The use of a two-step removal protocol and optimized culture conditions improve development and quality of zona free mouse embryos

Weihong Fan[1], Misato Homma[1], Renliang Xu[2], Hiroki Kunii[1], Hanako Bai[3], Manabu Kawahara[3] Toshikazu Kawaguchi[2], Masashi Takahashi[3]

Affiliations:

[1] Graduate School of Agriculture, Hokkaido University, Hokkaido 060-8589, Japan

[2] Faculty of Environmental Earth Science, Hokkaido University, Hokkaido 060-8628, Japan

[3] Research Faculty of Agriculture, Hokkaido University, Hokkaido 060-8589, Japan

Corresponding author:

Masashi Takahashi, Email address: mmasashi@anim.agr.hokudai.ac.jp

Abstract

The zona pellucida (ZP) plays an important role in both the fertilization and embryonic development. For the successful handling of early stage blastomeres for differentiation analysis, the production of identical twins or quadruplets, nuclear transfer or gene introduction requires the removal of the ZP (ZPR). Although single use of either acidic Tyrode's solution or pronase are commonly used for ZPR, long-term exposure to these agents can result in the inhibition of development with the collapse of the three-dimensional blastomere structure. Here, we demonstrate the benefits of using a two-step combined ZPR method, which relies upon a customized well-of-well (cWOW) system with smaller well size, on developmental competence and the quality of the zona free (ZF) mouse embryos.

We first isolated 2-cell embryos using acid Tyrode's solution and then cultured these embryos using either commercially available or cWOW, which had a smaller microwell size. The rate of blastocyst was significantly increased by use of cWOW when compared to other culture systems. Then we evaluated the use of a two-step ZPR protocol, relying on acid Tyrode's solution and proteinase K, and subsequent culture in the cWOW system. Although acid Tyrode's solution treatment alone reduced ZPR time, blastomere morphology became wrinkled, significant decrease in blastocyst rate associated with increased number of apoptotic cells and increased expression of apoptosis-related genes were observed. Using proteinase K alone increased ZPR time and significantly decreased the blastocyst rate, but did not induce an increase in apoptotic cell number or apoptosis-related gene expression. In contrast, two-step method significantly reduced ZPR time and improved blastocyst rate by increasing the total number of cells in these wells an reducing the number of apoptotic cells in these experiments. These results suggest that the two-step ZPR protocol is beneficial for reducing the toxic effects of zona removal on ZF embryo development and quality when combined with a suitable culture system.

Keywords: Apoptosis; Embryo development; Customized WOW; Two-step combined zona pellucida removal

1. Introduction

The zona pellucida (ZP), mixed structure of glycoproteins produced by mammals, surrounds follicular oocytes, ovulated eggs, and preimplantation embryos [1]. The mouse ZP is composed of several glycoproteins (ZP1, ZP2, ZP3), all encoding a relatively conserved recognition domain which contributes to oocyte/sperm interactions during the initial phases of fertilization [2].

The *in vitro* production (IVP) of mammalian embryos has been significantly improved over the last several decades and is now widely used in reproductive technologies in several different ways, including in assisted reproductive technologies (ART), for both humans and animals designed to facilitate breeding and development, production of transgenic animals, and cloning [3]. The ZP is often manipulated during IVP procedures with these changes including ZP thinning and complete or partial ZP removal (ZPR) and these changes are made to facilitate embryo applications such as chimera generation [4], transgenic procedures [5], blastomere separation [6], handmade cloning [7], and RNA interference experiments [8].

ZPR protocols are commonly performed as a single use of acid Tyrode's solution or pronase on their own [9, 10] with both options being reasonably popular due to their simplicity and relative cost-effectiveness [9]. Given this, several efficient ZPR protocols have been developed for a variety of species, including humans [9], cows [11] and pigs [12] by considering of the suitable chemical composition of each zona pellucida. However, some chemical (i.e., acid Tyrode's solution or pronase) treatments used for ZPR can reduce developmental competence as a result of their toxicity [13] with a long time exposure time for complete ZPR to prevent insufficient digestion [9], or excessive digestion time [14]. This means that there is still a need for a stable, high efficiency, low toxicity ZPR protocol to produce embryos suitable for ART and research into embryonic development and differentiation.

A previous study reported cellular association at the ZF 4-cell stage embryos importantly affects the further differentiation to inner cell mass (ICM) and trophectoderm (TE) when using a multiplate system influencing subsequent embryonic development in

mice [15]. To date, there have been several ZF embryo culture systems developed including glass oviducts, WOW, all designed to improve outcomes [16, 17]. However, these systems have not yet been evaluated for ZF embryonic development. In this study, we developed a customized WOW system designed to accommodate the smaller size of ZF embryos and provide an optimal environment for murine ZF embryo development. We also attempted to develop a two-step method suitable for development of ZF embryo by using our customized well-of-well (cWOW) system which well size is suitable for mouse embryos.

