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Original Article

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Does Embryonic Culture Environment Affect Ploidy Rates in ART Cycles: A Single Center Study in UK

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Abstract

Background: The purpose of the current study was to assess whether embryonic culture conditions has an impact on embryo ploidy in a preimplantation genetic testing for aneuploidy (PGT-A) cycle.

Methods: In this retrospective single center cohort study, a total of 1099 blastocysts from 278 PGT-A cycles were analyzed. The generated blastocysts were biopsied on days 5 and 6. Inseminated oocytes were allocated in different incubators (benchtop and time lapse) and assisted zona hatching was performed on day 3 of embryo development to facilitate the biopsy process which was performed on days 5 and 6 (blastocyst stage).

Results: The average age across the groups was 38.7±3.6 years and the total number of mature eggs was 2912 which were randomly distributed across both incubators. The euploidy rate obtained from both groups showed a higher proportion of euploid embryos in the TLM incubator (37.03%, 95% CI 31.9-42.1) compared to those cultured in the BT incubator (30.4%, 95% CI 23.1-37.7). Regression analysis showed that female age remains to be the key variable driving euploidy rates (0.85, 95% CI 0.82-0.88) although incubator type could be an important covariable (0.54, 95% CI 0.45-0.59). A subgroup analysis of 74 single euploid embryo transfers showed comparable pregnancy and live birth rates.

Conclusion: This large cohort study demonstrates that uninterrupted controlled culture environment provides increased probability to develop euploid embryo in a PGT-A cycle. However, further evaluation is required to assess how environmental culture conditions at a cellular level could affect epigenetic mechanisms in embryo development and higher aneuploidy rate.

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Introduction

Based on the statistics of United Kingdom's Human Fertilisation and Embryology Authority (HFEA), one in six couples require assisted reproductive technology (ART) in the UK. Amongst these couples, over half on average have experienced repeated failed cycles, as reproductive success is dependent on age. Maximizing the reproductive outcome has been the primary aim of ART cycles. Interventions such as preimplantation genetic testing for aneuploidy (PGT-A) have been introduced to optimize outcomes and decrease the time interval to delivery. Traditionally, selecting a morphologically viable embryo for transfer is one of the most challenging aspects in an IVF cycle (1). However, conventional morphological assessment of preimplantation embryos is the gold standard amongst embryologists in spite of its subjective nature (1). Some fertility clinics have an internal system in place to minimize variations in grades amongst their prac-

titioners and in some cases are also involved in an external system to minimize inter-laboratory variation (1).

Most aneuploidies are meiotic in origin and known to be associated with maternal age. Other chromosome abnormalities occur post activation, during the resumption of meiosis II and subsequent mitoses, leading to complex abnormalities, polyploidy, and mosaicism in these embryos (2). These aneuploidies have a poor correlation to embryo development and subsequent blastocyst formation can still be achieved (3). With PGT-A, the ploidy status of embryo can be known, thus facilitating the selection of an euploid embryo in an IVF cycle. Screening techniques such as array-based comparative genetic hybridization (aCGH) or next generation sequencing (NGS) have been favorable for trophectoderm biopsy which allow extended culture of embryos to the blastocysts stage (3, 4).

However, experimental evidence and clinical data suggest that a number of treatment related factors may affect the incidence of chromosomal imbalance in embryos with exclusion of age (3, 5-7). Factors such as culture conditions, gamete manipulation, high oxygen tension during culture, immaturity, and post maturity of oocytes at the point of fertilization (3, 5, 7, 8) can possibly induce gene expression and chromosomal abnormalities. Some studies have also shown that ovulation induction protocol used for standard IVF may increase the frequency of meiotic errors in eggs, thus secondarily increasing aneuploidy in the resulting embryos (3, 9, 10).

Therefore, laboratory culture conditions affect the incidence of chromosomal imbalances in embryos across different centers (5). Some studies have shown a significant increase in clinical pregnancy rate (CPR) and live birth rate (LBR) from embryos cultured in time lapse monitoring (TLM) incubators compared to a standard incubator (7, 11, 12).

