Developmental potential of zona pellucida–free oocytes obtained following mild in vitro fertilization

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Objective: To determine the developmental potential of oocytes in which the zona pellucida was damaged and subsequently removed, producing "zona-free" (ZF) oocytes that were cultured until the blastocyst stage.
Design: ZF eggs from cycles with more than one oocytes retrieved (n = 97) were compared with zona-intact (ZI) oocytes originating from the same patient.
Setting: Private infertility clinic.
Patient(s): Infertile patients (n = 135) undergoing minimal ovarian stimulation or natural-cycle in vitro fertilization treatment during 2010–2012.
Intervention(s): ZF oocytes undergoing intracytoplasmic sperm injection (ICSI) fertilization, blastocyst culture, elective vitrification, and subsequent single vitrified-thawed blastocyst transfer (SVBT).
Main Outcome Measure(s): Rate of fertilization, cleavage, and blastocyst development. Live birth rate and neonatal outcome in subsequent SVBT cycles.
Result(s): There were no significant differences in fertilization (77% vs. 77%), cleavage (75% vs. 75%), or blastocyst development rates (39% vs. 32%) between the internally controlled ZF and ZI groups, respectively. Survival after thawing (90% vs. 100%) and live birth rates (37% vs. 36%) per thawed blastocyst were also similar. Newborns originating from all ZF and ZI oocytes had a similar gestational age at delivery (38.3 ± 3.7 wk vs. 39.5 ± 1.5 wk) and birth weight (3,115 ± 946 g vs. 3,010 ± 441 g).
Conclusion(s): Our retrospective comparative study suggests that ZF eggs could be as successfully fertilized and cultured until the blastocyst stage as ZI control eggs without adversely affecting subsequent pregnancy rates and basic neonatal outcome. (Fertil Steril® 2014;102:1602–7. ©2014 by American Society for Reproductive Medicine.)
Key Words: Zona pellucida, blastocyst culture, minimal ovarian stimulation, in vitro fertilization

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In recent years, increasing interest has emerged in milder approaches to in vitro fertilization (IVF), including minimal ovarian stimulation and natural-cycle IVF (ncIVF) (1, 2). These treatment modalities have become especially widespread in Japan owing to a high proportion of advanced-age infertile women, the increased use of single-embryo transfer (SET) policy (3), and the efforts of specialist centers that have been developing these innovative treatment protocols for more than two decades (4–6). Compared with conventional ovarian stimulation, with the above-mentioned mild approaches the expected egg yield is considerably lower, meaning that usually only a single or fewer than eight oocytes can be retrieved (7). Therefore, in the setting of minimal ovarian stimulation, it is imperative to fully utilize the developmental potential of each harvested egg to avoid the physical and psychologic burden of cycle cancellation for the individual patient.

In 1991 it was first described that human oocytes devoid of their zona pellucida could be successfully fertilized by intracytoplasmic sperm injection (ICSI) and cultured until blastocyst stage. This is of importance because in most centers, eggs with damaged zona pellucida are generally discarded (8). A few subsequent case

Received April 17, 2014; revised August 6, 2014; accepted August 18, 2014; published online September 23, 2014.
S.U. has nothing to disclose. D.B. has nothing to disclose. K.U. has nothing to disclose. T. Okimura has nothing to disclose. T. Okuno has nothing to disclose. T.K. has nothing to disclose. K.K. has nothing to disclose.
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Fertility and Sterility® Vol. 102, No. 6, December 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.08.025

reports described early pregnancies and a single live birth originating from blastocysts cultured from zona-free (ZF) mature oocytes (9, 10). In the latter report, obtained blastocysts were electively vitrified, suggesting that ZF blastocysts could withstand the freezing-thawing procedure and still conserve their developmental potential (10). Although these single case reports were encouraging there are no larger case series available that could lend support to the problem-free culturing of ZF eggs (and cryopreservation of resulting blastocysts) as an option when zona damage occurs in the embryology laboratory. Moreover, data are extremely scarce on neonatal outcome of resulting offspring. Therefore the objective of the present retrospective internally controlled study was to evaluate the reproductive potential of eggs in which the zona pellucida was damaged and subsequently removed, producing ZF oocytes that were cultured until the blastocyst stage. We also report on basic neonatal outcome following transfer of resulting ZF embryos from a single center handling a large number of mild IVF cycles.