2. Material & Methods

2.1. Animals and Ethical approval

ICR male and female mice were used for all experiments with feeding a standard diet and housed in a controlled environment with a 12 h day: night cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee at Hokkaido University, and were conducted in accordance with the guidelines set out in the Hokkaido University Regulations on Animal Experimentation (approval number 19 (77)-2).

2.2. Oocyte collection and in vitro fertilization

Oocytes were collected using standard methods and superovulation was induced by injecting 5 IU PMSG (ASKA Pharmaceutical, Japan) and then 5 IU hCG (ASKA Pharmaceutical, Japan) 48 h later.

Spermatozoa were collected from cauda epididymis of male mice and preincubated in the droplets of HTF at 5% CO_2 and 37 °C for 1.5 h. Oocytes were collected from oviduct 15 h after hCG injection and then transferred to preprepared HTF droplets. These HTF droplets were then mixed with the preincubated spermatozoa to facilitated IVF. After 6 h of insemination, zygotes were transferred into droplets of M16 medium for *in vitro* culture. Zygotes that reached the 2-cell stage after 24 h were used for experiments.

2.3. Establishment of culture systems for ZF 2-cell embryos

ZF 2-cell embryos were produced by incubating the zona intact embryos in acid Tyrode's solution (Sigma-Aldrich) and incubated for 60 s. ZF embryos were then cultured in a (1) conventional microdroplet culture system using a flat bottom 60 mm × 15 Petri dish (Corning, NY), (2) a commercial WOW dish (DNP, Japan) or (3) cWOW system by making wells manually in a 60 mm × 15 mm Petri dish (Suppl. Fig). The well size of commercial WOW and cWOW was evaluated using color 3D laser microscopy observation (VK-9710, KEYENCE, Japan). Embryos were cultured for 2.5 days at 5% CO_2 and 37 °C and rate of blastocyst was evaluated.

2.4. Evaluation of the ZPR methods

After collection of 2-cell embryos, ZP was removed using one of the following methods until a complete zona digestion: (1) incubation in acid Tyrode's solution for approximately 1 min (2) incubation in 0.5% proteinase K solution (Fujifilm Wako, Japan) for approximately 6 min and (3) two-step combination in acid Tyrode's solution for approximately 40s followed by several seconds in 0.5% proteinase K solution. Precise ZPR time was recorded. After washed in M2 medium and M16 medium, ZF embryos were transferred into droplets of the M16 medium and cultured in the cWOW system for 2.5 days at 5% CO_2 and 37 °C. The blastocysts were used for gene expression analysis, TCN, and apoptotic cell detection.

2.5. RNA Extraction and Quantitative PCR

Five blastocysts were collected and used for RNA collection and cDNA synthesis using Super Prep Cell Lysis & RT Kit for qPCR (TOYOBO, Japan) according to the manufacturer's instructions. The primers used in these evaluations are listed in Suppl Table 1 and qPCR was carried out by a Light Cycler Nano (Roche Diagnostics, Switzerland) using Thunderbird SYBR qPCR Mix (TOYOBO). These assays were designed to evaluate the mRNA expression of apoptosis-related genes (*Caspase 3* and *Bax)* was calculated using the ΔΔCt method, with *GAPDH* as the reference gene.

2.6. Detection of DNA fragmentation using TUNEL assay

Apoptotic cells were detected using the MEBSTAIN Apoptosis Kit Direct (MBL, Japan) according to the manufacturer's instructions. Briefly, the blastocysts were fixed in 4% paraformaldehyde (Fujifilm Wako, Japan) in PBS for 1 h and then washed three times with 0.2% PVA-PBS before being permeabilized using 0.2% Triton X-100 in PBS for 1 h. Blastocysts were then washed three times with 0.2% PVA-PBS and the fragmented DNA was labeled with fluorescein-dUTP for 1 h. Blastocysts were then washed three times in PVA-PBS before being incubated in 1μg/mL Hoechst 33342 solution (Sigma-Aldrich) for nuclei staining. Fluorescence of the fragmented DNA was detected using LAS X on a DMi8 fluorescence microscope (Leica Microsystems, Germany) and the apoptotic index per blastocyst was calculated as follows: (TUNEL-positive cells/total cell number) × 100.

2.7. Statistical analysis

All data are representative of at least three replicates and statistically significant differences were identified using one-way analysis of variance (ANOVA)-Tukey's multiple range test implemented in GraphPad Prism 7 software (LA Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of different culture systems on the development of ZF embryos

In the initial study, we used the acid Tyrode's solution-treated ZF embryos to evaluate the different culture systems, including flat microdroplet, WOW, and cWOW (Fig. 1, Suppl. Fig) on their development. We first compared the microwell sizes from the WOW and cWOW by 3D surface scanning and revealed that the average diameter of the microwells in the cWOW system were significantly smaller than those of the commercial WOW (Fig. 1C). However, the depth of these wells was not significantly different in these two systems (Fig. 1D). The blastocyst rate was also shown to be significantly lower in the microdroplet and WOW when compared to that of the cWOW (Suppl Table 2).

