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Sibling oocytes cultured in a time-lapse versus benchtop incubator: how time-lapse incubators improve blastocyst development and euploid rate

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Summary

The aim was to study whether a limited exposure of embryos outside the incubator has an effect on embryo development, blastocyst quality and euploid outcomes. This retrospective study was performed at ART Fertility Clinics, Abu Dhabi, United Arab Emirates (UAE) between March 2018 and April 2020 and included 796 mature sibling oocytes that were split randomly between two incubators after intracytoplasmic sperm injection (ICSI): an EmbryoScope™ (ES) incubator and a benchtop incubator, G185 K-SYSTEMS (KS). The fertilization, cleavage, embryo/blastocyst qualities, useable blastocyst and euploid rates were assessed to evaluate the incubator performance. In total, 503 (63.2%) mature oocytes were cultured in the EmbryoScope and 293 (36.8%) in the K-SYSTEMS. No differences were observed in fertilization rate (79.3% vs 78.8%, $P = 0.932$), cleavage rate (98.5% vs 99.1%, $P = 0.676$) and embryo quality on Day 3 ($P = 0.543$) between both incubators, respectively. Embryos cultured in the EmbryoScope, had a significantly higher chance of being biopsied (64.8% vs 49.6%, $P < 0.001$). Moreover, a significantly higher blastocyst biopsy rate was observed on Day 5 in the EmbryoScope (67.8% vs 57.0%, $P = 0.037$), with a highly significant increased euploid rate (63.5% vs 37.4%, $P = 0.001$) and improved blastocyst quality ($P = 0.008$). We found that exposure of embryos outside the incubator may negatively affect the *in vitro* blastocyst development and euploid rate on Day 5.

Introduction

The goal of reproductive medicine specialists is to increase pregnancy rates and achieve singleton live births in a shorter time, while minimizing the number of transferred embryos to a couple undergoing assisted reproductive treatment (ART; Ubaldi *et al*., 2015).

The laboratory environment is one of the crucial influencing elements of the delicate equation to a successful cycle. It has been established that optimal IVF laboratory conditions are a key requirement in optimizing the chances of having a good-quality embryo to transfer (Wale and Gardner, 2016). Stable gas concentrations and temperature, well designed culture medium, minimum light exposure, controlled pH and osmolarity and elimination of volatile organic compounds (VOC) in the laboratory, are examples of conditions that are in need of constant optimal quality control to ensure proper embryo development (Cairo Consensus Group, 2020). Another important decision in an IVF laboratory is the choice of the incubator. Each incubator has its benefits and negative aspects as well (Vajta *et al*., 2021). Benchtop incubators show efficacy in terms of culture due to the small chamber that allows fast recovery of gases and temperature after lid opening (Swain, 2014). Conversely, time-lapse technology (TLT) incubators allow automated images to be captured without embryo disturbance during incubation.

Many studies have been published comparing efficiency (Sciorio *et al*., 2018), safety (Nakahara *et al*., 2010; Cruz *et al*., 2011; Kirkegaard *et al*., 2012) and outcome performance (Mascarenhas *et al*., 2019; Kalleas *et al*., 2022) between benchtop and TLT incubators. Although TLT incubators have shown better quality embryos when compared with benchtop incubators (Alhelou *et al*., 2018; Sciorio *et al*., 2018; Kalleas *et al*., 2022), some studies did not find any difference in embryo quality between both incubators (Nakahara *et al*., 2010; Cruz *et al*., 2011; Park *et al*., 2015).

Higher implantation (Alhelou *et al*., 2018), pregnancy (Rubio *et al*., 2014), ongoing pregnancy (Ueno *et al*., 2019) and live birth rates (Mascarenhas *et al*., 2019; Kalleas *et al*., 2022) have been observed with the use of TLT incubators, although similar pregnancy outcomes have been reported in many other studies (Cruz *et al*., 2011; Kahraman *et al*., 2012; Kirkegaard *et al*., 2012; Park *et al*., 2015; Barberet *et al*., 2018). Consequently, a recent Cochrane review

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(Armstrong *et al.*, 2019) on TLT incubators confirmed the lack of clinical data to choose TLT incubators over benchtop incubators for embryo culture. Interestingly, no publication has explored a possible difference in euploid rate between these two incubators.

