, the cell boundaries reappear with a few small cells or fragments, resulting in incomplete compaction. We observed that the occurrence of the incomplete compaction process increased in embryos that compacted before the 8-cell stage (Table 5). Previous studies by Wrenzycki *et al.,* and Rizos *et al*., demonstrate that intercellular structures are altered in *in vitro* produced embryos, most likely due to a reduction in the expression of genes responsible for compaction and cell-cell adhesion, such as CX43 (gap junction alpha-1 protein) and CDH1 (cadherin-1), respectively (Wrenzycki *et al*., 1996, Rizos *et al*., 2003). As shown in Table 5, incomplete compaction process was the more observed in ZP-free embryos, while ZP-intact embryos showed similar rate. This result indicates expression of other junctional proteins might be affect depend on their intercellular structures during cleavage stage. similarly occurred in about 20 % of both ZP-free and intact embryos, which may have not changed genes related junctional proteins by ZP removal.

Our study compared spent time to develop into the blastocyst and OG stages according to compaction process before or after 8-cell stage. We found that the embryos that reached compaction before the 8-cell stage took longer time to develop into the blastocyst and OG stages compared to embryos with compaction after the 8-cell stage in both ZP-free and ZP-intact groups. Despite the sample size of outgrowth rate and compaction patterns was too small to be used in comparisons, only less than 20 % (18/102 and 17/108) of embryos developed into blastocysts in ZP-free and intact groups. The compaction process is the one of important morphokinetic parameters to choose good quality of embryos for better clinical outcomes.

In present study, we tried to demonstrate the effects of morphokinetics including the timing of compaction and presence or absence of ZP during embryogenesis on the implantation process using embryos transfer. However, we only showed the implantation abilities of the embryos regardless of the presence or absence of ZP. Because the number of embryos compacted before 8-cell stage was not enough to make any significance. In the next study, implantation abilities of embryos by compaction timing would be demonstrated to give us more insights for clinical applications.

TABLE 6

IMPLANTATION RATES IN ZP-FREE OR -INTACT EMBRYO TRANSFERRED MICE

	ZP-free embryos	**ZP-intact embryos**
Implantation rate on 7.5 dpc	63.3 ± 20.3% (n=6)	43.3 ± 20.3% (n=6)

Conclusions

Collectively, our study monitored and evaluated the *in vitro* developmental competence of embryos with or without ZP using a time-lapse monitoring system. Our results demonstrate that the modification of ZP could be useful for various purposes without decreasing the developmental competence of mouse 2-cell embryos. Furthermore, we hypothesize that the appearance of compaction after the 8-cell stage in ZP-intact mouse embryos may be a positive indicator of developmental competence and pregnancy rate with the appropriate timing of compaction.

Materials and Methods

The overall scheme of this study is presented in Fig. 1.

Hormonal stimulation

Experimental animal protocols were approved by the Eulji University Institutional Animal Care and Use Committee (EUIACUC 12-19). Outbred ICR (Institute of Cancer Research) female mice (6 to 8 weeks old) were superovulated by the intraperitoneal injection of 5 IU serum gonadotropin from a pregnant mare (PMSG; Sigma, USA) followed by injection with 5 IU human chorionic gonadotropin (hCG; Sigma) 46 h later. Superovulated female mice were then mated with sexually mature males (8 to 20 weeks

old) and euthanized via cervical dislocation 46 h after hCG injection. The morning of the vaginal plugging day was designated as 0.5 days post coitum (dpc). In this study, ten independent experiments were carried out to monitor embryonic development using a time-lapse monitoring system. A total of 210 embryos, including 102 ZP-free and 108 ZP-intact embryos, were used in this study.

Embryo culture and the preparation of ZP-free embryos

Mouse 2-cell embryos were collected by oviduct flushing on 1.5 dpc and cultured in Quinn's Advantage Blastocyst Medium (QABM; SAGE/Origio, Denmark) containing serum protein substitute (SPS; SAGE/Origio) in 5% CO_2 at 37°C. The ZP of two-cell embryos was removed by treatment with acid Tyrode's solution (Sigma, USA) and immediately washed with QABM, then transferred into a WOW (well-of-well) dish for time-lapse monitoring. For the outgrowth (OG) of blastocysts, the culture media was replaced with Dulbecco's modified Eagle medium (DMEM; Welgene, Korea) containing 10% fetal bovine serum on the third day of *in vitro* culture (4.5 dpc). The outgrowth of blastocysts was monitored for three days (from 4.5 dpc to 7.5 dpc)

