

From the Klinik für Gynäkologie und Geburtshilfe

(Director: Prof. Dr. med. Nicolai Maass)

at the University Medical Centre Schleswig-Holstein, Campus Kiel at Kiel University

**CRITICAL ANALYSIS OF BLASTOCYST CULTURE IN IN-VITRO FERTILIZATION
AND EMBRYO TRANSFER**

Dissertation

to acquire the doctoral degree (Dr. med.)

at the Faculty of Medicine

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presented by

ANUPAMA DEENADAYAL DASARI (INDIA)

Kiel (2020)

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1. Introduction

In 1977, Steptoe and Edwards successfully carried out the pioneering work of developing a human embryo outside the human body. On 25th July 1978, the world's first In Vitro Fertilization (IVF) baby, Louise Brown was born. The first German IVF baby was born at the University Department of Obstetrics and Gynaecology in Erlangen in 1982, followed by the University Hospital of Schleswig Holstein, Luebeck and Kiel in the same year. Since then, more than 8 million IVF babies have been born all over the world. Over the years, there have been numerous developments in the form of improvement of stimulation schemes, culture systems, oocyte pick up methods, embryo transfer and new freezing techniques. Several patients with male factor infertility were benefited with the development of Intra Cytoplasmic Sperm Injection (ICSI) in the 90s. Both IVF and ICSI together are referred to as Assisted Reproductive Technology (ART). With the evolution of ART, there was parallel increase in the pregnancy rates world-wide. In Germany the number of oocyte retrievals have increased from 742 in 1982 to 64,441 in 2018 (Figure 1) with 4.5% of the births (12,813) in 2018 in Germany being from ART treatments(1).

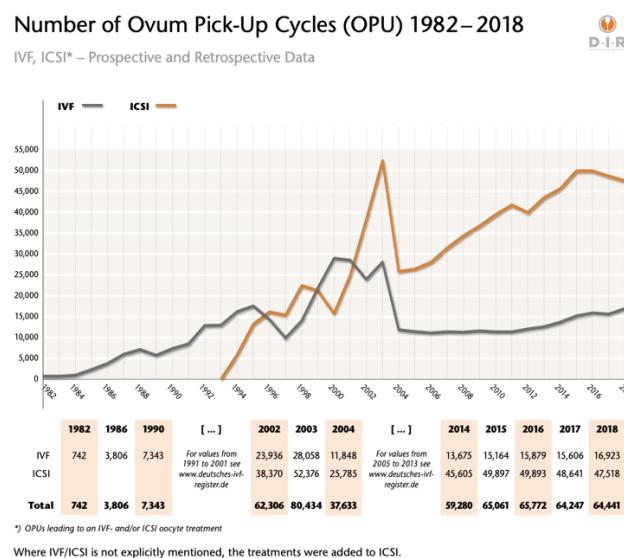


Figure 1: Number of Assisted Reproductive Technology cycles in Germany from 1982 to 2018. Adapted from the German IVF-Registry (D.I.R) 2018, Journal of Reproductive Medicine and Endocrinology

In 1988 it was hypothesised that extending culture of the embryo beyond the typical 2–3 days in vitro, to 5 days (blastocyst culture) would help identify those embryos capable of converting their development from stored maternal messages to an activated embryonic genome (2). Hence, assisting in transferring only those embryos capable of implantation and pregnancy. Culture media failed to take into account the changing physiology and requirements of the embryo and it was not until 1996 that a human blastocyst could be cultured successfully in-vitro. Since then, blastocyst culture has become the standard for the most successful labs internationally.

While most countries laws or guidelines allow all fertilized oocytes to develop in culture, allowing the developmentally competent embryos to be transferred or frozen, the ART legislation of Germany - Embryo Protection Act (ESchG- Embryonen-Schutz-Gesetz), 1990 bans embryo selection. The act states that only that many fertilized oocytes are allowed to be developed in culture that are going to be transferred. Hence, if a couple desires 2 embryos to be transferred and had 10 oocytes retrieved and 8 fertilized, then, only 2 fertilized oocytes could be cultured. The remaining 6 fertilized oocytes could be frozen or discarded or a combination of both could be performed.