MATERIALS AND METHODS

Participants and Study Design

The retrospective study period was from May 2010 to August 2012 (2 years 4 months), including all patient cycles (n = 135) performed at Kato Ladies Clinic, Tokyo, Japan, involving the retrieval of an oocyte where the ZP was found to be damaged, resulting in an egg that was fertilized with ICSI and cultured without its envelope (ZF eggs). In those study cycles where more than one egg was retrieved (n = 97), the outcome of the ZF group was compared with the rest of the zona-intact (ZI) oocytes obtained from the same patient. The outcome of those study cycles (n = 38) where only a single ZF egg was obtained was also presented separately without any control group. The fate of all vitrified blastocysts was followed until December 2013 (allowing for subsequent vitrified-thawed embryo transfer cycles). All resulting pregnancies were followed until delivery. Institutional Review Board was obtained for the present study (IRB approval no. 13-21).

Minimal Stimulation and Natural Cycle IVF Protocol

All patients undergoing IVF treatment at our center received detailed information about the proposed treatment option and provided written informed consents. Additionally those patients who had ZF eggs were also informed accordingly and agreed to their use. A clomiphene citrate (CC)–based minimal stimulation protocol was used in the majority of cycles, whereas unstimulated natural-cycle IVF was used in a smaller proportion of cases. Details of the CC-based minimal stimulation protocol were described previously (5). Briefly, CC (50–100 mg/d) was administrated orally with an extended regimen from cycle day 3 until the day before inducing final oocyte maturation. hMG or recombinant FSH was added in the form of injections (50–150 IU every other day) or nasal spray to obtain one to four mature follicles. Monitoring involving ultrasound scans and hormonal profiles (E_2, LH, and P) was usually started on day 8 and continued every other day until triggering day. Ovulation triggering was performed with a GnRH agonist, buserelin (Suprecur, 600 μg), administered in a nasal spray form. In the natural-cycle IVF protocol, the only pharmaceutical intervention consisted of inducing the final oocyte maturation with a GnRH agonist. Monitoring consisted of ultrasound scans and hormonal profiles (E_2, LH, and P) and was usually started in the morning of day 10–12 according to the patient's cycle length. When the leading follicle reached 18 mm with a concomitant E_2 level $\geq$250 pg/mL, oocyte retrieval was scheduled. Oocyte retrieval was usually performed 30–34 hours after triggering, though in some cases where the start of the LH surge was detected it was performed 26–30 hours after triggering (11).

