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Long-term outcomes of freeze-all strategy: A retrospective analysis from a single ART center in Japan

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Abstract

Purpose: To demonstrate the benefits of the freeze-all strategy for in vitro fertilization treatment based on retrospective analyses.

Methods: Post-thaw embryo survival rates of slow-frozen embryos in 294 cycles and vitrified embryos in 12 195 cycles were assessed. Progesterone (P4) and estradiol (E2) levels per mature oocyte by age category were assessed in 9081 cycles and pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer by P4 level were assessed in 1535 cycles.

Results: The survival rates of frozen-thawed embryos were 92.5% with slow freezing and 99.1% with vitrification. P4 levels on the day of human chorionic gonadotropin (hCG) injection showed a trend toward an increase with age. The pregnancy rate per mature oocyte with fresh embryo transfer decreased dependently upon P4 level, while that with frozen-thawed embryo transfer was not affected by P4 level. The pregnancy rates with frozen-thawed embryo transfer were higher than those with fresh embryo transfer in patients aged 42 years or younger.

Conclusions: The freeze-all strategy is a valuable treatment option which allows the separation of an embryo transfer cycle from an oocyte retrieval cycle, especially for patients with high P4 levels at oocyte retrieval and patients of advanced maternal age.

KEYWORDS
in vitro fertilization, freeze-all strategy, vitrification, frozen-thawed embryo transfer, progesterone level

1 | INTRODUCTION

The freeze-all strategy is a modality consisting of an ovarian stimulation cycle in which oocytes are retrieved and inseminated, and all the generated embryos are cryopreserved. Subsequently, the cryopreserved embryos can be thawed and transferred after the priming of the endometrium primarily by hormone replacement or in other cases using the natural menstrual cycle.[1] Since the freeze-all strategy was initially reported in the 1990s,[2] the number of frozen-thawed embryo transfers and the resulting number of births have been increasing.[1,3] The evolution of cryopreservation-thawing techniques is a major contributor to the increased frozen-thawed embryo transfers. Cryopreservation was first successful in 1972 using mouse embryos,[4,5] and subsequently in the 1980s, cryopreservation techniques expand to human embryos. The development of vitrification in 1985[6] and the subsequent continuous improvements have

led to the establishment of the current cryopreservation procedure. Especially, the advent of the Cryotop® (Kitazato Corporation, Fuji, Japan) method using a less toxic vitrification medium and providing the ultrarapid speed of cooling and warming has allowed the cryopreservation of human unfertilized oocytes that used to be associated with low post-thaw survival rates and low normal fertilization rates. Another reason for the increased number of frozen-thawed embryo transfers is a report saying that the elevation of the progesterone (P4) level on the day of the human chorionic gonadotropin (hCG) injection administered as a part of controlled ovary stimulation leads to decreased implantation rates with fresh embryo transfer.[7,8] With these reports, there was an impetus to cryopreserve all embryos and transfer the thawed embryos after endometrial preparation/natural cycle in patients with high P4 levels at oocyte retrieval. The freeze-all modality has been reported to not only improve implantation rates but also prevent OHSS.[9-12]

Increasingly women of advanced maternal age have sought fertility treatment as a result of social changes, such as the tendencies to marry or have children later in life. Ovarian age is a major determinant of the oocyte reserve, which can be determined using AMH[13] and generally requires prolonged duration of stimulation. How to overcome these difficulties and lead women of advanced maternal age to pregnancy is a challenge for physicians.

Frozen-thawed embryo transfer may have numerous advantages; however, there are as yet few IVF centers that apply the freeze-all strategy to all patients. This study analyzed P4 and estradiol (E2) levels per mature oocyte, frozen-thawed embryo survival rate, and pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer in patients treated at our IVF center by age category to demonstrate the benefits of the freeze-all strategy especially in patients of advanced maternal age.

2 | MATERIALS AND METHODS

2.1 | Patients

Post-thaw embryo survival rate was assessed in 294 cycles in which slow-frozen embryos were thawed and 12 195 cycles in which vitrified embryos were thawed between 2008 and 2016 at our IVF center, except for the following cycles: (a) cycles in which non-pronuclear embryos were thawed (cycles in which both pronuclear and blastocyst embryos were thawed were excluded from the study), (b) cycles in which embryos cryopreserved by other methods than slow freezing or vitrification, and (c) cycles in which embryos derived from delayed intracytoplasmic sperm injection (ICSI) were thawed.