3.2. Effect of ZPR methods on the blastomere morphology

There was no significant difference in treatment time between the acid Tyrode's solution and the two-step combined method (Table 1). In contrast, proteinase K required a significantly longer treatment time than the other two methods to completely remove the ZP ($P < 0.0001$). Just after ZPR by acid Tyrode's treatment, several blastomeres showing wrinkles on the surface were observed compared with ZI embryos (Fig. 2A, B). In contrast, no obvious wrinkles were observed by proteinase K, but loosening and detachment of blastomeres were clearly observed (Fig. 2B) than other ZPR methods (black arrows) and ZI embryos(Fig.2 A, C, D). ZF embryos obtained using the two-step method showed no morphological and attachment abnormalities of the blastomeres like ZI embryos(Fig. 2A, D).

3.3. Effect of ZPR on embryo development and quality

We then went on to investigate the effect of each of these ZPR methods on embryonic development by culturing ZF 2-cell embryos from each protocol in the cWOW system.

The rate of blastocyst was significantly decreased ($P< 0.05$) when embryos were treated with acid Tyrode's solution and proteinase K when compared with embryos with an intact zona. In contrast, the blastocyst rate was significantly increased in the two-step combined method embryos when compared to either the acid Tyrode's solution or proteinase K embryos (Table 2).

We then assessed blastocyst quality by evaluating the total cell number (TCN) in both the ZI and ZF blastocysts using Hoechst staining (Fig. 3A, B). TCN of blastocyst was significantly decreased both by acid Tyrode's solution and proteinase K when compared with ZI embryos ($P< 0.01$). In contrast, TCN was significantly increased in two-step method compared with both acid Tyrode's solution and proteinase K ($P< 0.05$), and not significantly different with ZI. (Fig. 3B).

Next, we assessed the apoptotic index of the ZF embryos produced by each of ZPR methods using TUNEL staining (Fig. 3A, C). The apoptotic index significantly increased in

response to treatment with acid Tyrode's solution ($P < 0.0001$), but there were no significant differences in the apoptotic indexes of the proteinase K or two-step combined method ZF embryos when compared with the ZI embryos (Fig. 3C). We also examined the effect of ZPR on the expression of specific apoptosis-related genes (*Bax, Caspase3*). This evaluation revealed no significant differences in the expression of *Bax* mRNA in acid Tyrode's solution treated ZF embryos when compared with ZI embryos; however, *Bax* expression was significantly upregulated in the acid Tyrode's solution group when compared to either the proteinase K or two-step combined method embryos ($P < 0.001$) (Fig. 3D). *Caspase 3* expression was highest ($P < 0.0001$) in the blastocysts from the acid Tyrode's solution ZF embryos when compared with the other ZPR methods; however, there were no significant differences in *Caspase 3* expression in either the proteinase K or two-step combined method groups (Fig. 3E).

4.Discussion

Establishing a more robust method for ZPR and improving the overall culture system for ZF embryos is critical to improve the quality and broadening their application in ART and embryology. Here, we describe a novel method for ZPR with reduced toxicity. This protocol uses a two-step combined method for ZPR and improves the development of ZF embryos with reducing the number of apoptotic cells in the blastocyst. This method was further improved by the application of a cWOW system with smaller well sizes that are more compatible with ZF embryos.

One of the most important roles of the ZP during early embryonic development is the maintenance of the three-dimensional (3D) structure of and intracellular contacts within the blastomere. Without ZP, divided blastomeres often adopt two-dimensional forms (Fig. 1E) and retain this structure until compacted within a flat bottom dish. This indicates that the weakening of 3D-contacts between the blastomeres affects further development, differentiation and embryo quality (Suppl Table 2). A previous study revealed that WOW improves development in both bovine ZI and ZF embryos [17]. Although the effectiveness of WOW for mouse embryos and its impact on their developmental competence has not yet

been reported, our results show that the blastocyst rate for ZF embryos in the flat microdroplet system was very low (Suppl Table 2). In contrast, the use of a commercially available WOW system improved the blastocyst rate in these embryos, but cWOW, which had a smaller well bottom area, was shown to be the more efficient system for ZF culture. This cWOW produced a significant increase in the blastocyst rate compared to commercial WOW which microwell size affects development. In general, when we compare the size of the ZF mouse embryos (approximately 100 μm) with the diameter of commercial WOW plates (approximately 280 μm) we can see that these wells are likely to be too wide. In contrast, our cWOW microwells had a diameter of approximately 170 μm which may provide more suitable conditions for cell division and the maintenance of the 3D blastomere structures, allowing for improved development and differentiation.

