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# Creation, effects on embryo quality, and clinical outcomes of a new embryo culture medium with 31 optimized components derived from human oviduct fluid: A prospective multicenter randomized trial

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## Abstract

**Purpose:** Our aim is to make an ideal embryo culture medium close to human oviduct fluid (HOF) components, and to evaluate the quality of this medium with embryo quality and clinical outcomes in assisted reproductive technology (ART) by a prospective randomized controlled trial (RCT).

**Methods:** Study I: HOF was collected laparoscopically from patients ($n$ = 28) with normal pelvic findings. According to HOF analysis results, the new medium "HiGROW OVIT®" (OVIT) was designed. Study II: Embryos (2 pronuclei (2PN) = 9633) were assigned from 1435 patients. The blastulation rate (BR), good BR (gBR), utilized (transferred/cryo-preserved) BR (uBR), pregnancy rate (PR), and miscarriage rate (MR) were compared between the OVIT and control groups by RCT.

**Results:** The novel medium 'OVIT' was produced according to 31 HOF components. The concentrations of essential amino acids (e-AAs) were lower in OVIT than in current media, yet the opposite was true for ne-AA concentrations. gBR and uBR were higher in the OVIT group than in the control group. In the older female group, gBT and uBR were significantly higher in the OVIT group.

**Conclusions:** The novel medium 'OVIT' was produced according to HOF data. The OVIT had significantly better embryo quality and clinical outcomes than the current media.

**KEYWORDS**
assisted reproductive technics, culture media, embryo, in vitro fertilization, oviduct

## 1 | INTRODUCTION

Historical evaluations of studies on in vitro fertilization (IVF) and embryo transfer (ET) continuously increased the number of successful pregnancies using assisted reproductive technology (ART).[1] Although culture conditions were recognized as key points for obtaining high-quality embryos and successful pregnancies,[1] an optimal culture medium formulation, which closely resembles natural human oviduct fluid (HOF), has not been obtained yet.

In the early 1970s, according to the theory of "back to nature," compositional analyses of oviducts and uterine fluids collected during laparotomy demonstrated the presence of albumin, globulin, inorganic salts, glucose,[2] and seven elements of inorganic salts.[3] Based on the composition of human tubal fluid (HTF), a modified version of Tyrode's solution,[4] the "HTF" medium was created.[5] Data on laparoscopically collected tubal and uterine fluids have shown that three components had variable concentrations throughout the tube and uterus, suggesting that the nutritious environment of the embryo may change according to the embryo growth phase.[6] Accordingly, "sequential media" was proposed.[7,8] Subsequent reports have shown that sequential blastocyst stage ET medium is advantageous compared with the current cleavage stage ET media.[9-12] However, the advantages of the former remain controversial.[13-17]

In contrast, according to the theory of the "let embryo choose," which states that an embryo may choose and receive the necessary nourishment from surrounding components, a "single-step medium" comprising 10 essential components selected by a computer-assisted algorithm[18] that was developed into the simplex optimization medium (SOM) and potassium-simplex optimization medium (KSOM) was designed.[19] A single-step medium does not need to be changed during embryo culture; accordingly, laboratory work, along with the stress of changing media on embryos, is reduced. Currently, clinical evaluations of sequential and single media[20-24] have shown similar efficacy rates between the aforementioned media; therefore, the choice of a medium is based on the internal preference and experience of facilities.

The knowledge of ART has progressed throughout the years; however, there are some concerns regarding the health of children conceived by ART, including major birth defects,[25] rare congenital disorders,[26] epigenetic alterations,[27,28] and delayed physical, psychomotor, and intellectual development.[29] Some studies[26,28] have suggested that culture conditions can affect such alterations.

This study aimed to investigate the possibility of producing an optimized culture medium in which constituents closely mimic the constituents of HOF, evaluate the clinical effectiveness of the novel medium of this study and quality of the related cultured human

embryos, and compare the clinical outcomes of this medium with those of the current commercial media.

## 2 | MATERIALS AND METHOD

### 2.1 | Study I

Study I attempted to design a sufficient embryo culture medium to resolve the concerns regarding ART by the Japan Society for Ova Research (JSOR). Study I aimed to collect and analyze HOFs between August 2006 and May 2010. All patients provided signed informed consent for participation in this study, and the study was approved by the institutional review boards of each center in which this study was conducted and JSOR (approval number: 2007915). Patients also provided consent for the use of an assay comprising human surplus embryos, which was approved by the ethical review board of the Japanese Institution for Standardizing Assisted Reproductive Technology (JISART) (approval number: 2012-05). The use of an assay using mouse embryos was approved by the ethics committees of the care and use of experimental animals at the Niigata University in Japan and Fuso Pharmaceutical Industries, Ltd., located in Osaka, Japan.