P4 and E2 levels per mature oocyte by age category were assessed in a total of 9081 cycles between 2008 and 2016, except for the following cycles: (a) cycles in which oocytes were retrieved only for oocyte cryopreservation, (b) cycles in which oocytes were repeatedly retrieved (ie, cycles in which the patients insufficiently responded to GnRH agonist trigger and oocytes were retrieved again after hCG injection), and (c) cycles involving embryos derived by delayed ICSI.

Pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer by P4 level on the day of hCG injection were assessed in a total of 1535 cycles in which embryos were created by IVF and transferred as single day 3 embryo in the IVF cycle or a later endometrial preparation cycle, between 2008 and 2016 at our IVF center, except for the following cycles: (a) cycles in which oocytes were retrieved only for oocyte cryopreservation, (b) cycles involving embryos derived from re-retrieved oocytes, (c) cycles involving embryos derived by delayed ICSI, and (d) cycles in which refrozen embryos were thawed.

The age categories used for comparisons included ≤30 years, ≥31 and ≤35 years, ≥36 and ≤39 years, ≥40 and ≤42 years, and ≥43 years.

2.2 | Controlled ovarian stimulation protocol

The gonadotropins used for controlled ovarian stimulation (COS) were human menopausal gonadotropin (Ferring Pharmaceuticals, Tokyo, Japan or ASKA Pharmaceutical, Tokyo, Japan) or recombinant FSH (Gonal-f®; Merck Serono, Tokyo, Japan). The long protocol started with administration of nasal buserelin 600 µg/day (Buserecur®; Fuji Pharma, Tokyo, Japan) on the prestimulation cycle day 21. The short protocol started with administration of nasal buserelin 600 µg/d on the stimulation cycle day 3. In both the long and short protocols, a GnRH agonist was daily administered until the day of hCG injection. For the GnRH antagonist protocol, alternate-day (in principle) administration of a GnRH antagonist (ganirelix [Ganirest®, MSD, Tokyo, Japan] or cetrorelix [Cetrotide®, Merck Serono]) began when follicular size reached a diameter of 14 to 16 mm or when a premature luteinizing hormone (LH) surge was suspected based on blood LH levels, followed by final oocyte maturation with a GnRH agonist or hCG (3000-5000 IU). After 34-36 hours of hCG injection, the mature oocytes were retrieved transvaginally, depending on follicular diameter, serum E2 and AMH levels, and patient's age. The retrieved oocytes were then fertilized by conventional IVF or ICSI. Split insemination was selected in cases of ≤6 metaphase II (MII) oocytes or oligozoospermia (defined as sperm concentrations post swim up ≤10 × 10^6/mL). Fresh embryo transfer was conducted, if all the following criteria were met: (a) serum E2 level on the day of hCG injection was ≤6000 pg/mL, (b) the serum P4 level on the day of hCG injection was ≤1.5 ng/mL, and (c) no signs of OHSS were noticed after oocyte retrieval.

2.3 | Embryo culture and cryopreservation

For fresh embryo transfer, the embryos were individually cultured in 15 µL drops of Onestep Medium® (NAKA ivf medium®, Nakamedical, Tokyo, Japan) until day 3 embryo. For frozen-thawed embryo transfer, the embryos were cryopreserved by slow freezing or vitrification at the pronuclear state, and in later cycles, the cryopreserved pronuclear embryos were thawed and individually cultured in 15 µL drops of Onestep Medium® until day 3 embryo. Embryo Freezing Pack and Embryo Thawing Pack (Origio) were used for slow freezing,

and Vitrification Kits VT101 and VT102 (Kitazato BioPharma) were used for vitrification.