Our hypothesis is that uninterrupted culture condition does not affect euploidy rate. In this study, the ploidy rate in embryos cultured in an uninterrupted closed system such as TLM incubator versus interrupted benchtop incubator was retrospectively compared. This study, to our knowledge, is the first to compare embryo ploidy status in a single center laboratory where there is no variation in gamete manipulation and culture conditions, except the type of incubator used.

Methods

Study design and participants: In this retrospective single center study, 148 patients were recruited between December 2018 to September 2019 at the Centre for Reproductive and Genetic Health, London, UK. The clinical variables for patient inclusion were PGT-A cycle, age, history of at least two failed previous IVF attempts, advanced maternal age, recurrent miscarriage, and previous aneuploidy or severe male factor infertility. A total of 3588 eggs were collected, 2912 metaphase II eggs were inseminated with intracytoplasmic sperm injection (ICSI)/intracytoplasmic morphologically selected sperm injection (IMSI), and blastocysts were distributed across the incubators (Benchtop; n=373, TLM; n=726). Ethics approval was obtained by the internal research committee (IRB) for this study and all procedures have been previously validated and practiced in accordance to licence from the HFEA.

Transvaginal oocyte retrieval was performed 37 hr after human chorionic gonadotropin (HCG) injection (10,000 *IU*) or agonist trigger (buserelin 0.5 *ml*/1 *ml*, Suprefact®). Cumulus oocyte complex was separated from the follicular fluid and cultured in 1 *ml* of fertilization media (Sage; CooperSurgical, Denmark and ORIGIO; CooperSurgical, Denmark) in a controlled humidified em-cell chamber of 37.5℃ and 6% CO_2. All oocytes collected were subsequently cultured in a pre-equilibrated 5 well dish containing fertilization media (Sage; CooperSurgical, Denmark and ORIGIO; CooperSurgical, Denmark) with oil (Vitrolife, Denmark).

Oocytes denudation was performed 3-4 *hr* post egg collection, initially using a 130 *µm* pasteur pipette with Cumulase media to remove the cumulus oocyte complex. Further denudation was achieved using a 140 *µm* sterile pipette (Swemed Bio, India). Denuded oocytes were separated based on stages of maturation (MII, MI, and GV). The oocytes reaching metaphase II stage were subjected to intracytoplasmic sperm injection (ICSI) or intracytoplasmic morphologically selected sperm injection (IMSI) 4-5 *hr* after egg collection (40 to 41 *hr* post HCG).

After ICSI or IMSI, oocytes were randomly distributed either by patients' preference or predominately by laboratory workflow, placed in a pre-equilibrated 5 well dish containing 0.5 *ml* of Sage single-step media overlaid with 0.3 to 0.5 *ml* of oil and cultured in a standard benchtop incubator

with low oxygen concentration (MINC, Cook Medical, Australia). Alternatively, in a pre-equilibrated Embryoscope slide containing 12 wells of 20-23 μl of media, they were overlaid with 1.3 ml of OVOIL (Vitrolife, Denmark), and cultured in the TLM incubator (Embryoscope™, conditions of 37 ℃, 5.5% CO_2, 5% O_2, and 89.5% N2). Fertilization was assessed by examining the presence of two pronuclei, 15.5 to 17.5 *hr* post injection.

Embryos cultured in a standard benchtop incubator (interrupted culture): After ICSI, oocytes cultured in a standard incubator at 37 ℃ and pre-mixed gas with low oxygen level (89% Nitrogen, 5.5% CO_2) were subjected to daily observation on day 1 (fertilization assessment), day 2, 3, 5, and 6. Embryos were removed from the incubator for static observations and assessments which were carried out in a humidified chamber of 37°*C* and 6.0% CO_2. In addition, embryos were co-cultured in a 5 well dish depending on the number of fertilized embryos. On day 3 of development, embryos were removed and placed in HEPES buffered medium (ORIGIO, CooperSurgical, Denmark) for assisted hatching and placed in a new pre-equilibrated dish of Sage single step media and cultured to blastocysts stage (day 5 or 6).