Three hospitals in JSOR and one research institute in a pharmaceutical manufacturing company participated in this study. All patients were between the ages of 26 and 41 years old and had regular menstruation cycles. The patients on whom laparoscopy was conducted were diagnosed with uterine diseases and infertility with unknown factors in three hospitals. HOF was collected from each patient using a silicone catheter (Kitazato Corporation, Shizuoka, Japan) for a "gamete intra-fallopian tube transfer" under laparoscopy. At the same time, the peritoneal fluid was collected as a reference for the oviduct fluid. Those samples were analyzed individually, and the average and standard deviation (SD) were calculated separately for HOF from ovulation to day 2 (midcycle) and from day 3 to day 12 (luteal phase). The average and SD of all the collection periods (midcycle to luteal phase; day 0 to 12) were also calculated for HOF and peritoneal fluid. The menstrual phase of each patient was determined by pelvic ultrasound examinations, hormonal evaluations, and basal body temperature. Any samples contaminated with blood were excluded from analysis. Patients with endometriosis, severe pelvic adhesion, ovarian tumor, or chlamydiosis were also excluded. Laparoscopy was performed under insufflation with $CO_2$ and/or $N_2O$ in the abdominal cavity. Related side effects on HOF components were assessed using Liquid Chromatography (LC)-Mass Spectrometry (MS)/MS and ion chromatography (IC). No side effects were recognized. Collected HOF samples were stored at −80°C until the analysis at the research institute of Fuso Pharmaceutical Industries, Ltd.

Twenty-one amino acids (AAs) were derivatized using an AccQ Tag Ultra derivatization kit (Waters) and analyzed using the LC-MS/MS system, comprising the Acquity Ultra Performance (UP) LC system (Waters), combined with the ACQUITY UPLC Ethylene Bridged Hybrid (BEH) C18 column (Waters) and 3200 Quadropule Ion Trap (QTRAP) system (AB Sciex). Six inorganic salts and three organic acids were analyzed using the IC system (ICS-2000; Dionex), with IonPac AS18 column (Dionex) for anions and organic acids and IonPac CS16 column (Dionex) for cations. Carbohydrates were analyzed on the LC-MS/MS system, comprising the Acquity UPLC system (Waters), combined with the ACQUITY UPLC BEH Amide column (Waters) and 3200 QTRAP system (AB Sciex).

We estimated the osmolarity of HOF from the concentrations of the 31 components we measured (Table 1). To adjust osmolarity to a level suitable for embryo development in vitro (about 250–300 mOsm/kg),[30,31] prototype media with osmolarity of 285 mOsm/kg were prepared by multiplying the concentration of each component we measured by 0.87.[32] Subsequently, the concentrations of energy substrates and inorganic salts were examined. These culture media were examined using mouse embryo assays, and important components, including potassium and phosphate, were examined by response surface methodology, which is an experiment designed to efficiently examine the optimal concentration of each component, including interaction between components in mouse embryos.[1,33] Then, HiGROW OVIT® medium (OVIT) with an osmolarity of 265 mOsm/kg was developed with lower concentrations of glucose, sodium, potassium, and phosphate than the prototype media and was compared the differences of components from CSCM (Irvine Scientific).

### 2.2 | Study II

In study II, the efficacy of the novel one-step medium "OVIT" was assessed using a prospective multicenter randomized clinical trial that was prospectively registered on September 2017 at the Japanese Trial Registry (UMIN 000033115) after obtaining protocol approval from the institutional ethical review boards of the Yokota Maternity Hospital, Gunnma; Sendai ART Clinic, Miyagi; Tawara IVF Clinic, Sizuoka; Ork Sumiyoshi Sanfujinka, Osaka; and St. Luke Clinic, Oita, which were all located in Japan. We did not change the eligibility criteria, methods, or measured outcomes following study initiation.

All ART procedures were previously described[34]; the corresponding procedural methods were minimally arranged according to individual center protocols as follows: oocyte collection was performed after controlled ovarian stimulation with urinary and/or recombinant human menopausal gonadotropin (HMG)/follicular stimulating hormone (FSH) under gonadotropin suppression by a gonadotropin-releasing hormone (Gn-RH) antagonist or Gn-RH agonist. Luteinizing hormone (LH) surges were induced using a Gn-RH agonist and/or a human chorionic gonadotropin (HCG) injection 36 h before oocyte collection. The embryos introduced by intracytoplasmic sperm injection (ICSI) from surgically collected sperm were excluded from this procedure. ET had a distribution ratio of 28.9% for fresh cycles and 71.1% for frozen-thawed cycles and was performed by a single-embryo transfer. Surplus embryos remaining from ET and freeze-all