2.4 | Day 3 embryo transfer

All frozen-thawed embryo transfers were performed in artificial hormone replacement cycles, according to the endometrial preparation protocol using transdermal estradiol (Estrana®, Hisamitsu Pharmaceutical, Saga, Japan) in combination with chlormadinone acetate (Lutoral®, Fuji Pharma). Estradiol treatment started on the second or third day of the artificial hormone replacement cycle and endometrial thickness was assessed on the 9th to 11th day of the cycle. If the endometrium was ≥7 mm, the frozen-thawed embryo transfer was scheduled. Administration of chlormadinone acetate 6 mg/d started on the 15th day of the cycle. The transfer of cleavage-stage embryos was performed on day 3, counting the day on which administration of chlormadinone acetate started as day 0. If a pregnancy occurred, transdermal estradiol 2.16 mg/every 2 days and chlormadinone acetate 6 mg/d were administered during the first 9 weeks of gestation.

Pregnancy was determined by urinary hCG measurement and transvaginal ultrasound scan. When the urinary hCG level at 14 days after embryo transfer was ≥50 IU/mL, the pregnancy test was considered to be positive and 1 week later, transvaginal ultrasound scan was performed to confirm the presence of an intrauterine gestational sac.

2.5 | Hormone assays

During the ovary stimulation cycle, serum FSH, LH, and E2 levels were measured in house, on several occasions including the day of hCG injection on which serum P4 level was additionally measured, using an electrochemiluminescence immunoassay (Cobas® e 411 plus, Roche Diagnostics KK, Tokyo, Japan).

3 | RESULTS

3.1 | Post-thaw embryo survival rates with slow freezing and vitrification

The mean annual post-thaw survival rate in 1756 embryos from 294 cycles, cryopreserved by slow freezing between 2008 and 2012 was 92.5%. The mean annual post-thaw survival rate in 42 189 embryos from 12 195 cycles, cryopreserved by vitrification between 2012 and 2016 was 99.1%, which was significantly higher than that with slow freezing ($P < 0.001$, Table 1).

3.2 | Serum P4 and E2 levels on the day of hCG injection and fertilization rates by age category

Serum P4 and E2 levels on the day of hCG injection were compared among different age categories in 9081 cycles after COS between 2008 and 2016. The comparisons between different age categories showed that the serum P4 and E2 levels per mature oocyte

TABLE 1 Post-thaw embryo survival rates with slow freezing and vitrification

	Slow freezing	Vitrification	p-value[a]
Patient age (y)	36.3 ± 3.7	38.3 ± 4.1	NA
No. of cycles (n)	294	12 195	NA
No. of thawed embryos (n)	1756	42 189	NA
No. of embryos survived after thawing (n)	1630	41 797	NA
Survival rate (%)	92.5 ± 12.8	99.1 ± 5.9	<0.001[b]

NA, not applicable.

Post-thaw embryo survival rate was assessed in cycles in which cryopreserved pronuclear embryos were thawed between 2008 and 2016 at our IVF center, except for the following cycles:

(1) cycles in which both pronuclear and blastocyst embryos were thawed.

(2) cycles in which embryos cryopreserved by other methods than slow freezing or vitrification were thawed.

(3) cycles in which pronuclear embryos created at two or more occasions (including embryos cryopreserved in two aliquots) were thawed in 2013 or later.

(4) cycles in which some of the embryos retrieved were lost.

(5) cycles in which cryopreserved embryos were thawed for two-step embryo transfer.

(6) cycles in which embryos derived by delayed ICSI were thawed (unknown for delayed ICSI embryos that were derived in 2012 or earlier and thawed in 2013 or later).

[a]Comparisons of post-thaw embryo survival rates between slow freezing and vitrification were assessed using Mann-Whitney U test.

[b]Statistically significant difference ($P < 0.05$).

significantly increased with advancing age (all $P < 0.001$, except for the P4 level between age category of ≥40 and ≤42 and 43≥: $P < 0.05$). The fertilization rates in younger patients in age categories of ≤30 years and ≥31 and ≤35 years were significantly higher than those in older patients in age category of ≥36 and ≤39 years ($P = 0.006$ and 0.048, respectively) and in age category of ≥40 and ≤42 years (both $P < 0.001$, Table 2).

3.3 | Pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer by serum P4 level on the day of hCG injection

The pregnancy rate with fresh embryo transfer was 25.2% in patients with low P4 levels on the day of hCG injection, which declined with the increase in P4 level. On the other hand, the pregnancy rate with frozen-thawed embryo transfer was not affected by P4 level. The comparisons of pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer between different serum P4 level groups showed that the pregnancy rates with frozen-thawed embryo transfer were significantly higher than those with fresh embryo transfer in patients with P4 levels of $1 \leq P4 < 1.5$ ($P < 0.001$) and $1.5 \leq P4 < 2$ ($P = 0.016$) on the day of hCG injection.