The duration and circumstances of the exposure of the embryos outside the incubator during embryo evaluation and medium changeover, as required with embryos cultured in benchtop incubators, may contribute to the differences observed in embryological and clinical outcomes. Although stable culture conditions – mimicking physiological conditions – should be guaranteed, it has been shown that increased culture condition variations have a considerable negative effect on the number of blastocysts obtained, as well as on pregnancy and implantation rates (Krasnopolskaya *et al.*, 2019), thereby questioning their influence on the ploidy status of the generated blastocysts.

Therefore, the objective of this study was to compare the development, useable blastocyst rate, quality and ploidy outcomes of sibling embryos cultured in TLT and benchtop incubators. This was to observe if the differences between both incubation systems had an effect on the final embryo developmental outcome.

Materials and methods

This study was approved by the Research Ethics Committee of ART Fertility Clinics LLC, Abu Dhabi, UAE (REC Reference number – REFA055).

Study design

This retrospective observational sibling oocyte study was performed at ART Fertility Clinics, Abu Dhabi, UAE, between March 2018 and April 2020 and included 796 mature oocytes injected from 42 stimulation cycles. Patients with maternal age between 18 and 45 years old, and who underwent ovarian stimulation for intracytoplasmic sperm injection (ICSI) and preimplantation genetic testing for aneuploidies (PGT-A) through next generation sequencing (NGS) on trophectoderm biopsies due to primary or secondary infertility, were included. Indications to perform PGT-A were the following: advanced maternal age (AMA) if the patient was over 35 years old (9), recurrent miscarriage (5), implantation failures (1), severe male factor (12) and cases in which genetic testing was performed according to the patient's request (15). With the objective to have comparable groups, only patients with at least 16 fresh mature oocytes subjected to ICSI were included. Oocytes after injection were transferred randomly to the EmbryoScope slide until completion of one full dish; 12 oocytes were cultured in an EmbryoScope™ incubator (Vitrolife, Sweden) and the remaining oocytes were allocated for culture in a K-SYSTEMS G185© incubator (Cooper Surgical, Denmark). Cycles with testicular sperm extraction (TESE) or microTESE were excluded from the study. All included patients had a systemic progesterone level <1.5 ng/ml on the day of trigger for final oocyte maturation.

Research questions

The primary endpoint was to analyze the useable blastocyst rate after culturing sibling oocytes in the two different types of incubators. The useable blastocyst rate was calculated as the number of biopsied blastocysts on Days 5, 6 and 7 per number of normally fertilized zygotes in each arm of the study.

Secondary endpoints comprised fertilization and cleavage rates, embryo qualities on Day 3 and on the day of trophectoderm biopsy (Day 5, 6 or 7), and euploid rate per incubator. Fertilization was calculated as the number of normally fertilized zygotes (2PN) per number of injected oocytes. The cleavage rate was calculated as the number of cleaved embryos on Day 3 per 2PN. The euploid rate was defined as the number of euploid blastocysts per number of biopsied blastocysts in each group.

Ovarian stimulation protocol and oocyte retrieval

Ovarian stimulation was performed by standard protocols, either gonadotropin-releasing hormone (GnRH) agonist or GnRH antagonist protocols, using recombinant follicle-stimulating hormone (recFSH) or human menopausal gonadotropin (HMG) as stimulation medication. The dosage of the stimulation medication was chosen according to the ovarian reserve parameters (La Marca and Sunkara, 2014). Final oocyte maturation was achieved by administration of either 5000 or 10,000 IU hCG, 0.3 mg of GnRH agonist (Triptorelin) or dual trigger (hCG and GnRH analogue), as soon as ≥3 follicles ≥17 mm were present. Oocyte retrieval (OR) was scheduled for 36 h after the trigger for the final oocyte maturation. Oocytes were collected in Quinn's Advantage Medium with HEPES, (SAGE, Målov, Denmark) supplemented with human serum albumin (HSA; Vitrolife, Göteborg, Sweden; HTF-HSA) and washed in Global Total LP medium for fertilization (CooperSurgical Inc., USA) after which they were cultured at 37°C, 6% CO_2 and 5% O_2 until denudation.