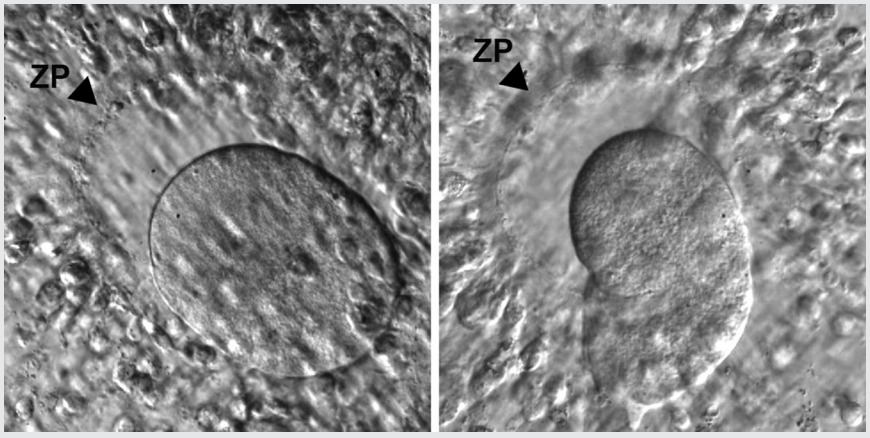
Oocyte Retrieval and Fertilization

Oocyte retrieval was performed without general anesthesia with the use of a 21–22-gauge fine needle (Kitazato) with an aspiration pressure of 300–330 mm Hg. Follicular flushing was not used during retrieval. Oocyte maturity was checked immediately after egg retrieval. In our study cases, we did not observe any "intrinsically" absent ZP. Rather the ZP was found to be fractured and the oocyte was protruding through its ruptured envelope (Fig. 1). Denuding was performed with a hand-drawn pipette with the use of a hyalorunidase (Sigma Chemical) containing in-house–made human tubal fluid medium with HEPES. Despite the gentle removal of surrounding cumulus cells the oocyte escaped spontaneously and was cultured further without its envelope. To avoid polyspermy, all ZF eggs were fertilized with the use of ICSI performed 5 hours after oocyte retrieval, whereas ZI eggs could be fertilized with conventional insemination or ICSI. During the ICSI procedure, the oocyte was handled gently with extra care to avoid any damage to its oolemma. The location of meiotic spindle was confirmed beforehand with polarized light microscopy (IX-Robopolar; Olympus). Supplemental Video 1 (available online at www.fertstert.org) shows observation of the spindle and the ICSI procedure performed on a ZF oocyte. Fertilization assessment was done 16–20 hours after insemination. From day 1 to day 3, normally fertilized two-pronuclei (2PN) zygotes were cultured in drops of 20 μL Quinn Advantage Protein Plus cleavage medium (Sage) covered by mineral oil in Falcon 1008 dishes (Becton Dickinson Labware). Following this, the embryos were transferred to Quinn Advantage Protein Plus Blastocyst medium (Sage) from day 4 to day 6. ZF embryos were always cultured individually to avoid the risk of chimera formation. All embryos were cultured at 37°C under the gas phase of 5% O_2, 5% CO_2, and 90% N_2 with 100% humidity in water-jacket small multigas incubators or dry desktop incubators (Astec). The liquid nitrogen was produced by a N_2 generator system in a 10,000-class clean room environment.

Embryo Culture, Frozen-thawed Cycles, and Embryo Transfer Procedure

During the study period, only SETs were performed in our center, and an exclusive SET policy was strictly observed.

FIGURE 1



Oocytes protruding from a partially ruptured zona pellucida (ZP) surrounded by cumulus cells.

Ueno. Zona-free oocyte culture. Fertil Steril 2014.

Patients were counseled accordingly before their treatment started about the risk of multiple pregnancies, the benefits of an SET, and the possibility of embryo cryopreservation. In all study and most control cycles, embryos were cultured to blastocyst stage and electively vitrified for subsequent use in vitrified-thawed blastocyst transfer cycles. Details of the vitrification method with the use of the Cryotop (Kitazato) were described previously (12). If several embryos were available (from both ZF and ZI eggs), the best one for embryo transfer was selected according to its morphologic quality regardless of the presence of zona pellucida. Vitrified-thawed embryo transfers were performed in spontaneous natural or hormonal replacement cycles. All embryo transfer procedures were performed under vaginal ultrasound guidance with the use of a specially designed soft catheter (Kitazato) by placing a single embryo in minimal volume to the mid-uterine cavity (13).

Outcome Measures and Definitions

The primary outcome was the fertilization, cleavage, and blastocyst formation rate per inseminated egg. The secondary outcome was subsequent live birth rate per thawed blastocyst. An intrauterine gestational sac revealed by ultrasound scan ~4 weeks after transfer was considered to be a clinical pregnancy. Live birth was defined as delivery at ≥22 weeks of pregnancy. Metric variables were analyzed with the use of independent *t* test. Nominal variables were analyzed with the use of conditional logistic regression analysis (in case of nonindependent matched samples) or the chi-square test (for independent samples). $P<.05$ was considered to be statistically significant.