Although previous research used porcine ZI embryos to evaluate the effects of microwell size on embryonic development, they clearly demonstrate that the blastocyst rate was also affected by microwell size [18] and the findings are in agreement with our study. The lack of ZP is likely to affect both the contact and communication between blastomeres, affecting their compaction at the morula stage and preventing their progression to the blastocyst stage. Therefore, the size of the microwell could play an important role throughout the process of culturing ZF embryos (Fig. 1E). Taken together this supports our hypothesis that our cWOW is better suited to mouse ZF embryo development.

Previous studies have established that bovine zona removal is most effectively achieved using 0.2% proteinase K for 5 min and that this process exhibited low toxicity [19]. In contrast, treatment with 0.5% proteinase K took approximately 6.8 min to completely remove the ZP in mouse embryos suggesting that it is not as efficient in mice. However, despite the differences in digestion time between species this digestion did not induce any visible damage to the blastomere membranes. However, we cleared that enzymatic treatment increases the loose of cell-to-cell contacts and blastomere detachment when compared to other ZPR methods. In addition, another study showed that prolonged exposure time to proteinase K damages the ICM/TE structures and disturbs subsequent implantation in human blastocysts [14]. Therefore, enzymatical ZPR may digest the proteins on the blastomere

surface needed to maintain the cell-to-cell contacts necessary for cell growth and differentiation and induce a decrease in blastocyst rate and total cell number.

Acid Tyrode's solution is also commonly used in ART, and has been applied to assisted hatching and blastomere separation in humans; however, its toxicity remains controversial. In humans, ZF blastocyst produced using acid Tyrode's solution demonstrated increased implantation rates, resulting in more successful pregnancies in patients with poor prognosis [20]. However, the toxicity of acid Tyrode's solution causes damage to the embryos and separation of the blastomeres during cleavage [13]. This has led to the development of several zona thinning techniques which rely on the partial digestion of the outer layer of the ZP. For example, partial and circumferential zona thinning has been described in several animal experiments [21]. Here, blastocyst rate and total cell number were significantly decreased following treatment with acid Tyrode's solution despite its shorter treatment time. Morphological changes including the development of wrinkles on the blastomere surface were clearly observed.

Acid Tyrode's solution-treated ZF blastocyst showed increased apoptotic cells and higher expression of caspase 3 which is consistent with previous study reported [22]. Given this we went on to evaluate the expression of *Bax,* which acts as a cell death inducer, and *Caspase3* as candidate genes for evaluating apoptosis in response to each of these ZPR protocols. Exposure of ZF embryos to pH (2.5) increased the toxicity of acid Tyrode's solution. While acid Tyrode's solution induced an increase in apoptosis proteinase K-treated bovine embryos did not experience any increase in apoptosis [23]. This was similar to our results, which showed that proteinase K treatment did not affect *Caspase 3* or *Bax* expression, and significantly decreased the apoptotic index in these embryos when compared to their ZI embryo counterparts. In addition, there was also no significant difference in the apoptosis index of ZF embryos after combined two step processing.

The two-step combined method used a 40s exposure to acid Tyrode's solution followed by several seconds of proteinase K treatment to remove the ZP from 2-cell embryos. This method demonstrated a significant increase in blastocyst rate and total cell number and reduced cellular apoptosis when compared to the other ZPR treatments, reducing the toxic

effects of low pH on these embryos.

In conclusion, we propose a novel two-step combination method for ZP removal and recommend that this is combined with a customized WOW culture system to improve the development of ZF embryos in mice. This study provides an efficient approach for culturing ZF embryos for IVP procedures.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgements

Funding: This study was supported by a Grant-in-Aid of Scientific Research from the Tojuro Iijima Foundation for Food Science and Technology. We would like to thank Editage (www.editage.com) for English language editing.

References

[1] P.M. Wassarman, E.S. Litscher, Mammalian fertilization: the egg's multifunctional zona pellucida, Int. J. Dev. Biol. 52 (2008) 665-676. https://doi.org/10.1387/ijdb.072524pw.

[2] S.J. Conner, L. Lefièvre, D.C. Hughes, C.L.R. Barratt, Cracking the egg: increased complexity in the zona pellucida, Hum. Reprod. 20 (2005) 1148-1152. https://doi.org/10.1093/humrep/deh835.

[3] Y. Sjunnesson, In vitro fertilisation in domestic mammals-a brief overview, Ups. J. Med. Sci. 125 (2020) 68-76. http://doi.org/10.1080/03009734.2019.1697911.

[4] A.K. Tarkowski, Mouse chimaeras developed from fused eggs, Nature. 190 (1961) 857-60. https://doi.org/10.1038/190857a0.

[5] A. Nagy, J. Rossant, R. Nagy, W. Abramow-Newerly, J.C. Roder, Derivation of completely cell culture-derived mice from early-passage embryonic stem cells, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 8424-8428. https://doi.org/10.1073/pnas.90.18.8424.