Embryos cultured in a TLM incubator (Uninterrupted culture): After ICSI/IMSI, injected oocytes were individually cultured in microwells of 23-25 μl of pre-equilibrated Sage single step media and placed in a time lapse incubator (37°*C*, 5.5% CO_2, 5.0% O_2, and balanced N2-Embryocope; Vitrolife, Denmark) until day 5 and 6 of embryo development. The EmbryoViewer software recorded the precise developmental timing images every 14-15 *min* from different focal planes (Embryocope; Vitrolife, Denmark). The developmental events were annotated by the embryologist starting from day 1 (fertilization assessment), day 2, day 3, day 5, and day 6.

The culture dish was removed on day 3 in order to perform assisted hatching (AH) and this was quickly placed into the time lapse incubator and remained uninterrupted until day 5 or 6 to prepare for biopsy.

Assisted hatching (AH): AH was performed on day 3 post injection for all PGT-A cycles using a non-contact 1.48 *um* diode laser (Research Instruments Ltd (RI), UK) for zona drilling.

Blastocyst biopsy and cryopreservation: All embryos reaching blastocyst stage were biopsied on day 5 and 6 after ICSI/IMSI for PGT-A cycle. Hatching blastocysts were individually placed on numbered dishes containing HEPES buffered medium (ORIGIO; CooperSurgical, Denmark). Maximum of five to eight trophectoderm cells were removed from each blastocyst using a non-contact 1.48 μm diode laser (Research Instruments Ltd (RI), UK) and sent for genetic analysis. Embryos were vitrified after biopsy with blastocyst vitrification media (Cook Medical, Australia).

Embryo scoring and blastocyst conversion (classification): All embryos were graded prior to trophictoderm biopsy using the 20x magnification Olympus X71 inverted microscope. Embryos were graded (AA, B+A, excellent=1, B+B+, AB+, B+B-, B-B+, good, B-B-, B+B-, average=2, and B-B-, B+C, poor=3) according to modified Gardner and Cornell's group scoring system. Graded 1 embryos had tightly packed cells of the inner cell mass (ICM) and a trophectoderm (TE) with many healthy cells forming a cohesive epithelium. In graded 2 embryos, the ICM had large cells, not tightly packed or cells making up a cellular bridge and, in some cases, small fragments within the ICM and a TE with average to few cells but healthy and large in size. Graded 3 embryos had visible but loose cells making up the ICM which could be fragmented and a TE of few very large or unevenly distributed cells. Most embryos were fully expanded and on very few occasions, full hatched at the time of biopsy.

Next generation sequencing (NGS): All trophectoderm cells were analyzed in a single external genetics laboratory (CooperGenomics, UK) using next generation sequencing (VQSERIES). This technology enables the analysis of whole genome amplification, testing for both aneuploidy and monogenic diseases simultaneously (10). The PGT-A results were reported as Euploidy (normal chromosome compliments), Aneuploidy (presence of monosomy, trisomy, and complex aneuploidy), and Mosaicism (high and low mosaic levels). Mosaicism is classified when 30% to 70% of the cells from the biopsied blastocyst showed aneuploidy which is further classified into low (30 to 50%) and high (50% to 70%) mosaic levels.

Statistical analysis: The analyzed embryos were classified into two groups, according to their culture conditions (benchtop interrupted *vs.* TLM uninterrupted incubator). Shapiro-Wilk test was used to analyze the normal distribution of the continuous variables in the two groups. Normally distributed variables were further compared using

student's t-test, while the Mann-Whitney U test was performed to compare differences between groups when the dependent variable was not normally distributed. Chi-square tests were used to compare categorical variables. Aneuploidy status was fitted to a logistic regression model in order to analyze the effect of possible confounding factors such as female age, type of incubator, number of oocytes collected, and blastocyst quality.