Insemination and embryo culture

Obtained cumulus–oocyte complexes (COCs) were denuded (Hyase, Vitrolife) 3 h after OR and ICSI were performed on all mature (MII) oocytes 40 h after trigger (Palermo *et al.*, 1992; Vandenberghe *et al.*, 2021). After injection, sibling oocytes were cultured in an EmbryoScope or K-SYSTEMS (37°C, 6% CO_2, 5% O_2). According to our clinical practice, only one EmbryoScope slide (~12 oocytes) is used per patient, and the remaining oocytes are cultured in a K-SYSTEMS incubator. For the EmbryoScope, the first 12 injected oocytes were placed individually on the EmbryoScope slide with 12 culture drops of 25 µl overlaid with 1.4 ml of oil (Ovoil®; Vitrolife), and for the K-SYSTEMS, oocytes were placed individually in a culture dish containing eight culture drops of 25 µl, overlaid with 8 ml of oil. Single Step Medium (Global®Total®LP, CooperSurgical Inc.) was used for both arms of the study. The average ICSI time per case was 30 min.

For the EmbryoScope, Embryo Viewer Software© was used to evaluate zygotes and embryos without removing the dish from optimal conditions. The fertilization (16–18 h post-injection) and embryo evaluation on Day 3 (68 h), and Days 5, 6 and 7 (116, 140 and 164 h, respectively) were assessed under an inverted microscope for embryos cultured in the K-SYSTEMS incubator and according to the Vienna Consensus. In both groups, zygote/embryo evaluation was performed at the same time and by the same operator, as well as medium refreshment on Day 3 of embryo development, to sustain development up to Day 7. For the EmbryoScope, 20 µl of culture medium was removed from each culture drop and replenished with 20 µl overnight pre-equilibrated Global Total LP medium. For the K-SYSTEMS, embryos were transferred individually to a fresh GPS® Dishware (CooperSurgical LLC) that was pre-equilibrated overnight. Embryos were cultured until the blastocyst stage (Day 5, 6 or 7) and biopsied once the blastocyst was graded ≥ BL3CC (Gardner and Schoolcraft, 1999). On Days 5/6/7, embryos from both incubators were checked twice a day at the same time; blastocysts incubated in the EmbryoScope were verified on the screen of the incubator, via EmbryoViewer

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and blastocysts in the K-SYSTEMS were removed from the incubator and evaluated under a microscope. Morphokinetics were not considered for blastocyst biopsy selection in the EmbryoScope arm.

Embryo quality

Embryo quality on Day 3 was evaluated according to the number of blastomeres, symmetry, fragmentation, multinucleation and presence of vacuoles. The scoring grade was determined according to a combination of all five classical morphological assessments described above and divided into four categories of embryo qualities: excellent or top quality [embryo quality 1 (EQ1)]: ≥7 blastomeres, fragmentation ≤10%, equal blastomere size, no vacuoles or multinucleation; moderate quality (EQ2): ≥6 blastomeres, fragmentation until 20%, similar blastomere size, and multinucleation and vacuoles observed in <50% of the blastomeres; poor quality (EQ3): embryos with ≥4 blastomeres, fragmentation between 21–50%, unequal blastomere size, several normal nuclei or smaller fragmented nuclei visible in <50% of the blastomeres and vacuolated blastomeres, with ≥50% small vacuoles; and bad quality (EQ4): embryos with >50% of fragmentation, unequal cell size, multinucleation and large vacuoles seen in >50% of the blastomeres.

Biopsied blastocysts were graded based on the inner cell mass (ICM) and trophectoderm (TE) morphology and expansion stage (Gardner and Schoolcraft, 1999). The scoring grades were divided into four categories of embryos: excellent or top quality (EQ1): AA, AB, BA; good quality (EQ2): BB; moderate quality (EQ3) AC, BC, CA, CB, CC; and poor quality (EQ4) AD, BD, CD, DA, DB, DC, DD. For statistical purposes, embryo quality was dichotomized: good quality (GQ = EQ1 + EQ2) and poor quality (PQ = EQ3 + EQ4).

Blastocyst biopsy and NGS

Trophectoderm biopsy was performed on expanded blastocysts (≥BL3) on Day 5, 6 or 7. The biopsy technique and assessment of ploidy status were performed as previously described (De Munck *et al.*, 2020). Blastocysts were vitrified 1 h after the biopsy procedure according to the manufacturer's protocol (Kitazato© Vitrification).