[6] M. Maemura, H. Taketsuru, Y. Nakajima, R. Shao, A. Kakihara, J. Nogami, Y. Ohkawa, Y.I. Tsukada, Totipotency of mouse zygotes extends to single blastomeres of embryos at the four-cell stage, Sci. Rep. 11 (2021) 11167. https://doi.org/10.1038/s41598-021-90653-1.

[7] R. Li, J. Miao, Z. Wang, Production of Genetically Engineered Porcine Embryos by Handmade Cloning, Methods. Mol. Biol. 1874 (2019) 347-360. https://doi.org/10.1007/978-1-4939-8831-0_20.

[8] S. Ikeda, M. Sugimoto, S. Kume, Lipofection of siRNA into bovine 8-16-cell stage embryos using zona removal and the well-of-the-well culture system, J. Reprod. Dev. 64 (2018) 199-202. https://doi.org/10.1262/jrd.2017-137.

[9] K. Yano, C. Yano, T. Kubo, I. Ohashi, N. Maeda, T. Fukaya, Chemical zona pellucida thinning with acidified Tyrode's solution: comparison between partial and circumferential techniques, J. Assist. Reprod. Genet. 24 (2007) 471-5. https://doi.org/10.1007/s10815-007-9131-z.

[10] A.R. Menino, Jr., R.W. Wright, Jr., Effect of pronase treatment, microdissection, and zona pellucida removal on the development of porcine embryos and blastomeres in vitro, Biol. Reprod. 28 (1983) 433-46. https://doi.org/10.1095/biolreprod28.2.433.

[11] G. Vajta, I.M. Lewis, P. Hyttel, G.A. Thouas, A.O. Trounson, Somatic cell cloning without micromanipulators, Cloning. 3 (2001) 89-95. https://doi.org/10.1089/15204550152475590.

[12] P.J. Booth, S.J. Tan, P. Holm, H. Callesen, Application of the zona-free manipulation technique to porcine somatic nuclear transfer, Cloning. Stem. Cells, 3 (2001) 191-7. https://doi.org/10.1089/15362300152725909.

[13] J. Nichols, R.L. Gardner, Effect of damage to the zona pellucida on development of preimplantation embryos in the mouse, Hum. Reprod. 4 (1989) 180-7. https://doi.org/10.1093/oxfordjournals.humrep.a136868.

[14] C.Y. Fong, A. Bongso, H. Sathananthan, J. Ho, S.C. Ng, Ultrastructural observations of enzymatically treated human blastocysts: zona-free blastocyst transfer and rescue of blastocysts with hatching difficulties, Hum. Reprod. 16 (2001) 540-6. https://doi.org/10.1093/humrep/16.3.540.

[15] H. Suzuki, M. Togashi, J. Adachi, Y. Toyoda, Developmental ability of zona-free mouse

embryos is influenced by cell association at the 4-cell stage, Biol. Reprod. 53 (1995) 78-83. https://doi.org/10.1095/biolreprod53.1.78.
[16] G.A. Thouas, G.M. Jones, A.O. Trounson, The 'GO' system--a novel method of microculture for in vitro development of mouse zygotes to the blastocyst stage, Reproduction. 126 (2003) 161-9. https://doi.org/10.1530/rep.0.1260161.
[17] G. Vajta, T.T. Peura, P. Holm, A. Páldi, T. Greve, A.O. Trounson, H. Callesen, New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system, Mol. Reprod. Dev. 55 (2000) 256-64. https://doi.org/10.1002/(SICI)1098-2795(200003)55:3<256::AID-MRD3>3.0.CO;2-7.
[18] M. Taka, H. Iwayama, Y. Fukui, Effect of the well of the well (WOW) system on in vitro culture for porcine embryos after intracytoplasmic sperm injection, J. Reprod. Dev. 51 (2005) 533-7. https://doi.org/10.1262/jrd.17005.
[19] A. Velásquez, J. Manríquez, F. Castro, L. Rodríguez-Alvarez, Effect of zona pellucida removal on early development of in vitro produced bovine embryos, Arch. Med. Vet. 45 (2013) 7-15. http://dx.doi.org/10.4067/S0301-732X2013000100003.
[20] R.T. Mansour, C.A. Rhodes, M.A. Aboulghar, G.I. Serour, A. Kamal, Transfer of zona-free embryos improves outcome in poor prognosis patients: a prospective randomized controlled study, Hum. Reprod. 15 (2000) 1061-4. https://doi.org/10.1093/humrep/15.5.1061.
[21] J.W. Gordon, U. Dapunt, Restoration of normal implantation rates in mouse embryos with a hatching impairment by use of a new method of assisted hatching, Fertil. Steril. 59 (1993) 1302-7. https://doi.org/10.1016/s0015-0282(16)55994-9.
[22] A.G. Porter, R.U. Jänicke, Emerging roles of caspase-3 in apoptosis, Cell. Death. Differ. 6 (1999) 99-104. https://doi.org/10.1038/sj.cdd.4400476.
[23] E. Gómez, J.N. Caamaño, P. Bermejo-Alvarez, C. Díez, M. Muñoz, D. Martín, S. Carrocera, A. Gutiérrez-Adán, Gene expression in early expanded parthenogenetic and in vitro fertilized bovine blastocysts, J. Reprod. Dev. 55 (2009) 607-14. https://doi.org/10.1262/jrd.09-077m.