Results

The total number of analyzed blastocysts were 1099, of which 390/1099 (35.4%) were chromosomally normal, 666/1099 (60.6%) were chromosomally abnormal, and 43/1099 (3.9%) were mosaic. The remaining blastocysts (0.1%) failed to produce a result and were not re-biopsied at the time of the data retrieval. Table 1 illustrates the patient demographics of the studied cohort.

Culture performance in different incubators: Table 2 demonstrates the key laboratory parameters assessed for each culture condition in two incubators. Female patient age showed no statistical difference in both groups (p=0.0563).

Fertilization rate was calculated individually per cycle and the mean was compared against culture conditions. Fertilization rates were statistically similar when oocytes were cultured overnight in a standard benchtop conventional tri-gas incubator (77.7%, 95% CI 74.2-81.2) compared to TLM incubator (79.7%, 95% CI 75.8-83.5) (p=0.696). Similarly, blastocyst conversion was compared and showed no statistical significance among incubators (51.8% *vs.* 48.9%, p=0.548). Although the percentage of mature eggs was higher in the TLM group, all oocytes were incubated in standard benchtop incubators prior to insemination.

Embryo quality at blastocyst stage: Embryo quality was compared between both incubators and the obtained data was divided in three categories of "Excellent", "Good", and "Poor". Table 2 shows the statistical association found between embryo quality and type of incubator (p<0.001). Analysis of adjusted residuals (z-scores) showed a statistically significant proportion of "excellent" quality embryos in the TLM incubator when compared to the benchtop (34.5% *vs.* 21.4%). Type I error was then controlled and significance of p-value was adjusted to 0.01. Only the proportion of "excellent" embryos was found to be different among both incubators (p=0.001).

Comparison of genetic mechanisms after culturing embryos in different systems: Overall, embryo biopsy was predominantly performed on day 5 (667/1099,60.7%). Looking at each incubator, the same principle applies when comparing proportion of embryos biopsied on each day (Conventional 225/373 *vs.* TLM 435/726). Comparison of euploidy rate shows this parameter to be significantly lower in embryos cultured in conventional tri-gas incubator (30.4%, 95% CI, 23.1-37.7) as compared to TLM (37.03%, 95% CI, 31.9-42.1) (p=0.048). A post hoc power analysis was conducted on these findings and revealed a power of 60.3%. To con-

Table 1. Patient demographics

Variable	MINC (n=102)	ESCOPE (n=176)	p-value
Female age (years)	39.2±3.4	38.4±3.7	0.053 **
Male age (years)	40.5±6.6	41.2±6.7	0.764 **
AMH	15.4±12.3	17.3±12.4	0.116 **
FSH	7.5±3.2	7.4±2.9	0.984 **
E2	226±173.6	216.2±260.7	0.083 **
AFC	14.9±9.0	16.1±10.5	0.502 **
BMI (female)	23.4±4.2	23.1±3.7	0.515 **
Time of infertility (months)	31.1±22.6	32.5±23.0	0.610 **
Previous pregnancy (n)	1.8±2.1	1.4±1.5	0.293 **
Previous miscarriage	1.2±1.7	0.8±1.3	0.186 **
Previous LB (n)	0.3±0.5	0.3±0.6	0.522 **
Sperm concentration (*M/ml*)	70.2±50.3	38.3±58.3	0.779 **
Motility (%)	57.9±15.2	57.4±16.6	0.397 *
Normal forms (%)	4.82±2.3	5.2±3.2	0.589 **

* Mann-Whitney U-test
** t-student

Table 2. Laboratory outcomes

		MINC	ESCOPE	p-value
Number of collected eggs		11.2±6.3	13.9±7.7	0.006 *
Number of to oocytes MII		9.3±5.1	11.1±5.7	0.010 *
Fertilisation rate		77.7 (74.2-81.2)	79.7 (75.8-83.5)	0.696 *
Blastulation rate		51.8 (49.6-59.1)	48.9 (49.4-56.5)	0.548 *
Embryo quality				
Embryo quality	Excellent	21.71582 (n=81)	31.12948 (n=226)	<0.0001 **
	Good	62.19839 (n=232)	55.64738 (n=404)	
	Poor	16.19839 (n=60)	13.22314 (n=96)	
Euploid rate		30.4 (23.1-37.7)	37.03 (31.9-42.1)	0.0488 *
Aneuploidy rate		67.8 (56.4-79.2)	56.7 (50.8-62.6)	0.070 *
Mosaicism rate		4.2 (1.5-6.9)	3.4 (1.8-5)	0.833 *