The comparisons of fertilization rates between different serum P4 level groups showed that there were no significant differences

TABLE 2 Serum P4 and E2 levels per mature oocyte and Fertilization rates by age category

Patient age (y)	≦30	31-35	36-39	40-42	43≦
No. of cycles (n)	1045	3296	3036	1436	268
Antral follicle count (n)	18.3 ± 8.3	16.7 ± 8.2	14.3 ± 7.1	12.8 ± 6.6	11.4 ± 5.1
Days of stimulation (d)	13.2 ± 2.2	13.5 ± 2.4	13.8 ± 2.4	14.4 ± 2.4	14.8 ± 2.7
Serum E2 (pg/mL)	11 068.5 ± 5980.0	9856.7 ± 5614.9	8851.1 ± 4891.2	8365.2 ± 4732.6	7791.3 ± 4313.8
Serum P4 (ng/mL)	2.6 ± 2.2	2.6 ± 2.3	2.6 ± 2.7	2.8 ± 2.5	2.7 ± 3.0
Retrieved oocytes (n)	21.8 ± 11.1	18.4 ± 10.7	15.1 ± 9.1	12.7 ± 7.9	10.9 ± 6.5
Mature oocytes (n)	17.8 ± 9.7	14.7 ± 9.3	11.8 ± 7.9	9.7 ± 6.8	8.1 ± 5.6
E2 per mature oocyte (pg/mL)[a]	765.8 ± 628.0[f]	860.0 ± 888.4[f]	958.4 ± 701.2[f]	1090.1 ± 877.3[f]	1244.2 ± 941.2[f]
P4 per mature oocyte (ng/mL)[b]	0.22 ± 0.9[f]	0.27 ± 0.6[f]	0.33 ± 0.5[f]	0.44 ± 0.8[f]	0.49 ± 0.8[f]
Fertilization rate (%)[c]	78.5 ± 16.9	77.4 ± 18.1	76.0 ± 19.5[d]	74.6 ± 20.8[e]	73.7 ± 22.4

P4 and E2 levels per mature oocyte by age category were assessed in IVF cycles conducted between 2008 and 2016 (for antral follicle count, between 2011 and 2016) at our IVF center, except for the following cycles:
(1) cycles in which oocytes were retrieved only for oocyte cryopreservation.
(2) cycles in which oocytes were repeatedly retrieved.
(3) cycles in which oocytes were derived by delayed ICSI.
Serum E2 and P4 levels were measured on the day of hCG injection.
[a]Comparisons of serum E2 per mature oocyte between age categories were assessed using Steel-Dwass test. Significant differences were observed between any two age categories (all $P < 0.001$).
[b]Comparisons of serum P4 per mature oocyte between age categories were assessed using Steel-Dwass test. Significant differences were observed between any two age categories (all $P < 0.001$, except for 40-42 vs 43≦: $P < 0.05$).
[c]Comparisons of fertilization rates between age categories were assessed using Steel-Dwass test.
[d]Significantly different from the fertilization rates in age≦30 and age 31-35 ($P = 0.006$ and 0.048, respectively).
[e]Significantly different from the fertilization rates in age≦30 and age 31-35 (both $P < 0.001$).
[f]Statistically significant difference ($P < 0.05$).

except for some groups (P4 < 1 [Fresh] vs 5 ≦ P4 [Freeze all]; P4 < 1 [Fresh] vs 2 ≦ P4 < 5 [Freeze all]; P4 < 1 [Freeze all] vs 5 ≦ P4 [Freeze all]; P4 < 1 [Freeze all] vs 2 ≦ P4 < 5 [Freeze all]; Table 3).

3.4 | Pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer by age category

Comparisons of pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer among different age categories showed that the pregnancy rate with frozen-thawed embryo transfer was higher than that with fresh embryo transfer in age categories of ≤30 years, ≥31 and ≤35 years, ≥36 and ≤39 years, and ≥40 and ≤42 years, and lower in an age category of ≥43 years. The statistically significant difference between pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer was seen in age category of ≥36 and ≤39 years ($P = 0.018$, Table 4).