Figure/ Table legends

Figure 1. Comparison of microwell sizes in the commercial and customized WOW systems. The microwell size in each culture system was determined using color 3D laser microscopy. A: Commercial WOW system B: Customized WOW system (scale bar: 750 μm). C: The diameter of each of the microwells in each culture system. D: Depth of the microwells in each culture system. E: Enlarged image of the morphological differences in the ZF embryos cultured in each culture system (scale bar: 50 μm). The results are reported as the mean ± S. E. M., and the Student's *t*-test was used for all statistical analyses. Asterisks indicate significant differences (**** $P < 0.0001$).

Figure 2. Effect of different zona pellucida removal methods on the morphology of the resultant blastomeres. The blastomere membranes from ZI and ZF embryos were observed using a phase-contrast microscope. White arrows indicate wrinkles on the blastomere surface while black arrows indicate the blastomere attachment status. Scale bar: 150 μm (left) , 50 μm (right).

Figure 3. Effect of different zona pellucida removal methods on the total number of cells and their apoptotic status within the mouse blastocysts. A: Apoptotic blastomeres within the murine blastocysts were detected using the TUNEL assay (green). Nuclei were stained with Hoechst (blue) to help visualize the individual blastomeres and apoptotic cells were counted in both the ZI and ZF blastocysts produced by different ZPR methods. Scale bar: 25 μm. B: Total cell number. C: Apoptosis index. D, E: Differences in *Bax* (D) and *Caspase3* (E) transcription in blastocysts produced using different ZPR methods. The results are reported as the mean ± SEM, and one-way ANOVA was used to analyze at least three replicates for each experiment. Asterisks indicate a significant difference, B: Total cell number (a *vs.* b: $P < 0.01$); C, E (apoptosis index and *Caspase 3* mRNA expression): a *vs.* b: $P < 0.0001$; D: a *vs.* c: $P < 0.05$, b *vs.* c: $P < 0.001$.

Table 1. Treatment time to complete zona removal. A: Exposure time needed to facilitate total ZPR. The results are shown as the mean ± SEM, and one-way ANOVA was used to analyze at least three replicates. Different letters indicate significant differences (a *vs.* b: $P < 0.0001$).

Table 2. Effect of different methods of zona pellucida removal on the development of zona free embryos. Zona Free (Acid Tyrode's Solution): Embryos where the ZP was removed at the 2-cell stage using acid Tyrode's solution. ZF (Proteinase K): Embryos where the ZP was removed at the 2-cell stage using proteinase K. ZF (two-step combined method): Embryos where the ZP were removed at the 2-cell stage using a combined two-step method. The data are expressed as the mean ± SEM, and one-way ANOVA was used to analyze the data. Different letters indicate statistical differences (a *vs.* b: $P< 0.01$).

Supplementary Figure. Workflow describing the method of zona pellucida removal and ZF embryo culture. The 2-cell embryos were treated with acid Tyrode's solution until the ZP was completely removed. Zona intact 2-cell embryos were used as the control and ZF embryos were cultured using microdroplet (1), WOW (2), or cWOW (3) systems. These wells were manually produced using an aggregation needle and paraffin oil and each microwell contained a single ZF embryo at the bottom of the dish. ZF: Zona free.

Supplementary Table 1. Primers used in the qPCR analysis.

Supplementary Table 2. Effect of different culture systems on the development of zona free mouse embryos

Flat microdroplet: Embryos were removed from the ZP using acid Tyrode's solution and cultured in a flat microdroplet system. WOW: Embryos were removed from the ZP using acid Tyrode's solution and cultured in the WOW system. Customized WOW: Embryos were removed from the ZP by acid Tyrode's solution and cultured in a cWOW system. The data are expressed as the mean ± SEM, and one-way ANOVA was used to analyze the

data. Different letters indicate statistical differences (a *vs.* b: $P < 0.0001$, a *vs.* c: $P < 0.0001$, b *vs.* c: $P < 0.05$).

Table 1. Treatment time to complete zona removal

Treatment	No. of replicates	Time required for complete zona removal (Min, Mean ± S. E. M.)
Acid Tyrode's Solution	5	1.2 ± 0.17[a]
Proteinase K	5	6.8 ± 0.60[b]
Two-step	5	1.4 ± 0.15[a]

Table 2. Effect of different methods of zona pellucida removal on the development of zona free embryos

Treatment	No. of replicates	No. of embryos cultured	No. of Blastocysts (Mean ± S. E. M.)
Zona intact	5	110	95 (86.3 ± 2.2[a])
Zona Free			
Acid Tyrode's Solution	5	148	103 (69.6 ± 2.3[b])
Proteinase K	5	122	78 (63.9 ± 7.4[b])
Two-step	5	90	69 (76.7 ± 2.2[a])

Highlights:

- Zona pellucida (ZP) removal decreased embryo development.
- Customized WOW system with smaller microwells improved ZP-free embryo development.
- Acid Tyrode treatment shortened ZP removal time, but decreased blastocyst rate with increased apoptosis.
- Proteinase K increased ZP removal time with blastomere dissociation and decreased blastocyst rate.
- Combined use of acid Tyrode and proteinase K improved development with low toxicity.