Statistical analysis

Data analysis was performed using the R Computational Statistical Framework [Ref1: https://www.r-project.org/] with IDE RStudio [Ref2: https://rstudio.com/]. Cycle characteristics were described using mean ± standard deviation (SD), minimum and maximum values. Outcomes were inspected initially using Bland–Altman plots, as well as computing and visualizing Spearman's coefficients and their statistical significance. The Mann–Whitney statistical test was applied to selected outcome variables of mean per cent per cycle to compare the two incubators (EmbryoScope versus K-SYSTEMS), using the 'wilcox.test' function. Differences in embryo development and outcome in each arm were analyzed using the chi-squared test (or Fisher's exact test). All the significance tests were two-tailed and the significance level was set at $P < 0.05$. Figures were generated using R library ggplot2 (https://ggplot2.tidyverse.org/) 'geom_smooth' function, suppressing the representation of standard error (SE).

This retrospective study included 796 embryos of which 63.2% were placed in an EmbryoScope ($n = 503$) and 36.8% in K-SYSTEMS ($n = 293$) incubator considering a 1:1.7 sample ratio. The euploid rates using both types of incubators were assumed to be 60% vs 70% and 50% vs 60% with 0.10 effect size, considering a 0.05 margin of error ($Z_{\alpha/2}$). Our study calculated power was 0.8131 and 0.7812, respectively, that is acceptable to prove the significant results (Serdar *et al.*, 2021).

Table 1. Cycle characteristics ($n = 42$)

	Mean ± SD	Minimum–Maximum
Age (years)	31.4 ± 6.3	21–45
BMI (kg/m^2)	26.5 ± 4.8	17.63–38.78
AMH (ng/ml)	6.03 ± 4.2	1.81–23
Duration of stimulation (days)	10.2 ± 1.3	8–13
P4 on trigger day (ng/ml)	0.76 ± 0.3	0.08–1.43
Total sperm count (million)	131.3 ± 152.7	0.04–571.9
Total progressive motility (million)	63.5 ± 86.4	0–366.02
COCs retrieved	26.3 ± 10.0	16–66
Mature oocytes – MII	21.3 ± 7.1	16–43

Results are presented as mean ± SD per cycle. AMH: anti-Müllerian hormone (ng/ml); BMI: body mass index (kg/m^2); COC: cumulus–oocyte complex; P4: progesterone.

Results

In total, 796 mature sibling oocytes from 42 patients were assigned for culture in two different incubators (EmbryoScope and K-SYSTEMS) and their embryo development was analyzed. Table 1 describes the cycle characteristics of all patients included in this study. The indications for PGT-A were AMA (21.4%), repeated implantation failure (2.4%), recurrent miscarriages (11.9%), male factor (28.6%) and elective PGT-A in 35.7% of the cases.

Out of 796 mature oocytes included in the study, 503 were cultured in an EmbryoScope (63.2%) and the remaining 293 sibling oocytes were cultured in K-SYSTEMS (36.8%; Table 2). The fertilization (79.3% vs 78.8%, $P = 0.932$) and cleavage rates (98.5% vs 99.1%, $P = 0.676$) were not different between both incubators. However, the total useable blastocyst rate was significantly higher for embryos cultured in the EmbryoScope (64.8% vs 49.6%, $P < 0.001$), mainly because a higher percentage of blastocysts was biopsied on Day 5 from the EmbryoScope (67.8% vs 57.0%, $P = 0.037$). There was no difference in the total euploid rate between the EmbryoScope and K-SYSTEMS (59.9% vs 50.4%, $P = 0.314$); however, a significantly higher euploid rate was observed for blastocysts cultured and biopsied on Day 5 from the EmbryoScope (63.5% vs 37.4%, $P = 0.001$). The distribution of the useable blastocyst rate is shown in Figure 1. The useable blastocyst rate was also calculated per age in both the EmbryoScope ($P = 0.005$, aR2: 0.1606) and K-SYSTEMS ($P = 0.028$, aR2: 0.093; Figure 2). Advanced maternal age showed a negative correlation with the useable blastocyst rate in both incubators. For all age categories, the EmbryoScope showed a highly statistically increased useable blastocyst rate compared with K-SYSTEMS ($P < 0.0001$).