RESULTS

During the study period, ZF oocytes were retrieved and fertilized in 135 cycles. Of these, in 97 cycles more than one egg was retrieved, allowing us to create an internally controlled study and control group by comparing the ZF and ZI eggs originating from the same patient. The outcome of 38 cycles with only a single retrieved ZF egg is presented separately.

For the internally controlled same-patient ZF and ZI groups, there were no significant differences in fertilization (77% vs. 77%; $P=1.0$), embryo cleavage rate (75% vs. 75%; $P=1.0$), rate of cycles reaching the blastocyst stage (39% vs. 32%; $P=.11$), or proportion of cycles where a good-quality blastocysts could be electively vitrified (28% vs. 26%; $P=.37$). For the "single-egg" ZF group, these rates were 92%, 90%, 34%, and 29%, respectively. Embryologic laboratory outcome data are summarized in Table 1. Cleavage-stage embryos in the study group displayed three typical patterns of blastomere contact at a 4-cell stage, which are shown in Figure 2.

During the follow-up period, totals of 27 and 36 blastocysts were thawed for subsequent use in the ZF and ZI groups, respectively. The survival rates after thawing were, respectively, 90% and 100% for the internally controlled same-patient groups. Clinical pregnancy (37% vs. 44%) and live birth rates (37% vs. 36%) per thawed blastocyst were also similar. Reproductive outcome data of all subsequent SVBT cycles are summarized in Table 2. During the follow-up period, totals of nine and 17 live births were obtained in both groups. Newborns originating from ZF and ZI oocytes had a similar gestational age at delivery (38.3 ± 3.7 wk vs. 39.5 ± 1.5 wk; $P=.40$) and birth weight (3,115 ± 946 g vs. 3,010 ± 441 g; $P=.77$). One major malformation (palatoschisis and hypospadias together with extremely low birth weight) occurred in the ZF group.

DISCUSSION

In this retrospective internally controlled study, we found that ZF mature human oocytes could be successfully fertilized with the use of ICSI, cultured until the blastocyst stage, and cryopreserved with the use of vitrification for subsequent

TABLE 1

Laboratory outcomes of zona-free (ZF) and zona-intact (ZI) oocytes.

	Study group			
		From same patient		
Data collected	ZF (1 egg)	ZF (>1 eggs)	ZI (>1 eggs)	*P* value[a]
ICSI cycles, n	38	97		–
Patient age, y	40.1 ± 3.3	39.3 ± 4.3		–
Stimulation protocol (CC/natural)	25/13	95/2		–
Eggs retrieved	40	322		
Eggs inseminated	38	97	216	–
Fertilized (2PN), n (%)[c]	35 (92)	75 (77)	167 (77)	1.00[b]
Cleaved embryos, n (%)[c]	34 (90)	73 (75)	162 (75)	1.00[b]
Blastocysts, n (%)[c]	13 (34)	38 (39)	70 (32)	.11[b]
Frozen good-quality blastocysts, n (%)[c]	11 (29)	27 (28)	56 (26)	.37[b]

Note: Values are presented as mean ± SD or n (%). In the ZI group 18 and 4 embryos were transferred or frozen, respectively, at cleavage stage. 2PN = two pronuclei; ICSI = intracytoplasmic sperm injection.

[a] Comparison between ZF and ZI groups from the same patient in cycles with >1 retrieved egg.

[b] Conditional logistic regression analysis.

[c] Per egg inseminated.

Ueno. Zona-free oocyte culture. Fertil Steril 2014.

use. Basic neonatal outcome of resulting offspring was reassuring, suggesting that with appropriate handling the developmental potential of ZF eggs is not different from those with an intact zona pellucida.