* Mann-Whitney U-test
** Chi square Test

Table 3. Logistic model blastocyst quality regression

Logistic Model	Adjusted OR (95% CI)	p-value
Female age	0.85 (0.82-0.88)	<0.001
Eggs collected	1.21 (0.91-1.6)	0.679
Blastocyst quality	0.99 (0.98-1.01)	0.603
Incubator	0.54 (0.45-0.59)	0.039

firm and quantify the analysis for both incubators, PGT-A result of all tested embryos was adjusted to a logistic regression model. The following covariates were included: age of the patient (continuous, years), type of incubator (class variable, two states), oocytes retrieved (continuous, number), and blastocyst quality (optimal versus not; excellent and good were considered optimal). To evaluate a potential suppressor effect, correlation analysis between the covariates was performed and showed no significance at a 5% level. Results revealed that adjusted OR for female age and incubator types were associated with significance values below the 5% threshold (0.85, 95% CI, 0.82-0.88 and 0.54, 95% CI, 0.45-0.59, respectively; table 3).

Clinical outcomes of euploid single embryo transfers: The results of the subgroup analysis are summarized in supplementary table 1. A total of 74 euploid single embryo transfers were included. Considering the limited sample size, pregnancy rates were comparable amongst both incubation systems (benchtop 73.3% *vs.* TLM 75%, p=0.871). Moreover, differences in clinical (86% *vs.* 96%) or ongoing (63.3% *vs.* 72.7%) pregnancy rates were not reported at the 5% level.

Discussion

Selecting the best quality embryos based on morphology criterion has weak correlation with implantation due to low association with embryo ploidy status, which is essential to enhance implantation rates in IVF (13). PGT-A has been reported to improve clinical outcomes when genetically normal embryos are transferred (14). However, the European consortium on randomized controlled trials showed that chromosome screening of aneuploid human embryos does not increase pregnancy rates but reduces the time to pregnancy as well as decreases miscarriage rate (6).

In this study, euploidy rate was evaluated in different culture conditions comparing an interrupted (benchtop incubator) versus uninterrupted (TLM incubator) cultures in PGT-A patients only. Our results showed oocyte fertilization rate was statistically similar in both culture conditions. Similarly, blastocyst conversion was not different comparing both incubator systems. Comparison of euploidy rate demonstrated a statically significant reduction in embryos cultured in interrupted incubator (30.4%, 95% CI, 23.1-37.7) compared to uninterrupted incubator (37.03%, 95% CI, 31.9-42.1). Fitted regression analysis showed that female age was the key variable driving euploidy rates (0.85, 95% CI, 0.82-0.88; p<0.001) although incubator type could be an important covariable (0.54, 95% CI, 0.45-0.59; p=0.039).