**TABLE 1** The concentrations of inorganic salts, energy substrates, and amino acids in human oviduct fluid

| | | Human oviduct fluid | | | | | Human oviduct fluid | | Human peritoneal fluid[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Midcycle (Day 0[a]–2, $n = 21$) | | Luteal phase (Day 3–12, $n = 7$) | | | Midcycle to luteal phase (Day 0–12, $n = 28$) | | | | |
| | | Average (μM) | SD | Average (μM) | SD | $p^b$ | Average (μM)[c] | SD | Average (μM) | SD | $p^e$ |
| Inorganic salts | Sodium | 156137 | 25588 | 153223 | 11644 | 0.778 | 155352 | 22487 | 145139 | 6640 | 0.106 |
| | Potassium | 15832 | 7369 | 13723 | 2789 | 0.692 | 15264 | 6471 | 3425 | 422 | <0.001 |
| | Calcium | 1331 | 551 | 1133 | 228 | 0.651 | 1278 | 489 | 932 | 230 | <0.001 |
| | Magnesium | 510 | 272 | 599 | 143 | 0.094 | 534 | 245 | 338 | 144 | <0.001 |
| | Chlorine | 135966 | 24405 | 137405 | 14942 | 0.533 | 136339 | 22072 | 122113 | 6357 | 0.003 |
| | Phosphate | 2788 | 2137 | 1677 | 522 | 0.219 | 2500 | 1909 | 543 | 145 | <0.001 |
| Carbohydrate and organic acids | Glucose | 3429 | 1606 | 6159 | 1676 | 0.001 | 4164 | 2014 | 5246 | 890 | 0.004 |
| | Pyruvate | 209 | 161 | 106 | 62 | 0.019 | 182 | 148 | 75 | 16 | <0.001 |
| | Lactate | 5193 | 3052 | 3136 | 548 | 0.005 | 4660 | 2779 | 959 | 338 | <0.001 |
| | Citrate | 134 | 46 | 178 | 50 | 0.031 | 146 | 50 | 68 | 23 | <0.001 |
| Essential amino acids (e-AAs) | Valine | 115 | 65 | 149 | 49 | 0.208 | 124 | 63 | 132 | 28 | 0.248 |
| | Leucine | 92 | 62 | 95 | 28 | 0.348 | 93 | 55 | 72 | 17 | 0.367 |
| | Isoleucine | 40 | 28 | 42 | 15 | 0.348 | 41 | 25 | 31 | 8 | 0.376 |
| | Lysine | 215 | 143 | 160 | 59 | 0.730 | 201 | 129 | 118 | 21 | 0.005 |
| | Threonine | 126 | 70 | 123 | 50 | 0.937 | 125 | 65 | 92 | 23 | 0.060 |
| | Methionine | 27 | 19 | 21 | 4 | 0.874 | 25 | 17 | 17 | 3 | 0.017 |
| | Histidine | 58 | 35 | 67 | 31 | 0.439 | 61 | 34 | 60 | 15 | 0.400 |
| | Phenylalanine | 50 | 32 | 55 | 15 | 0.296 | 51 | 28 | 41 | 10 | 0.321 |
| | Tryptophan | 18 | 11 | 29 | 7 | 0.023 | 21 | 11 | 30 | 7 | 0.001 |
| | Arginine | 133 | 96 | 93 | 36 | 0.568 | 123 | 86 | 53 | 11 | <0.001 |
| | Cystine | 58 | 59 | 45 | 34 | 0.836 | 55 | 53 | 46 | 20 | 0.436 |
| | Tyrosine | 54 | 35 | 58 | 21 | 0.435 | 55 | 31 | 43 | 10 | 0.251 |
| | Glutamine | 426 | 173 | 542 | 116 | 0.036 | 455 | 167 | 504 | 100 | 0.067 |

TABLE 1 (Continued)