The time-lapse monitoring of embryo cultures

The *in vitro* embryonic development from 2-cell stage to OG was monitored using the Primo Vision time-lapse system (Vitrolife, Sweden) (Fig. 2). The camera for the time-lapse microscope was set to take a single image every 30 min for six days. Stages of embryo development were classified as 3-cell (t3), 4-cell (t4), 6-cell (t6), 8-cell (t8), morula (tMo), blastocyst formation (tB), expanded blastocyst (tEB), blastocyst hatching (tHB), and OG (tOG), as previously described (Minasi *et al*., 2016).

The evaluation of developmental competence and outgrowth between ZP-free and ZP-intact embryos

A total of 102 ZP-free and 108 ZP-intact embryos were observed in ten independent experiments and analyzed for this study. A comparative study by morphokinetics, including the mean cumulative times between cleavage stages, the blastulation rate on 4.5 dpc, and the proportion of embryos developing to outgrowth per blastocyst (OG/BL), was performed between ZP-free and ZP-intact embryos. In addition, the area of trophoblastic OG was measured on 7.5 dpc using Image J software (National Institutes of Health, USA).

The classification and evaluation by intercellular contact points of ZP-free embryos at the 4-cell stage

On 2.5 dpc, ZP-free embryos at the 4-cell stage were classified into three types according to the number of intercellular contacts. Embryos with three or more points of contact were classified, as shown in Fig. 3. Mean cumulative times and the rates of blastulation and OG were compared among these three types of blastomere alignments in ZP-free embryos at the 4-cell stage.

Evaluation of developmental competence and OG according to the appearance of the compaction process in ZP-free and ZP-intact embryos

ZP-free and ZP-intact embryos were assigned into two groups via time-lapse monitoring, based on compaction timing; embryos with compaction before the 8-cell stage and embryos with compaction after the 8-cell stage. The mean cumulative times from compaction to blastocyst were analyzed and the rates of blastulation and OG were compared between groups.

The localization of β-catenin and E-cadherin in compacting embryos by immunocytochemistry

To investigate the properties of ZP on the embryo compaction process, either ZP-intact or ZP-free two-cell embryos were *in vitro* cultured for 36-40 h. The compacting embryos were then fixed with 4% paraformaldehyde (Biosesang, Korea) for 30 min at room temperature (RT). Fixed embryos were washed with 0.1% PVA/PBS (Welgene, Korea) and permeabilized with 0.5% Triton X-100 for 30 min. After permeabilization, blocking was processed by incubating the embryos with 3% BSA/PBS for 30 min. The embryos were then incubated with either β-catenin (1:100, 712700, Invitrogen, CA, US) or E-cadherin (1:100, 3195s, Cell Signaling, MA, US) at 4°C overnight. The target proteins and nuclei were visualized by adding goat anti-rabbit Alexa Fluor 488 antibody (1:100, A11008, Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, US), respectively, for one hour at RT. The images were obtained using confocal microscopy (Leica TCS SP8, Wetzlar, Germany).

Evaluation of in vivo implantation potential in ZP-free and ZP-intact embryos

To investigate the properties of ZP on the implantation potential, either ZP-intact or ZP-free two-cell embryos were transferred on 3.5 dpc to the contralateral uterine horn of pseudopregnant recipients after mating with vasectomized male. Transferred mice were sacrificed and implantations in uterine horns were assessed after 5 days.

Statistical analysis

The statistical significance of the two-group comparisons was analyzed by Student's t-test or Fisher's exact test. One-way analysis of variance or a chi-square test was performed for multiple comparisons. P values < 0.05 were considered statistically significant.

Declarations

Ethics approval and consent to participate

Experimental animal protocols were approved by the Eulji University Institutional Animal Care and Use Committee (EUIACUC 12-19). All authors confirmed that all experiments were performed in accordance with relevant guidelines and regulations.

Availability of data and material

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The author(s) declare no conflict of interest.

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Authors' contributions

Kim, J: conception, experiment, writing, revision. Lee, J: conception, experiment, writing, revision. Choi, YJ: conception and experiment. Lee, TB: data acquisition and analysis. Jun, JH: conception, writing, revision, correspondence.

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