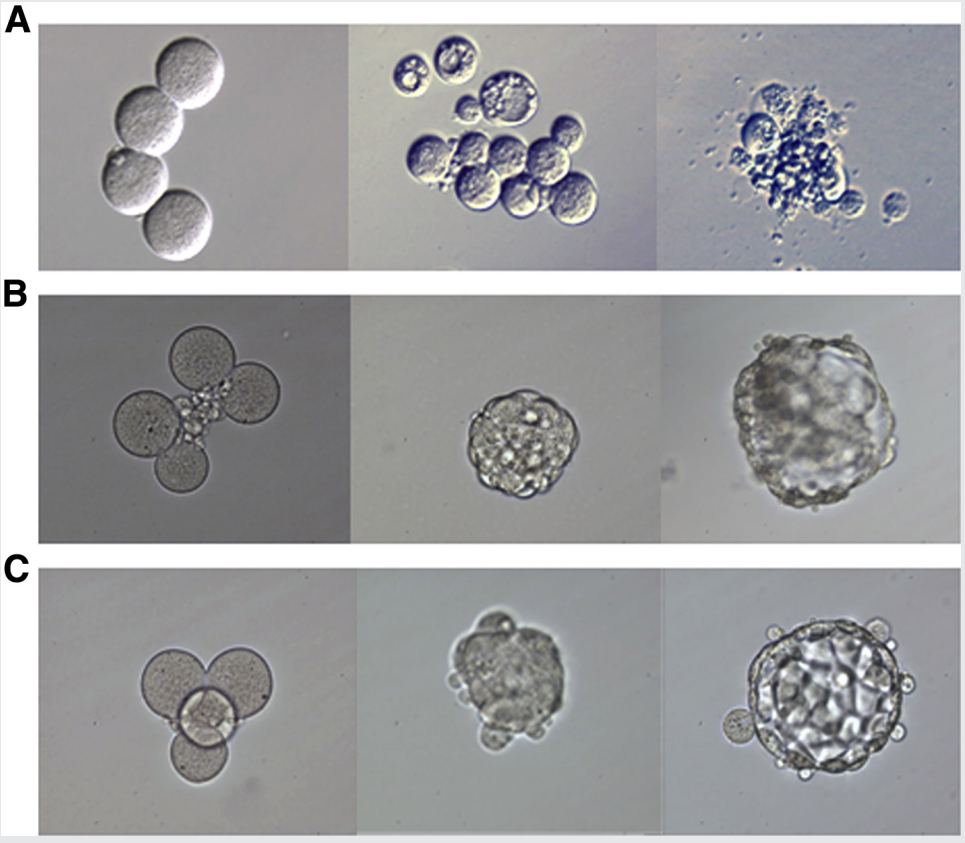
The ZP is a thick extracellular elastic coat surrounding the oocyte composed of a three-dimensional structure of interconnected sulphated glycoproteins. It has multiple in vivo functions which among others include protecting the oocyte/developing embryo, selecting a single fertilizing spermatozoon, and providing mechanical support by preventing blastomere separation before the compaction stage occurs (14). In the setting of IVF treatment, however, some of these functions might be redundant, because successful fertilization can be accomplished with the use of ICSI and protection against infectious agents is already provided by the strictly controlled setting of an embryologic laboratory. Nonetheless, the lack of mechanical support remains an important drawback because it increases the risk of blastomere separation and embryo degeneration before compaction occurs. It has been shown in mice that the developmental potential of ZF embryos directly correlates with the number of cell contacts observed in a 4-cell-stage embryo (15). Additionally, individual culturing of ZF embryos is always recommended to avoid aggregation of blastomeres from different embryos and chimera formation. Specifically designed culture dishes (such as the Well of the Well system) might provide an innovative solution to obtain the adequate culture conditions required for ZF embryos (16).