| | | Human oviduct fluid | | | | | Human oviduct fluid | | Human peritoneal fluid[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Midcycle (Day 0[a]–2, $n = 21$) | | Luteal phase (Day 3–12, $n = 7$) | | | Midcycle to luteal phase (Day 0–12, $n = 28$) | | | | |
| | | Average (μM) | SD | Average (μM) | SD | $p$[b] | Average (μM)[c] | SD | Average (μM) | SD | $p$[e] |
| Nonessential amino acids (ne-AAs) | Glycine | 1114 | 643 | 1137 | 570 | 0.750 | 1120 | 615 | 211 | 62 | <0.001 |
| | Alanine | 335 | 207 | 351 | 124 | 0.411 | 339 | 188 | 249 | 52 | 0.043 |
| | Glutamic acid | 670 | 454 | 508 | 183 | 0.678 | 630 | 407 | 16 | 5 | <0.001 |
| | Aspartic acid | 142 | 93 | 124 | 51 | 0.959 | 137 | 84 | 2 | 1 | <0.001 |
| | Asparagine | 16 | 6 | 23 | 16 | 0.405 | 18 | 10 | 45 | 11 | <0.001 |
| | Proline | 123 | 76 | 111 | 43 | 0.959 | 120 | 68 | 102 | 27 | 0.781 |
| | Serine | 196 | 123 | 216 | 144 | 0.640 | 201 | 126 | 95 | 24 | <0.001 |
| Other amino acid | Taurine | 1732 | 961 | 1265 | 522 | 0.326 | 1615 | 887 | 29 | 6 | <0.001 |
| Collection volume (μl) | | 6.8 | 3.8 | 6.1 | 4.7 | 0.425 | 6.6 | 4.0 | >1000 | – | – |
| Estimated osmolarity (mOsm/kg)[f] | | >324 | | >320 | | – | >323 | | >281 | | – |

[a]Day 0 is ovulation date.

[b]Midcycle versus Luteal phase (Wilcoxon rank sum test).

[c]Prototype media based on HOF were prepared by multiplying the concentration of each component we measured by 0.87.[32]

[d]Peritoneal fluid samples were analyzed to check on any contamination of peritoneal fluid into the oviduct fluid.

[e]Human oviduct fluid vs human peritoneal fluid (Wilcoxon rank sum test).

[f](Total analyte conc. (mM) + 25 (bicarbonate conc., mM)) × 0.92 (Osmotic coefficient[32]).

embryos to avoid ovarian hyperstimulation syndrome were frozen using vitrifaction method. In the next cycle, cryo-preserved embryos were thawed, and one embryo was transferred after preparing the endometrium using hormone replacement therapy (96.3%) or ovulation induction cycles (3.7%). Clinicians were unaware of the distribution of samples to the OVIT and control groups.

Normally fertilized oocytes with recognized two pronuclei (2PN) were equally distributed into the OVIT and control groups, indicating that, from every 2PN, one 2PN went to the OVIT droplet, and another went to the control medium droplet in different areas of the same incubating plate using the sealed envelope method. The control group medium was indicated to be CSC (Irvine Scientific Inc.) in two centers, QACM and QABM (Cooper Surgical Company) in two centers, and G-TL (Vitrolife AB) in one center.

In contrast to culture media, culture environments were similar between patients in both groups. Embryo cultures were prepared according to the instruction of manufacturers. Each embryo was individually cultured in one 30 μl droplet covered by mineral oil in gas environments of 5% $O_2$, 6% $CO_2$, and 89% $N_2$ in three centers and 5% $O_2$, 5% $CO_2$, and 90% $N_2$ in two centers. Cultures were checked microscopically by embryologists, that is, assessors, on days 1, 3, and 5 or 6 after fertilization. Embryos were cultured until the blastocyst stage and evaluated according to Gardner's classification.[35] Assessors were unaware of the randomization of embryos.

The blastulation rate (BR: blastocyst/2PN), good quality blastocyst rate (gBR: embryos with 3BB (Gardner's classification) or more/2PN), and utilized blastocyst rate (uBR: ET + cryopreservation/2PN) were compared between both groups.

The pregnancy rate (PR: recognition of gestational sac (GS) by ultrasonography/ET) and miscarriage rate (MR: number of miscarriage until 12 gestational weeks/GS) were compared between both groups.

The comparison of embryo quality between older patients (≥38) (40.3 ± 1.94: mean age ± SD) and younger patients (<38) (32.9 ± 3.08) was also a focus of this study. BR, gBR, uBR, PR, and MR were re-evaluated in older patients and younger patients.

### 2.3 | Sample size calculation

Sample size was calculated according to a difference in our data of usual BR of 50% in the control group compared 53% which we assumed in OVIT group. To achieve a power of 80% at a two-tailed significance level of 5%, 8712 blastocyst embryos were deemed to be required according to the chi-squared test (4,356 embryos in each group).

### 2.4 | Statistical analysis

In study I, statistical analysis was performed using the Wilcoxon rank sum test, whereas in study II, all data from the five participating centers were reported to the data analysis center of JSOR. Statistical analysis was performed using Statcel: The Useful Addin Forms on Excel, 3rd edition (OMS Publishing Ltd.). Categorical variables were analyzed using the chi-squared test. A $p$-value <0.05 was considered statistically significant.