Embryo quality at cleavage and biopsy stage is presented in Table 3. On Day 3, as well as for all biopsied blastocysts, no

Table 2. Fertilization and embryo development

	EmbryoScope		K-SYSTEMS		
	Mean %	SD/ SE*	Mean %	SD	*P*-value
Fertilization rate	79.3	10.3	78.8	19.11	0.932[a]
Cleavage rate	98.5	0.61	99.1	0.60	0.676[b]
Useable blastocyst rate	64.8	26.1	49.6	29.8	<0.001[a]
Day 5 useable BL	67.8	2.90	57.0	4.37	0.037[b]
Day 6 useable BL	31.0	2.87	39.8	4.32	0.084[b]
Day 7 useable BL	1.2	0.67	3.1	1.53	0.226[b]
Euploid rate	59.9	26.4	50.4	40.2	0.314[a]
Day 5 Euploid rate	63.5	29.0	37.4	44.1	0.001[a]
Day 6 Euploid rate	40.9	40.7	26.6	40.0	0.08[a]
Day 7 Euploid rate	0	0	3.6	17.1	0.182[a]

Calculation of useable blastocyst rate (number of biopsied blastocysts per number of normally fertilized oocytes), euploid rate (number of euploid blastocysts per number of biopsied blastocysts) and euploid rates per day of biopsy (5, 6 or 7). BL – blastocyst.
[a]Wilcoxon test (per cycle data).
[b]Chi-squared test or Fisher's exact test or post-hoc chi-squared test (embryo data).
*SD for mean % (per cycle), SE for proportion % (per embryo).

difference was observed in the embryo quality (GQ or PQ) between both incubators ($P = 0.543$ and $P = 0.151$, respectively). A significant difference in good-quality and poor-quality blastocysts biopsied on Day 5 and Day 6 was noted between embryos cultured in the EmbryoScope versus K-SYSTEMS, $P = 0.008$. The chance of good-quality or poor-quality cleavage stage embryos (Day 3) to develop into useable blastocysts, was calculated per incubator. Comparing the total useable blastocyst development, no difference was found between embryos originating from good-quality ($P = 0.083$) or poor-quality ($P = 0.111$) cleavage stage embryos. However, when stratifying according to the day of blastocyst development, poor-quality embryos on Day 3 showed a statistically significant superior blastocyst formation on Day 5 when cultured in the EmbryoScope (64.1% vs 39.1%), which was shifted to Day 6 for embryos cultured in K-SYSTEMS (35.9% vs 60.9%; $P = 0.005$). This difference in the day of blastocyst development was not observed for GQ cleavage stage embryos ($P = 0.916$).

Discussion

As the exposure of embryos outside the incubator may affect their development, this study aimed to evaluate the embryo development and ploidy rates when sibling oocytes were cultured in the EmbryoScope and K-SYSTEMS: a significantly higher total useable blastocyst rate (64.8% vs 49.6%, $P < 0.001$) and euploid rate on Day 5 was observed for embryos cultured in the EmbryoScope (63.5% vs 37.4%, $P = 0.001$). In addition to this, an increased blastocyst quality on Day 5 was observed after culture in the EmbryoScope ($P = 0.008$).

The optimization of ART outcomes is a long and complex journey that includes the couple's previous medical history, stimulation strategy, cycle plan, insemination method and embryo culture conditions. In the IVF laboratory, constant efforts are made to improve culture conditions (Wale and Gardner, 2016) and embryo selection with the objective of improving cycle outcomes. The incubator's choice is one relevant decision that possibly affects embryo development. Benchtop incubators are proven to be effective due to the fast recovery of temperature and gases, but a major limitation is a need to remove the culture dish from the incubator to perform embryo assessment (Swain, 2014). Although excluding Day 2 and Day 4 evaluations, the need to check fertilization, as well as performing a Day 3 changeover, induces a considerable amount of time outside the incubator during which time the embryos are exposed to suboptimal culture conditions (Zhang *et al.*, 2010). When TLT is considered, the continuous frames captured by a camera attached to the incubator, and subsequent video access by the embryologist, offer significantly reduced exposure of embryos to this suboptimal environment (Rubio *et al.*, 2014).