4 | DISCUSSION

To our knowledge, this is the first report from a single IVF center that has applied the freeze-all strategy to all patients and analyzed a large number of embryo transfers by patient's age category.

Before the advent of the vitrification technique, slow freezing had been mainly employed for the cryopreservation of embryos. The post-thaw embryo survival rate with slow freezing at our IVF center, despite exceeding 90%, is more than 5% lower than that with vitrification. Even such small difference may cause a grave concern to patients who have a difficulty in collecting oocytes, such as low responders and women of advanced age. The dissemination of vitrification has improved post-thaw embryo survival rates to ≥99%, and these high survival rates are expected to overcome the risk of cryodamage.

The age of women seeking fertility treatment continues to increase yearly. At our IVF center, about 50% of women undergoing assisted reproductive technology (ART) treatment are 40 years or older. The increased age of women is associated with various challenges such as decreased number of oocytes retrieved, low pregnancy rates, and high miscarriage rates. In patients of advanced age, serum P4 and E2 levels per mature oocyte are higher, probably because of the prolonged duration and higher FSH usage in COS required for the collection of sufficient mature oocytes. Since high P4 levels have been shown to result in low pregnancy rates with fresh embryo transfer, the freeze-all strategy allows the separation of an embryo transfer cycle from an oocyte retrieval cycle in such patients.

The comparisons of serum P4 and E2 levels per mature oocyte on the day of hCG injection and the fertilization rates between

TABLE 3 Pregnancy rates with fresh embryo transfer and frozen-thawed transfer by serum P4 level on the day of hCG injection

	Fresh	Freeze all	Fresh	Freeze all	Fresh	Freeze all
Serum P4 (ng/mL)	P4<1		1≤P4<1.5		1.5≤P4<2	
No. of cycles (n)	142	86	111	182	37	235
Patient age (y)	36.4 ± 3.4	35.4 ± 3.6	36.4 ± 3.9	35.7 ± 3.3	35.8 ± 3.3	35.2 ± 3.5
Antral follicle count (n)	6.4 ± 2.0	10.8 ± 5.9	6.9 ± 2.7	12.9 ± 5.3	8.7 ± 4.7	15.1 ± 6.3
Days of stimulation (d)	12.4 ± 2.6	14.0 ± 2.2	12.4 ± 2.3	14.4 ± 2.0	12.3 ± 1.8	14.9 ± 2.4
Serum E2 (pg/mL)	2917.9 ± 1428.8	5577.0 ± 3029.4	4526.7 ± 1734.5	7293.1 ± 2891.2	4663.5 ± 2042.6	8294.1 ± 2822.7
Retrieved oocytes (n)	7.3 ± 4.3	9.2 ± 5.6	9.4 ± 5.0	10.8 ± 5.3	10.6 ± 5.5	12.2 ± 6.0
Mature oocytes (n)	5.2 ± 3.7	6.9 ± 3.8	6.9 ± 4.5	8.0 ± 4.3	8.2 ± 5.0	8.9 ± 4.4
Fertilization rate (%)[a]	79.5 ± 22.6[b]	81.6 ± 16.2[c]	74.5 ± 23.3	75.7 ± 18.7	70.2 ± 21.7	77.4 ± 16.6
Implantation rate (%)	28.1 (39/139)	25.6 (22/86)	13.6 (15/110)	33.3 (60/180)	13.5 (5/37)	35.2 (82/233)
Pregnancy rate (%)	25.2 (35/139)	25.6 (22/86)	9.1 (10/110)	29.4 (53/180)	10.8 (4/37)	30.5 (71/233)
p-value for pregnancy rate[d]	1.000		<0.001[e]		0.016[e]	