## 3 | RESULTS

### 3.1 | Study I

Given that the aspirated HOF volume was very small, that is, approximately 6 μL, analytical methods for small samples were developed (Table 1, Table 2). Less turbid and clear samples collected during the midcycle and luteal phases (21 and 7 samples, respectively) were analyzed. The results showed a significant increase in glucose concentration and a significant decrease in pyruvate and lactate concentration from the midcycle to the luteal phase, although there were no significant differences in the other components, except for tryptophan and glutamine (Table 1). However, the concentrations of 20 out of 31 components in the HOF were significantly different from those in peritoneal fluid. In particular, the concentrations of potassium, phosphate, lactate, and glycine were about 5 times higher, and those of glutamic acid, aspartic acid, and taurine were about 40–70 times higher in HOF than in the peritoneal fluid (Table 1). Furthermore, the concentrations of the conventional commercial media did not coincide with the HOF due to the following reasons. Both essential AA (e-AA) and nonessential AA (ne-AA) components and concentrations were found in HOF. E-AAs had a concentration of 18–215 μM, while ne-AAs had a concentration of 16–1137 μM in HOF. Asparagine showed the lowest concentration (16 μM), while glycine showed the highest concentration (1137 μM). Additionally, high concentrations of taurine (1732 μM) were detected. The concentrations of other components were comparable to those of conventional media, except for potassium and phosphate. As for the ratio of OVIT components to current media components (Table 2), e-AA concentrations in HOF were approximately half of that in current media, except for isoleucine, cystine, and tryptophan. Ne-AA levels were up to 16.9 times higher in OVIT than in current media, especially glycine.

Finally, OVIT was designed (Table 2) according to HOF data and was commercialized in June 2017. Both prefilled human serum albumin (HSA) and HSA-free OVITs were commercialized, although no growth factor was contained.

Study II (Figure 1, Table 3).

Between July 2018 and June 2020, 11 984 cumulus-oocyte complex samples were obtained from 1435 patients all aged under 43 years. One day after IVF fertilization and/or ICSI, 2351 embryos were deemed ineligible to be included in the study due to abnormal microscopic findings. Consequently, the total number of eligible 2PN embryos was 9633, distributed into two groups, 4772 and 4814 in the OVIT and control groups, respectively (Figure 1).

Embryo quality was evaluated according to BR, gBR, and uBR (Table 3). The overall results of gBR and uBR were higher in the OVIT

**TABLE 2** Amino acid concentrations in culture media

| Supplier | | Human oviductal fluid | Somatic cell culture medium | Sequential step media | | Single step media | Ratio** | |
|---|---|---|---|---|---|---|---|---|
| | | Fuso | | SAGE | | Irvine | | |
| Brands of Medium | | OVIT | MEM[a] | QACM[b] | QABM[b] | CSC[b] | OVIT/QABM[b] | OVIT/CSC[b] |
| Essential amino acids (e-AAs) | Valine | 108 | 400 | 0 | 224 | 215 | 0.5 | 0.5 |
| | Leucine | 81 | 400 | 0 | 227 | 214 | 0.4 | 0.4 |
| | Isoleucine | 36 | 400 | 0 | 209 | 202 | 0.2 | 0.2 |
| | Lysine | 176 | 400 | 0 | 223 | 223 | 0.8 | 0.8 |
| | Threonine | 109 | 400 | 0 | 210 | 195 | 0.5 | 0.6 |
| | Methionine | 22 | 100 | 0 | 56 | 53 | 0.4 | 0.4 |
| | Histidine | 53 | 200 | 0 | 102 | 105 | 0.5 | 0.5 |
| | Phenylalanine | 45 | 200 | 0 | 106 | 106 | 0.4 | 0.4 |
| | Tryptophan | 18 | 50 | 0 | 28 | 26 | 0.6 | 0.7 |
| | Arginine | 108 | 600 | 0 | 313 | 281 | 0.3 | 0.4 |
| | Cysteine | 48 | 100 | 0 | 54 | 46 | 0.9 | 1.0 |
| | Tyrosine | 48 | 200 | 0 | 100 | 95 | 0.5 | 0.5 |
| Nonessential amino acids (ne-AAs) | Glutamine | 358* | 2000 | 0 | 0 | N.D. | N.D. | N.D. |
| | Glycine | 979 | 100 | 119 | 131 | 58 | 7.5 | 16.9 |
| | Alanine | 297 | 100 | 0 | 0 | 62 | N.D. | 4.8 |
| | Glutamic Acid | 550 | 100 | 0 | 0 | 46 | N.D. | 12.0 |
| | Aspartic Acid | 120 | 100 | 93 | 104 | 47 | 1.2 | 2.6 |
| | Asparagine | 15 | 100 | 112 | 124 | 57 | 0.1 | 0.3 |
| | Proline | 105 | 100 | 93 | 103 | 48 | 1.0 | 2.2 |
| | Serine | 176 | 100 | 107 | 123 | 55 | 1.4 | 3.2 |
| Other | Taurine | 1412 | 0 | 122 | 120 | 0 | 11.8 | N.D. |