Previous retrospective (Ueno *et al.*, 2019) and randomized controlled trials (Alhelou *et al.*, 2018; Barberet *et al.*, 2018) have described higher useable blastocyst rates in a TLT incubator compared with conventional incubators (51.0% vs 46.6%, $P < 0.05$; Ueno *et al.*, 2019), the total proportion of cryopreserved embryos (29.5% vs 24.8%, $P = 0.027$; Barberet *et al.*, 2018) and blastocyst formation (52.1% vs 46.3%, $P = 0.0022$; Alhelou *et al.*, 2018). These publications are in line with the results obtained in this study, as embryos cultured in EmbryoScope had a significantly higher chance to develop into blastocysts and be biopsied than the ones cultured in a K-SYSTEMS incubator (64.8% vs 49.6%, $P < 0.001$), especially on Day 5 (67.8% vs 57.0%, $P = 0.037$). The criteria for cycle inclusion in our study (≥16 mature oocytes for injection), resulted in the selectin of a 'good prognosis population' with a mean maternal age of 31.4 ± 6.3 years and a mean anti-Müllerian hormone (AMH) level of 6.03 ± 4.2 ng/ml, which may explain the generally increased blastocyst formation in our study compared with previous publications. Despite the fact that previous research using sibling oocytes failed to demonstrate the increased performance of TLT compared with conventional incubators for fertilization rate (57.5% vs 57.5%), GQ embryos on cleavage stage (36.0% vs 36.0%; Nakahara *et al.*, 2010), blastocyst rate (54.8% vs 50.6%; Cruz *et al.*, 2011), implantation rate (38.1% vs 30.4%, $P = 0.75$) and clinical pregnancy rate (36.8% vs 33.3%, $P = 1.0$; Kirkegaard *et al.*, 2012), the TLT incubator was proven to be safe, as no differences were found (Nakahara *et al.*, 2010; Cruz *et al.*, 2011; Kirkegaard *et al.*, 2012).

Even though the same numbers of embryos were cryopreserved in both incubation systems, Sciorio and colleagues (Sciorio *et al.*, 2018), using sibling oocytes, observed improved embryo quality on Day 2 and Day 3, when embryos were cultured in the EmbryoScope. This difference in Day 3 embryo quality was not observed in our study; however, a difference was observed in the potential of poor-quality Day 3 embryos to develop to the blastocyst stage. Poor-quality embryos on Day 3 developed significantly faster to blastocysts on Day 5, if cultured in the EmbryoScope rather than in K-SYSTEMS (on Day 5, 64.13% vs 39.13% and Day 6, 35.87% vs 60.87%, $P = 0.005$), confirming the advantage of a culture system with 'reduced disturbance' (Krasnopolskaya *et al.*, 2019). In addition to the difference in developmental speed, the EmbryoScope also showed an increased proportion of biopsied blastocysts with GQ on Day 5 compared with Day 6 (78.21% vs 62.5% for Day 5, 21.79% vs 37.5% for Day 6; $P = 0.008$).

Although the total euploid rate did not show any difference between the two incubators (59.9% vs 50.4%, $P = 0.314$), once the euploid rate was stratified per day of the biopsy, the EmbryoScope had a significantly higher euploid blastocyst rate on Day 5 compared with K-SYSTEMS (63.5% vs 37.4%, $P = 0.001$). This

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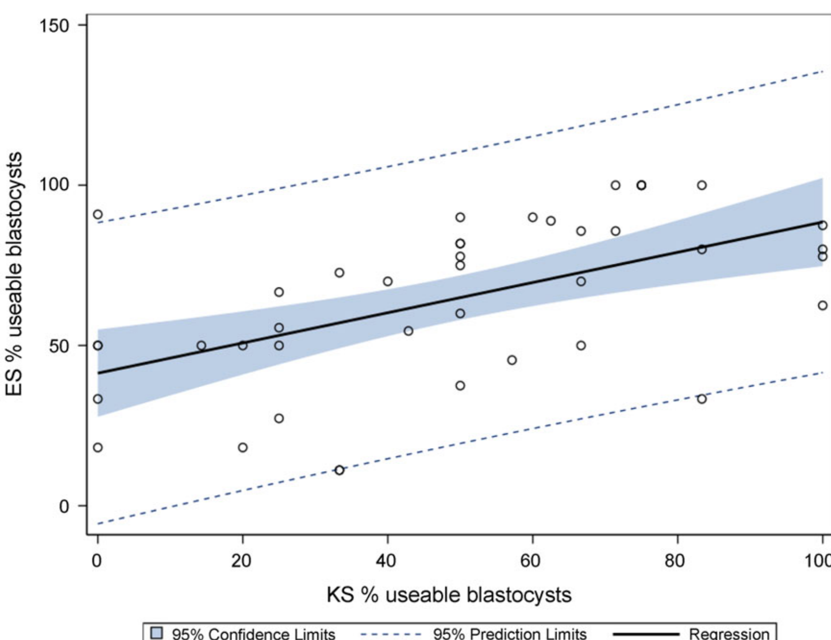

Figure 1. Useable blastocyst rate per cycle. Useable blastocyst rate (blastocysts biopsied/normally fertilized oocytes) per cycle, stratified per incubator (every dot represents one stimulation cycle) with 95% confidence limits. ES: EmbryoScope; KS: K-SYSTEMS.