	Fresh	Freeze all	Fresh	Freeze all
Serum P4 (ng/mL)	2≤P4<5		5≤P4	
No. of cycles (n)	1	627	0	114
Patient age (y)	40.0 ± 0	35.5 ± 3.5	—	35.7 ± 4.0
Antral follicle count (n)	—	18.1 ± 7.4	—	22.6 ± 9.1
Days of stimulation (d)	20.0 ± 0	15.1 ± 2.0	—	15.5 ± 2.0
Serum E2 (pg/mL)	3807.0 ± 0	10 273.5 ± 3768.4	—	11 997.4 ± 6050.1
Retrieved oocytes (n)	5.0 ± 0	13.6 ± 6.8	—	16.0 ± 9.5
Mature oocytes (n)	2.0 ± 0	10.3 ± 5.9	—	11.7 ± 7.1
Fertilization rate (%)[a]	50.0 ± 0	74.3 ± 17.8	—	71.5 ± 18.0
Implantation rate (%)	0.0 (0/1)	28.9 (180/622)	—	16.7 (19/114)
Pregnancy rate (%)	0.0 (0/1)	24.6 (153/622)	—	11.4 (13/114)
p-value for pregnancy rate[d]	NA	NA		

NA, not applicable.

Pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer by serum P4 level on the day of hCG injection were assessed in cycles in which IVF embryos were transferred as single day 3 embryo in the IVF cycle or a later endometrial preparation cycle at our IVF center, between 2008 and 2012 for fresh embryo transfer (for antral follicle count, between 2011 and 2012) or between 2013 and 2016 for frozen-thawed embryo transfer, except for the following cycles:

(1) cycles in which oocytes were retrieved only for oocyte cryopreservation.

(2) cycles in which oocytes were repeatedly retrieved or embryos derived from re-retrieved oocytes were transferred.

(3) cycles in which embryos were derived by delayed ICSI.

(4) cycles in which embryos derived by delayed ICSI were transferred.

(5) cycles in which refrozen pronuclear embryos or cleavage-stage embryos were thawed and transferred.

Serum E2 and P4 levels were measured on the day of hCG injection.

[a]Comparisons of fertilization rates between serum P4 level groups were assessed using Steel-Dwass test.

[b]Significantly different from the fertilization rates in 2≤P4<5 (Freeze all) and 5≤P4 (Freeze all) (both $P = 0.006$).

[c]Significantly different from the fertilization rates in 2≤P4<5 (Freeze all) and 5≤P4 (Freeze all) ($P = 0.005$ and $P = 0.001$, respectively).

[d]Comparisons of pregnancy rates between fresh embryo transfer and frozen-thawed embryo transfer by serum P4 level were assessed using Fisher's exact test.

[e]Statistically significant difference ($P < 0.05$).

different age categories (Table 2) showed that both P4 and E2 levels were significantly increased and the fertilization rates were significantly decreased in patients of advanced age, so that it seemed serum P4 and E2 levels on the day of hCG injection had affected on embryo quality. Nevertheless, since there were no significant differences in comparison of fertilization rate by serum P4 levels (Table 3), the decreases in fertilization rate shown in Table 2 were thought to be mainly caused by advancing age. There are reports that high serum E2 levels at oocyte retrieval compromised the quality of embryos derived from the oocytes and the resulting

TABLE 4 Pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer by age category

Patient age (y)	Fresh ≦30	Freeze all	Fresh 31-35	Freeze all	Fresh 36-39	Freeze all
No. of cycles (n)	15	118	102	487	119	484
Antral follicle count (n)	8.5 ± 2.6	18.3 ± 7.5	7.1 ± 2.4	16.5 ± 8.1	7.1 ± 3.4	15.9 ± 6.8
Days of stimulation (d)	11.7 ± 1.8	14.9 ± 2.2	12.5 ± 3.1	15.0 ± 2.3	12.2 ± 1.7	15.0 ± 1.9
Serum E2 (pg/mL)	4325.1 ± 1628.2	9891.7 ± 4214.0	3947.0 ± 1709.8	9348.6 ± 4370.5	3617.8 ± 1819.9	8912.3 ± 3701.5
Serum P4 (ng/mL)	0.97 ± 0.4	3.00 ± 2.1	1.03 ± 0.4	2.87 ± 3.1	1.01 ± 0.4	2.84 ± 2.9
Retrieved oocytes (n)	11.2 ± 4.7	14.2 ± 7.1	9.9 ± 5.2	12.3 ± 6.3	8.0 ± 4.8	12.0 ± 6.3
Mature oocytes (n)	7.8 ± 4.4	10.3 ± 5.7	7.4 ± 4.7	9.0 ± 4.9	5.8 ± 4.1	8.9 ± 4.9
Fertilization rate (%)	69.4 ± 23.9	74.1 ± 19.3	77.4 ± 24.4	74.5 ± 17.8	77.0 ± 21.4	75.8 ± 18.2
Implantation rate (%)	26.7 (4/15)	38.1 (45/118)	30.7 (31/101)	30.5 (148/485)	16.2 (19/117)	28.2 (135/478)
Pregnancy rate (%)	26.7 (4/15)	33.9 (40/118)	23.8 (24/101)	27.0 (131/485)	13.7 (16/117)	23.6 (113/478)
p-value for pregnancy rate[a]	0.773		0.537		0.018[b]	