*Note:* Values are presented in μM.MEM, minimum essential medium.*Contains glutamine derivative instead of glutamine. ** The concentrations of A· A in OVIT were compared with medium QABM[b,] and CSC[b]. [a]Eagle (1959).[60] [b]Morbeck et al. (2014).[61]

Abbreviations: CSC, continuous single culture; N.D., not described; QABM, Quinn's Advantage Blastocyst Medium; QACM, Quinn's Advantage Cleavage Medium.

group (20.1%, 47.0%) than in the control group of gBR (18.3%) and uBR (42.2%) ($p < 0.03$, $p < 0.001$).

Clinically, a single embryo transfer was performed under fresh and/or frozen-thawed ET cycles. The PR and MR of the OVIT (30.0% and 24.9%, respectively) and control groups (30.0% and 24.1%, respectively) were the same. Table 3 shows the results of each age group. In the under 38 years group, BR, gBR, PR, and MR were not significantly different between the OVIT and the control groups, yet uBR was significantly higher in the OVIT group (49.3%) than in the control group (45.4%) ($p < 0.003$). In the older age group, gBR and uBR were significantly higher in the OVIT group (14.6%, 43.3%) than in the control group (12.0%, 36.9%) ($p < 0.02$, $p < 0.001$), respectively. PR and MR were the same between both groups.

## 4 | DISCUSSION

Our study has demonstrated that, among the concentrations of the 31 components in HOF (Table 1), there was an increase in the concentration of glucose and a decrease in the concentrations of pyruvate and lactate in human oviductal fluid from midcycle to luteal phase. This phenomenon is similar to the results of Gardner et al.,[6] and is thought to be due to the utilization of glucose for ciliary movement and ovulatory activity in the oviduct, and the conversion of glucose to lactate by the cumulus cells. In addition, some of ne-AAs (glycine, alanine, and glutamic acid), glutamine, and taurine were detected in HOF at higher concentrations than in the peritoneal fluid. These amino acids are thought to be commonly abundant in the female reproductive tracts of mammals, since it has been reported that these amino acids are present in higher concentrations in various kinds of oviductal and uterine fluids compared to body fluids,[36-43] despite differences in animal species and collection time. These amino acid concentrations were also different between OVIT and current commercial media (Table 2). These ne-AAs, as well as glutamine and taurine, play a role in supporting cellular homeostasis, including osmotic and pH control,[43-45] and have been reported to promote embryo development in vitro.[46,47] These amino acids may have contributed to the fact that OVIT significantly increased

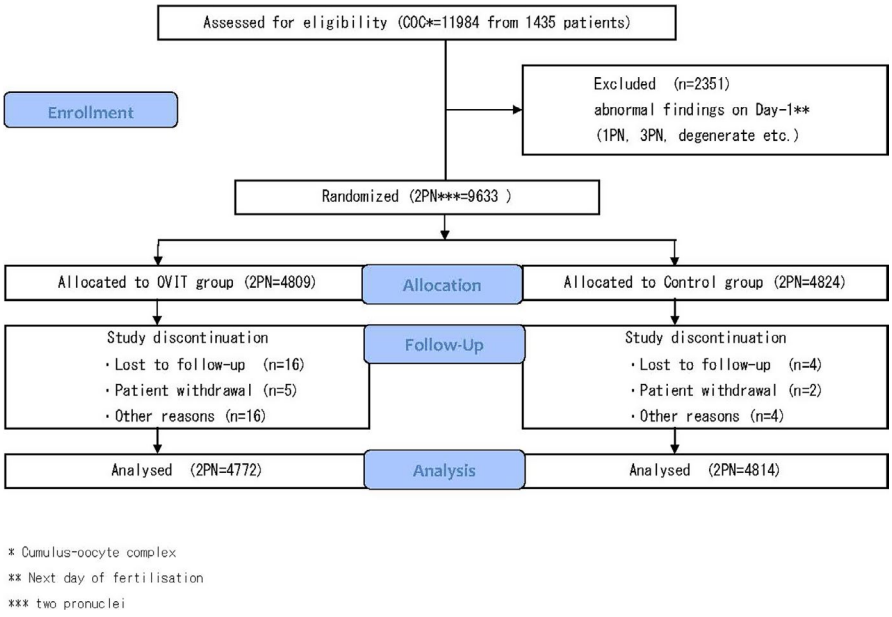


**FIGURE 1** Flow Diagram. *Cumulus-oocyte complex. **Next day of fertilization. ***Two pronuclei

**TABLE 3** Comparison of the overall and clinical outcomes, including the good blastocyst rate, utilized blastocyst rate, and pregnancy and miscarriage rates per embryo transfer cycles, between the old (≧38 years) and young (<38 years) age groups and OVIT and control groups