Previous case reports have already described that mature ZF human oocytes can be successfully fertilized through ICSI and could develop further to the blastocyst stage (8, 9, 17, 18). However, only one report had described the successful delivery of a healthy male newborn following fertilization of a ZF egg, blastocyst culture, and elective vitrification (10). In animal studies, there is some evidence showing that genetic mutations of ZP1–3 genes could lead to the formation of ZF eggs (19). In our series, we did not find any evidence of an intrinsically absent zona; instead, the main contributing factor was probably damage to the zona during oocyte retrieval. To avoid any iatrogenic damage, oocytes should always be handled with extra care, but our series suggests that if ZF eggs result these could be successfully cultured with outcomes similar to their ZI counterparts.

Our embryo transfer strategy was based on disengaging the embryo transfer from the stimulation phase, because our previous experience demonstrated that the minimal ovarian stimulation protocol was most effective when embryos were electively vitrified (preferably at blastocyst stage) and transferred in a delayed spontaneous or artificially prepared frozen embryo transfer cycle (6). This is also in line with recent evidence suggesting that segmentation of IVF treatment could overcome the impaired endometrial receptivity frequently occurring in stimulated cycles (20). The cryopreservation of ZF embryos was previously reported in only two case reports, one using the slow-freezing methodology and the most recent one using vitrification (8, 10). Our more extensive study has confirmed that (with an established vitrification method such as Cryotop) post-thawing survival rates and developmental potential are not significantly different from the control group, suggesting that the absence of the zona pellucida is not deleterious to successfully undergoing cryopreservation.

The number of live births in our study was not large (9 and 17 in the study and control groups, respectively), so further studies are still required to analyze the relationship between neonatal outcome and presence of zona pellucida. However, the above findings suggest that after adequate patient counseling and obtaining informed consent, blastocysts derived from ZF eggs could be transferred in a manner similar to their ZI counterparts. This is especially important in the setting of mild IVF where the number of available eggs is per se lower than with conventional ovarian stimulation protocols (on average 1.5 eggs at our center) (6).

FIGURE 2



Culture of zona-free oocytes. (**A**) Linearly arranged blastomeres: subsequently degenerated and did not reach blastocyst stage. (**B**) More closely positioned blastomeres: subsequently reached blastocyst stage but no pregnancy achieved. (**C**) Closely situated blastomeres: subsequently developed until blastocyst stage and live birth achieved.

Ueno. Zona-free oocyte culture. Fertil Steril 2014.

The main limitation of the present study is related to its retrospective nature and to a relatively small sample size. However, the rate at which the retrieval of ZF eggs occurs in daily clinical practice (at our center estimated to be 0.38% [170/44,460] per oocyte retrieval also including degenerated ZF eggs) means that only large IVF units could observe enough cases sufficient to produce comparative studies beyond simple case reports.

TABLE 2

Outcome of vitrified-thawed single-blastocyst transfers.

	Study group			
		From same patient		
Data collected	**ZF (1 egg)**	**ZF (> 1 eggs)**	**ZI (> 1 eggs)**	***P* value**[a]
Patient age, y	41.1 ± 2.2	36.7 ± 4.3	38.6 ± 4.2	.12[b]
Thawed blastocysts, n	8	19	36	–
Survived blastocysts, n (%)[d]	7 (88)	17 (90)	36 (100)	–
Clinical pregnancies, n (%)[d]	3 (38)	7 (37)	16 (44)	.59[c]
Live births, n (%)[c]	2 (25)	7 (37)	13 (36)	.96[c]

Note: Values are presented as mean ± SD or n (%). In the ZI group, four additional live births resulted from fresh/frozen cleavage-stage embryo transfers. Abbreviations as in Table 1.

[a] Comparison between ZF and ZI groups from the same patient in cycles with >1 retrieved egg.
[b] Independent *t* test.
[c] Chi-square test.
[d] Per thawed blastocyst.

Ueno. Zona-free oocyte culture. Fertil Steril 2014.

In conclusion, this retrospective study suggests that ZF eggs could be successfully fertilized and cultured until the blastocyst stage with efficiency similar to ZI control eggs without adversely affecting pregnancy rates and basic neonatal outcome.

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