Patient age (y)	Fresh 40-42	Freeze all	Fresh 43≦	Freeze all
No. of cycles (n)	47	141	8	14
Antral follicle count (n)	6.1 ± 2.4	18.3 ± 8.2	—	19.7 ± 6.1
Days of stimulation (d)	12.8 ± 2.2	14.6 ± 2.1	14.4 ± 2.6	14.5 ± 1.6
Serum E2 (pg/mL)	3740.5 ± 2064.8	9822.0 ± 4156.0	2421.8 ± 1447.7	10 486.8 ± 3858.9
Serum P4 (ng/mL)	1.11 ± 0.4	3.19 ± 2.7	0.93 ± 0.4	3.77 ± 2.3
Retrieved oocytes (n)	6.9 ± 3.4	16.0 ± 8.7	3.9 ± 3.0	19.6 ± 9.2
Mature oocytes (n)	5.0 ± 3.2	12.8 ± 7.5	2.3 ± 1.9	16.0 ± 8.5
Fertilization rate (%)	73.0 ± 22.9	77.6 ± 14.4	83.9 ± 21.7	77.6 ± 13.1
Implantation rate (%)	8.5 (4/47)	24.3 (34/140)	14.3 (1/7)	7.1 (1/14)
Pregnancy rate (%)	8.5 (4/47)	19.3 (27/140)	14.3 (1/7)	7.1 (1/14)
p-value for pregnancy rate[a]	0.112		1.000	

Pregnancy rates with fresh embryo transfer and frozen-thawed embryo transfer by age category were assessed in cycles in which IVF embryos were transferred as single day 3 embryo in the IVF cycles or later endometrial preparation cycles at our IVF center, between 2008 and 2012 for fresh embryo transfer (for antral follicle count, between 2011 and 2012) or between 2013 and 2016 for frozen-thawed embryo transfer, except for the following cycles:

(1) cycles in which oocytes were retrieved only for oocyte cryopreservation.
(2) cycles in which oocytes were repeatedly retrieved or embryos derived from re-retrieved oocytes were transferred.
(3) cycles in which embryos were derived by delayed ICSI.
(4) cycles in which embryos derived by delayed ICSI were transferred.
(5) cycles in which refrozen pronuclear embryos or cleavage-stage embryos were thawed and transferred.
(6) cycles in which no serum P4 levels at oocyte retrieval existed.
Serum E2 and P4 levels were measured on the day of hCG injection.
[a]Comparisons of pregnancy rates between fresh embryo transfer and frozen-thawed embryo transfer by age category were assessed using Fisher's exact test.
[b]Statistically significant difference ($P < 0.05$).

pregnancy rates[14-16]; however, the compromised embryo quality may be attributed to the negative effects of P4 levels increased concomitantly with the increase in E2 levels on the endometrial environment before fresh embryo transfer, rather than the direct effects of E2 on embryo quality.

This study showed that the pregnancy rate with frozen-thawed embryo transfer was higher than that with fresh embryo transfer in patients aged 42 years or younger, suggesting that frozen-thawed embryo transfer provided the endometrial environment more suitable for embryo transfer by reversing the increased hormone levels in oocyte retrieval cycles and then readjusting the hormone levels in later embryo transfer cycles.

Thus, the freeze-all strategy that allowed the separation of embryo transfer cycles from oocyte retrieval cycles provided improved

pregnancy rates in women aged 42 years and younger. If many mature oocytes can be collected with no concerns for adverse impacts on embryo transfer, the number of embryo transfers per oocyte retrieval should be increased, leading to the improvement in cumulative pregnancy rates.