| | Overall result | | | Maternal age <38 | | | Maternal age ≧38 | | |
|---|---|---|---|---|---|---|---|---|---|
| | OVIT group | Control group | *p*-value | OVIT group | Control group | *p*-value | OVIT group | Control group | *p*-value |
| Total number (N.) of 2PN | 4772 | 4814 | | 2968 | 2970 | | 1804 | 1844 | |
| N. of blastocyst | 2604 | 2533 | | 1720 | 1678 | | 884 | 855 | |
| (per 2PN)(BR) | 54.6% | 52.6% | 0.055 | 58.0% | 56.5% | 0.258 | 49.0% | 46.4% | 0.111 |
| N. of good blastocyst | 957 | 883 | | 693 | 661 | | 264 | 222 | |
| (per 2PN)(gBR) | 20.1% | 18.3% | 0.033 | 23.3% | 22.3% | 0.315 | 14.6% | 12.0% | 0.021 |
| N. of utilized blastocyst | 2244 | 2030 | | 1462 | 1349 | | 782 | 681 | |
| (per 2PN)(uBR) | 47.0% | 42.2% | <0.001 | 49.3% | 45.4% | 0.003 | 43.3% | 36.9% | <0.001 |
| Total number (N). of ET | 1175[a] | 1049[b] | | 679 | 601 | | 497 | 448 | |
| N. of pregnancies[c] | 353 | 315 | | 275 | 245 | | 78 | 70 | |
| (per ET)(PR) | 30.0% | 30.0% | 0.994 | 40.5% | 40.8% | 0.923 | 15.7% | 15.6% | 0.977 |
| N. of miscarriages[d] | 88 | 76 | | 64 | 57 | | 24 | 19 | |
| (per pregnancy) (MR) | 24.9% | 24.1% | 0.810 | 23.3% | 23.3% | 0.998 | 30.8% | 27.1% | 0.628 |

*Note:* The chi-squared test was used to calculate categorical variables.

Abbreviations: 2 PN, 2 pronuclei; ET, embryo transfer.

[a]Three cases of embryo transfer with unknown prognoses.

[b]Nine cases of ET with unknown prognoses.

[c]Pregnancies were diagnosed with ultrasonography.

[d]Number of miscarriages until 12 weeks of gestation.

the developmental rate of human embryos compared to the control medium in this study (Table 3). Several revolutionary findings in the field of ART have affected decades of research and increased the number of positive outcomes. However, the goal of ART is not only to achieve successful pregnancy but to ensure a healthy and thriving child. Accordingly, culture condition problems have been

receiving increasing attention. A report has argued that an ideal culture should be similar to the environment of a human oviduct.[48] Quinn et al. have presented their "HTF" medium based on the data of a human tubal fluid.[5] However, their data included only 10 components. Gardner et al. have examined three components in tubal and uterine fluid.[6] The components analyzed in these studies were seemingly insufficient for evaluating the whole composition and concentration variations of natural HOF. To design the new single step medium, each components and concentrations of HOF during from midcycle to luteal phase were needed. Our study initially aimed to assess HOF using modern analytic apparatus and techniques to analyze 31 components. The estimated osmolality of HOF is more than 320 mOsm/kg, which is comparable to that of the oviduct fluid from cattle (295–332 mOsm/kg) and pigs (318 mOsm/kg).[49,50] But it may not reflect the actual osmolarity because there are a variety of components in HOF that are not included for our calculations of osmolarity, and the HOF may have evaporated between collection to analysis due to low collection volume. The concentration of bicarbonate in the HOF, one of the most abundant solutes, is unknown (20 mM in human hydrosalpinx fluid[51]), but bicarbonate concentrations in other animal species (35–90 mM)[52,53] are higher than those we used for our calculations (25 mM). So, the actual osmolarity may be higher than our estimated value. In any case, the osmolarity of the culture media was set to 250–300 mOsm/kg, because it has been reported to be suitable for embryo culture in vitro,[31] and there is a risk of increased osmolarity during the preparation of culture drops and culture process.[54,55] Our new data enabled us to determine an ideal design for the culture medium. Additionally, the clinical effectiveness of the novel medium of this study on embryo quality was evaluated by performing a prospective multicenter randomized clinical trial that demonstrated excellent results.