Fig. 1 (Fan et al)

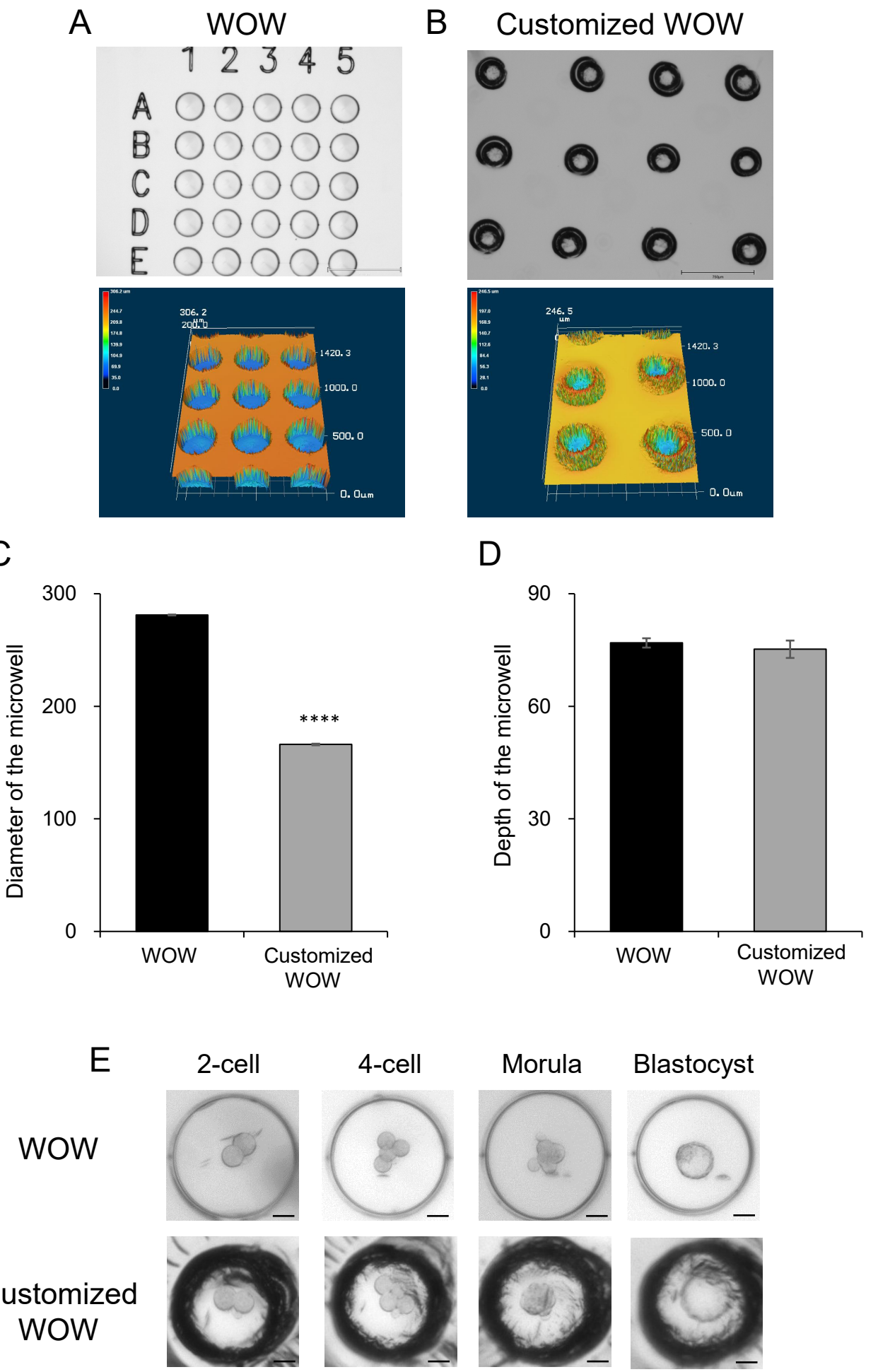
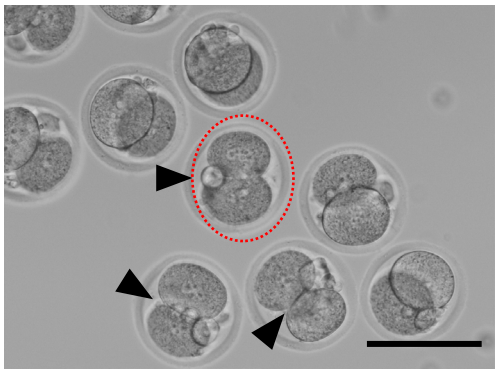