There is very limited research around the mech-

anism and periods of sensitivity during embryo development that are influenced by culture conditions following fertilization. While most studies focus on mitotic aneuploidies, a meta-analysis showed mitotic aneuploidies increased from 63% at the cleavage stage to 95% at the blastocyst stage (10), with no particular emphasis on post mitotic errors. Mantikou et al. (15) looked at the origin of mitotic aneuploidies, segregating these into different causative factors. One of the *in vitro* conditions highlighted was increased oxygen concentration which was shown to increase non-disjunction in early embryo division of non-disjunction-prone mouse embryos, indicating how laboratory settings can influence chromosome segregation (16). However, in our study, all embryos were cultured using incubator under low oxygen tension (5.5% O_2 interrupted and 5% O_2 uninterrupted). Another variable such as temperature can impact various aspects of gametes and embryo function. Almedia and Bolton (8) showed the effect of temperature on mitotic spindle repolarization and depolarization and possible embryo metabolism. A recent randomized controlled trial examined the effect of culture at either 37 or 36 degrees, demonstrating that embryos cultured at 37 degrees yielded higher cell numbers on day 3 of development and higher blastocyst and blastocyst quality when compared to culture at 36 degrees (9). Although this study showed a slight trend towards higher implantation rate (67.4% *vs.* 73.3%) for embryos cultured at 37 degrees, the incidence of aneuploidies was similar in both groups. In our study, the temperature of both incubators was set at 37 degrees; the only difference was that the interrupted incubator requires embryos to be analyzed on a daily basis for static observation and embryos belonging to approximately 4 different patients could be cultured in one compartment within the incubator. Our subgroup analysis on 74 euploid single embryo transfers showed no difference in implantation, clinical, or ongoing pregnancy rates when comparing both incubators.

Culture conditions and gamete manipulation are some of the treatment related factors that may affect the incidence of chromosomal imbalances in embryos (8, 11). A recent study by Munne et al. (5) reported large variation in euploid rates from oocyte donors between fertility centers. The average age of donor in the study was 25.5±3.03 years (range of 19-35 years), with average euploidy rate per center ranging from 39.5 to 82.5%. As such, this study indeed demonstrated that euploidy rate in oocyte donors is treatment center related. Studies have shown that suboptimal culture systems can affect gene expression and imprinting (Ho et al. 1994, 1995; Doherty et al. 2000; Fernandez-Gonzalez et al. 2010; Market Velker et al. 2010) demonstrating that embryonic chromosomal abnormality could be partly iatrogenic (5, 7).

The aim of this study was to compare the ploidy rate in an uninterrupted culture condition using time lapse incubator (EmbryoScope) with interrupted culture condition using benchtop conventional tri-gas incubator. While it was firstly hypothesized that there was no difference in ploidy rates between both culture conditions in the same laboratory setting, our study demonstrated a statistically significant difference between both culture conditions, thereby rejecting the null hypothesis. This study shows the importance of continuous maintenance of embryonic environmental conditions by constant maintenance of all parameters to reduce any potential effect on embryo development. It is apparent that not all embryos possess the ability to adequately adapt to the stress experienced *in vitro*, most probably due to inadequate number of oocytes and maternal risk factors.

Interventions like ovulation induction, in vitro fertilization, embryo culture, and cryopreservation of embryos are associated with changes in gene expression profiles and methylation patterns in the placenta and the birth weight of the offspring (17). In general, the mechanisms behind the changes in phenotype are unknown. In human IVF, different culture media were shown to induce different gene expressions in embryos and a significant difference in birth weight of the children born. Similarly, the oxygen concentration in the incubator will influence the gene expression in human embryos and this effect was dependent on the type of culture media used. This is the first study which indicates that TLM incubators increase the probability of obtaining an euploid embryo. It may be plausible that culture conditions may have an impact on embryo mitosis. Some of the limitations seen in our study is the sample size and the lack of sibling oocyte used.

Conclusion

Our study has demonstrated the effect of uninterrupted single step culture media on the euploidy rate in blastocysts possibly as a result of epigenetic changes as mentioned above. The impact of TLM on clinical outcomes in IVF is currently a

subject of intensive research.

Therefore, it seems that laboratory factors such as incubator type may cause temperature fluctuation which may induce cellular stress in embryos and possible mitotic errors.

Conflict of Interest

Authors declare no conflicts of interest.

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Supplementary

Supplementary Table 1. Clinical outcomes

ESET	Benchtop (n=30)	TLM (n=44)
Chemical pregnancy		
Yes	22 (73.33)	33 (75)
No	8 (26.67)	11 (25)
Clinical		
Yes	19 (86.36)	32 (96.97)
Anembryonic pregnancy	2 (2.09)	1 (3.03)
Ectopic	1 (4.55)	0
Final outcome		
LB	19 (63.33)	32 (72.72)