Originally, cell culture media were investigated for mammalian somatic cells, that is, ventricular heart beats.[56] Following the study of Ringer, several types of modified media were developed, such as the Krebs-Ringer-Bicarbonate medium,[57] Earle's medium,[58] and Ham's F-10.[59] Using HeLa cells and mouse L cells, Eagle has presented[60] the minimum essential medium (MEM), which packed AAs and vitamins in an easy-to-use form. Accordingly, most current media may have been based on MEM data (Table 2). Thus, historically, current culture medium compositions may have been based on the knowledge of mammalian somatic cells and cancer cells. There are a few media which components and concentrations were opened, however, fortunately, we could obtain the information of them in the papers (Eagle et al, Morbeck et al).[60,61] Therefore, component and concentration of the media which we used in OVIT group and control groups (CSC, QACM, QABM, and MEM) in our study were able to be compared (Table 2). In our study, various OVIT to current media ratios of different AAs have been reported (Table 2). E-AA concentrations in QABM and CSC were half of those in MEM; e-AAs demonstrated an OVIT to current media ratio of 0.2–1.0, indicating very low e-AA concentrations in OVIT. In contrast, concentrations of ne-AAs, except for glutamine, in single step media were half of those in MEM. The ratio of ne-AAs of OVIT to current media shows from 0.1 of asparagine to 16.9 of glycine. Culture medium composition balances should mimic that of natural HOF. Morbeck[61] found that culture media widely vary in compositions, suggesting that blastocyst development was dependent on culture media and protein presence.

Originally, culture conditions for embryos should be as close to the inside of a natural human oviduct atmosphere as possible.[1] Various differences have been demonstrated between children conceived by ART with different media.[62-67] Whether the current culture media compositions and concentrations are suitable and comfortable, and create a stress-free environment for embryo development requires careful assessment to address the essential physical and mental health of children conceived by ART.

The effectiveness of OVIT for ART was evaluated using a multicenter randomized clinical trial (Table 3). This study has shown that gBR and uBR were higher in the OVIT group than in the control group. However, there were no differences in BR, PR, and MR between the OVIT and control groups. gBR and uBR were significantly higher in the OVIT group than in the control group in old patients, yet only uBR was higher in the OVIT group than in the control group in young patients.

As countless factors affect pregnancy and miscarriage episodes, gBR and uBR may better reflect culture medium quality than PR and MR. Our study found that, in the older age group, embryo quality was significantly better in the OVIT group than in the control group (Table 3). In the clinical phase, we usually observe poor embryo quality in the older age group, indicating that, in patients with specific critical factors affecting fertility, medium quality should be considered. Our results indicate that cases with traditionally poor prognoses and particular fertility difficulties may better respond to OVIT than traditional media due to the physiologic status of the former being closer to the physiologic status of HOF than that of the latter.

While many embryos seemingly have good quality microscopically, the health of children conceived by ART must be considered and not PR and delivery rates only. Harper warns, "if we aim to change the design of culture media, we must carefully assess the risk of those changes, as well as the potential benefits".[68]

This study has several limitations. The condition of embryo cultures in ART fundamentally resembles the conditions of HOF. Although data were obtained from HOF, fluids were collected from infertile patients and not from completely healthy, fertile women. Additionally, to fulfil culture functions, unnatural substances, such as ethylenediaminetetraacetic acid, HSA, antibiotics, and altered salt concentrations, are needed. Therefore, compromises in medium composition should be made, and we are currently unable to produce a fully natural medium while simultaneously still reflecting the specific needs of embryos. Although ER, gER, and uER were better in the OVIT group than in the control groups, PR and ongoing PR were the same between both groups, suggesting that embryo quality may reflect culture conditions more sensitively than clinical results, which may be compromised by other factors. ART quality should be finally assessed by the health of children born by ART.

In conclusion, HOF components and AAs concentrations were analyzed, and the results indicated numerous differences between OVIT and current media, encouraging us to continue the development of the novel culture medium "OVIT." This medium provides a better culture condition and improves embryo quality compared with conventional media. Particularly, this study has found novel perspectives for older patients having difficulties in producing embryos of good quality and indicated the superior quality of OVIT compared with the quality of traditional media.

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## CONFLICT OF INTEREST

Takafumi Utsunomiya has nothing to disclose. The remaining authors have nothing to disclose.

## ETHICAL APPROVAL

This study was approved by the local ethics committee of St. Luke clinic (No.28).

## HUMAN/ANIMAL RIGHTS

The data were anonymized, and the requirement for informed consent was therefore waived.

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