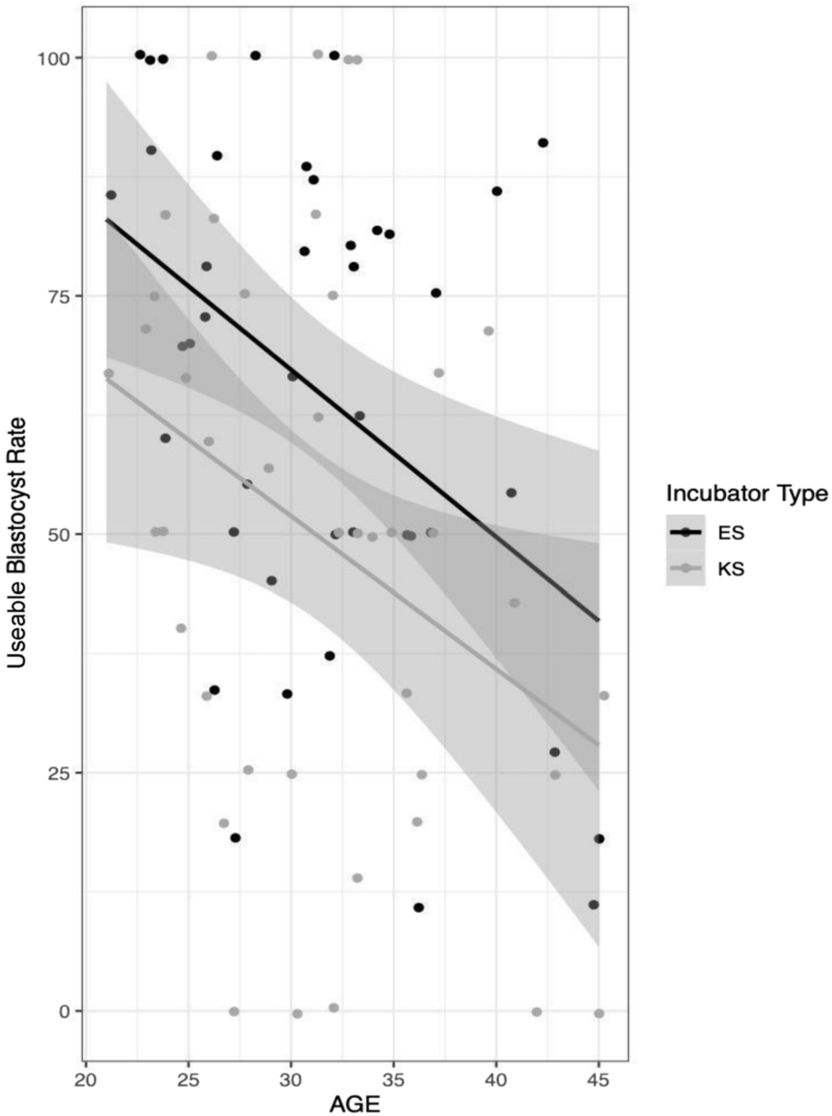

Figure 2. Useable blastocyst rate per age in EmbryoScope (ES) and K-SYSTEMS (KS). Distribution of useable blastocyst rate in both incubators per age (years) with darker area representing 95% CI.

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Table 3. Embryo quality and blastocyst formation

	EmbryoScope		K-SYSTEMS		
	n	%	N	%	*P*-value
Embryo quality on Day 3					
GQ D3	209	53.2	116	50.7	0.543
PQ D3	184	46.8	113	49.3	
Blastocyst quality at the time of biopsy					
GQ BL (D5/D6/D7)	180	69.8	80	62.5	0.151
PQ BL (D5/D6/D7)	78	30.2	48	37.5	
GQ BL D5	140	78.2	50	62.5	0.008
GQ BL D6	39	21.8	30	37.5	
PQ BL D5	35	46.1	23	52.3	0.511
PQ BL D6	41	54.0	21	47.7	
Blastocyst development from GQ Day 3 embryos					
GQ D3 vs Total BL	164	78.5	81	69.8	0.083
GQD3 vs BL D5	116	71.2	55	70.5	0.916
GQD3 vs BL D6	47	28.8	23	29.5	
Blastocyst development from PQ Day 3 embryos					
PQD3 vs Total BL	94	51.1	47	41.6	0.111
PQD3 vs BL D5	59	64.1	18	39.1	0.005
PQD3 vs BL D6	33	35.9	28	60.9	