In conclusion, the freeze-all strategy will surely become increasingly important, with an expected rise in the number of women of advanced age who seek fertility treatment. On the other hand, conventional fresh embryo transfer in the same cycle of COS is thought to have no advantage in consideration of the treatment-induced maternal hormonal environment during implantation.

The post-thaw embryo survival rates with slow freezing and vitrification and pregnancy rates with frozen-thawed embryos transfer and fresh embryos transfer, described above, were not results obtained from a comparative study as it was performed through long-term experience of clinical treatment. Therefore, the conditions of treatment procedures (eg, culture) were not completely identical among the cycles. Nevertheless, as we believe these differences had negligible impacts on the post-thaw embryo survival rates and pregnancy rates, statistical analyses on these results have been performed. It should be noted, however, that it is not only the difference between frozen-thawed embryos transfer and fresh embryos transfer that may affect the result of these analyses in view of this point.

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DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. *Human rights statements and informed consent*: All the procedures were followed in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and with the principles of the Helsinki Declaration of 1964 and its later amendments. This study was approved by the institutional review board of Asada Ladies Clinic. This is a retrospective study in patients who submitted informed consent for undergoing fertility treatment at our IVF center. *Animal studies:* This article does not contain any study with animal participants that have been performed by any of the authors.

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REFERENCES

1. Blockeel C, Drakopoulos P, Santos-Ribeiro S, Polyzos NP, Tournaye H. A fresh look at the freeze-all protocol: a SWOT analysis. *Hum Reprod*. 2016;31(3):491-497.
2. MacDougall MJ, Tan Sl, Jacobs HS. In vitro fertilization and the ovarian hyperstimulation syndrome. *Hum Reprod*. 1992;7(5):597-600.
3. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C. Clinical rationale for cryopreservation of entire embryo cohorts in lieu of fresh transfer. *Fertil Steril*. 2014;102(1):3-9.
4. Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos frozen to-196 and-269 degrees C. *Science*. 1972;178(4059):411-414.
5. Wilmut I. The effect of cooling rate, warming rate, cryoprotective agent and stage of development of survival of mouse embryos during freezing and thawing. *Life Sci II*. 1972;11(22):1071-1079.
6. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 C by vitrification. *Nature*. 1985;313(6003):573-575.
7. Bosch E, Labarta E, Crespo J, et al. Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for in vitro fertilization: analysis of over 4000 cycles. *Hum Reprod*. 2010;25(8):2092-2100.
8. Roque M, Valle M, Guimarães F, Sampaio M, Geber S. Freeze-all policy: fresh vs. frozen-thawed embryo transfer. *Fertil Steril*. 2015;103(5):1190-1193.
9. Devroey P, Polyzos NP, Blockeel C. An OHSS-Free Clinic by segmentation of IVF treatment. *Hum Reprod*. 2011;26(10):2593-2597.
10. Papanikolaou EG, Humaidan P, Polyzos N, et al. New algorithm for OHSS prevention. *Reprod Biol Endocrinol*. 2011;9:147.
11. Ortega-Hrepich C, Stoop D, Guzman L, et al. A "freeze-all" embryo strategy after in vitro maturation: a novel approach in women with polycystic ovary syndrome? *Fertil Steril*. 2013;100(4):1002–1007.e1.
12. D'Angelo A. Ovarian hyperstimulation syndrome prevention strategies: cryopreservation of all embryos. *Semin Reprod Med*. 2010;28(6):513–518.
13. La Marca A, Broekmans FJ, Volpe A, Fauser BC, Macklon NS. Anti-Müllerian hormone (AMH): what do we still need to know? *Hum Reprod*. 2009;24(9):2264–2275.
14. Forman R, Belaisch-Allart J, Fries N, Hazout A, Testart J, Frydman R. Evidence for an adverse effect of elevated serum estradiol concentrations on embryo implantation. *Fertil Steril*. 1988;49(1):118–122.
15. Simón C, Cano F, Valbuena D, Remohí J, Pellicer A. Implantation: Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. *Hum Reprod*. 1995;10(9):2432–2437.
16. Pereira N, Elias RT, Christos PJ, et al. Supraphysiologic estradiol is an independent predictor of low birth weight in full-term singletons born after fresh embryo transfer. *Hum Reprod*. 2017;32(7):1410–1417.