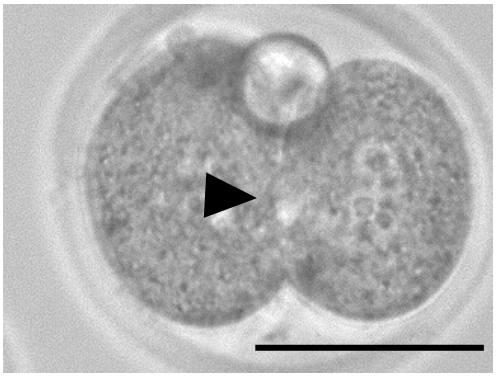
Fig. 2 (Fan et al)

A

Zona Intact

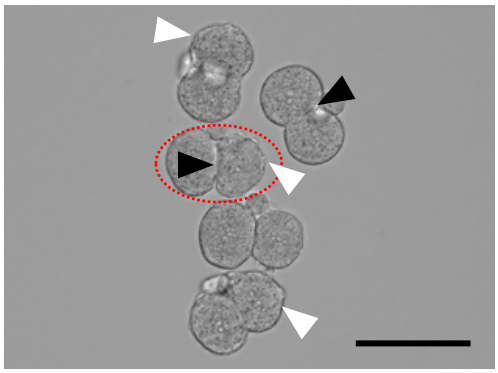


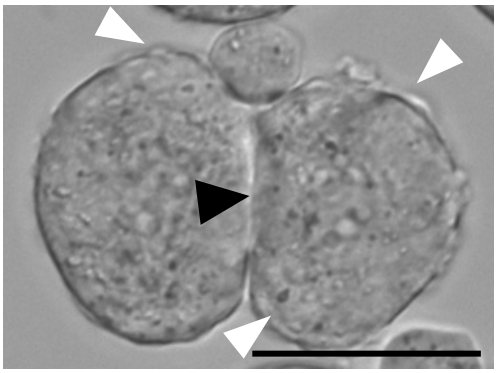
Enlarged



B

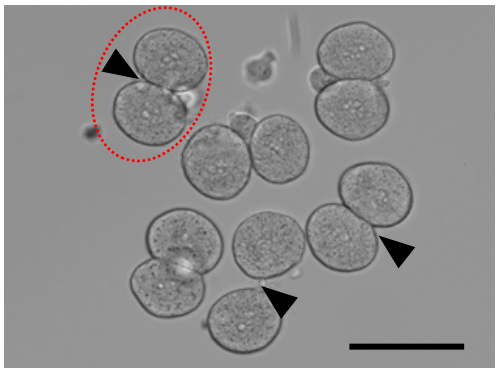
Acid Tyrode

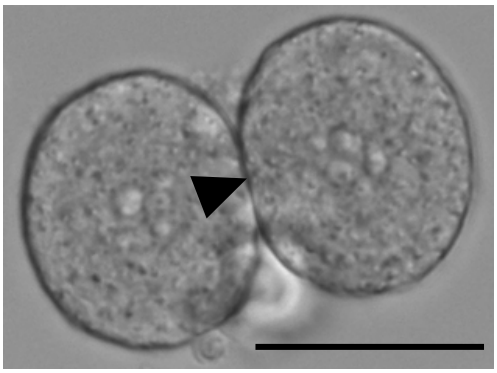




C

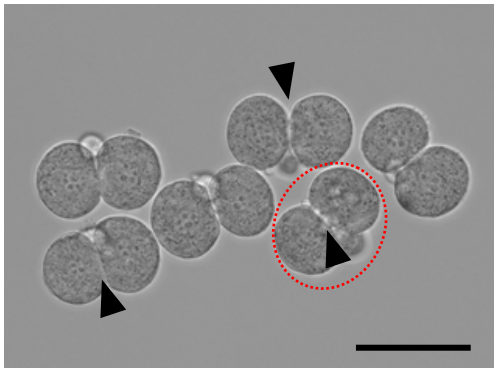
Proteinase K





D

Two-step



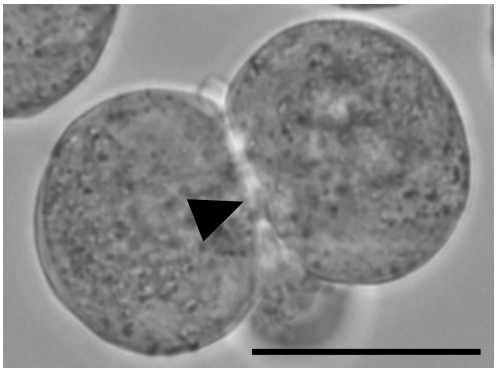


Fig. 3 (Fan et al)