Analysis of cleavage stage embryo quality and blastocyst quality at the time of biopsy between embryos cultured in the EmbryoScope and K-SYSTEMS. Day 7 development is not present due to insufficient data. D: day, GQ: good quality, PQ: poor quality, BL: blastocyst; chi-squared test or Fisher's exact test or post-hoc chi-squared test.

result can be ascribed to the fact that also a higher proportion of GQ blastocysts was observed on Day 5 in the EmbryoScope, as a negative correlation between embryo development and chromosomal content had previously been described (Magli *et al.*, 2007; Wang *et al.*, 2018). The advantage of culture in the EmbryoScope may become evident upon transfer, as previous meta-analyses confirmed the improved outcomes in cycles in which blastocysts were transferred on Day 5 of development rather than Day 6 (Bourdon *et al.*, 2019; Li *et al.*, 2020).

Advanced maternal age is one of the main infertility causes. Aged oocytes experience chromosome segregation errors during meiosis that can result in aneuploidy and poor oocyte quality (Mikwar *et al.*, 2020). Accordingly, maternal age plays a drastic influence on embryo development (Klein and Sauer, 2001). From our data, the EmbryoScope showed improved blastocyst development and euploid rates compared with K-SYSTEMS for all age ranges, reinforcing the advantage of TLT even in patients with AMA (Figure 2). Krasnopolskaya and colleagues (Krasnopolskaya *et al.*, 2019) pointed to the harmful effects on blastulation and pregnancy rate, mainly in patients with AMA, when unnecessary long manipulation of the culture was made on Day 3.

The enhanced culture environment due to the decreased exposure of embryos to a suboptimal environment outside the incubator during evaluation and medium changeover, is possibly one of the reasons for the improved results seen for embryos cultured in the EmbryoScope. Wale and Gardner (2016) reviewed previously that multiple stress factors of the *in vitro* culture system, such as pH and temperature oscillations and exposure to atmospheric oxygen (20%) may affect embryo development. Considering these stress factors, and even though TLT microwells were mainly designed for optimal optical visibility (Vajta *et al.*, 2021), the new generation of TLT incubators could be favourable for embryo development, as minimum manipulation of the culture dish is required (Wang *et al.*, 2001). In the present study, medium refreshment was performed on Day 3 for both incubators but decreased time outside the incubator was obtained for the EmbryoScope group as no microscopic evaluations were needed to check fertilization, cleavage and blastocyst development. Furthermore, to avoid the average ICSI duration and the possible effect on the developmental delay observed in one of the groups, embryo development was checked according to the order of injection. Despite the reduced time out of the incubator for embryos cultured in TLT, it must be highlighted that different incubators, culture dishes and quantity of oil were used in the two sibling arms, which may also contribute to the observed differences in blastocyst development, rather than the difference in time out of the incubator alone. Also, it is important to point out that the use of the Embryo Viewer in the EmbryoScope arm gives more time to the embryologist to evaluate blastocyst morphology and their real potential to be biopsied, which can explain the higher percentage of biopsied embryos on Day 5 and their improved quality.

The introduction of TLT incubators for embryo development and selection is still controversial due to non-concurring data on clinical and ongoing pregnancy, miscarriage and live birth between standard incubation and TLT (Armstrong *et al.*, 2019). Unfortunately, the outcome and performance of the euploid blastocysts were not followed in this retrospective study, as the number of patients was too low, and some patients performed 'mixed' double embryo transfers with blastocysts cultured in both incubators. Although no clinical benefit has been shown so far (Armstrong *et al.*, 2019), more data from randomized controlled trials is needed to compare clinical outcomes after the transfer of euploid blastocysts, obtained from different incubators. This retrospective study just starts to analyze important data that can be explored with future larger prospective randomized studies.

In summary, the presented data add valuable information for the improvement of blastocyst culture by decreasing embryo stress. Additionally, this study provides increased evidence to support the introduction of TLT incubators into the clinical routine. To conclude, exposure of embryos to a suboptimal environment when outside the incubator during evaluation and medium changeover, may negatively effect *in vitro* blastocyst development and euploid rates on Day 5.

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