In approximately 2008/2009 the so-called German Middleway (DMW- Deutscher Mittelweg) was used by many southern provinces of Germany whereby, if a couple wished, as many fertilized oocytes as necessary could be cultivated to enable identification of 2 viable embryos for transfer (3). Although complicated, many ART centres to adopted the DMW while planning a couple's treatment due to the multiple advantages of an extended culture of fertilized oocytes and enabling the identification of the most viable embryos on day 5 for transfer to improve pregnancy rates. In 2019 the German National Academy of Sciences Leopoldina and Union of the German Academies of Sciences published, "Reproductive medicine in Germany – towards an updated legal framework", in which it stated that many of the developments in ART now could not have been predicted in the 80s and hence, it is important to have new regulations with the best possible and least invasive treatments, giving due consideration to the rights of all parties, including the future children. Although there is a change word-wide to move to blastocyst transfers to improve pregnancy rates, it is still a topic of debate. Blastocyst transfer is surrounded

by controversy due to the advantages and disadvantages associated with it. Especially in Germany, with the strong ART Legislation and day 2/3 transfer being the precedent, it is necessary to critically evaluate the place of blastocyst transfer.

In the Reproductive Unit of the University Clinics of Schleswig Holstein, Kiel, Germany, the rule in the newly re-opened IVF Lab in 2014 was a day 2 or day 3 embryo transfer according to the ESchG. Hence, after performing the fertilization check on day 1, if 2 embryos we planned to be transferred, 2 were left in culture and the rest were frozen on day 1. Due to this, there was no reason to extend the culture to Day 5 except in rare cases. Eventually, we adopted the DMW which allowed for the culture of a minimal number of fertilized oocytes to day 5 to perform a successful Blastocyst transfer. Over 5 years, we increased the number of day 5 transfers while changing culture conditions and found an increase in our pregnancy rates.

It is the aim of the present study to critically analyse how day 2 or 3 embryo culture compared to day 5 embryo culture and transfer affected our pregnancy rates and to discuss the potential factors in our lab that improved embryo culture conditions and to discuss how we reached our present position of better pregnancy rates than before.

2. Methods

2.1 Patients

A prospective study was performed to analyse 1126 IVF/ICSI cycles performed between 1.2.2014 to 30.12.2018 at the University Reproductive medical unit in the UKSH (Universitätskliniken Schleswig-Holstein), Kiel, Germany to study the impact of blastocyst culture on pregnancy rates and what influences it.

The inclusion criteria were patients of the University Reproductive Medical Unit who underwent stimulation for oocyte retrieval, had oocyte retrieval by trans-vaginal follicular puncture, had fertilization after IVF/ICSI and had an embryo transfer in the same cycle. When an embryo transfer is performed in the same cycle as the stimulation, it is called a “fresh embryo transfer”.

The following patients were excluded from the study:

1. Patients who had stimulation in whom we could not retrieval any mature oocytes
2. Patients who did not have a fresh embryo transfer
3. Patients who had embryo arrest or abnormal embryos
4. Patients for social freezing and fertility preservation
5. Patients in whom ICSI was performed with testicular aspirated sperm and not with ejaculated sperm.

Out of the 1126 ART cycles, 791 had a day 2/ day 3 embryo transfer and 335 had a day 5 embryo transfer.

2.2 Ovarian stimulation and Oocyte retrieval

When a couple was selected for an IVF/ICSI cycle, controlled ovarian hyperstimulation was performed using recombinant FSH (Gonal-F, Bemfola, Rekovelle, Puregon oder Ovaleap) or urinary extracted HMG (Menogon, Pergoveris). Pituitary down-regulation was achieved by GnRH antagonist (Orgalutran (0,25mg/ 0,5ml)) from Day 6 of the stimulation and the dose (125-450 IU/day) was adjusted based on the ultrasound monitoring of follicular growth. During follicular monitoring,

when at least 3 of the follicles were >17mm in diameter, a HCG trigger of Ovitrelle (6500 IU) or alternatively Brevactid (5000/ 10.000 IU) was given. Trans-vaginal ultrasound guided oocyte retrieval either with or without anaesthesia was performed 36 hours after triggering with HCG. Oocyte retrievals were planned on Mondays, Wednesdays and Fridays while the embryo transfers were planned for day 2/3/5, depending on the previous discussion with the couple and the embryo development.

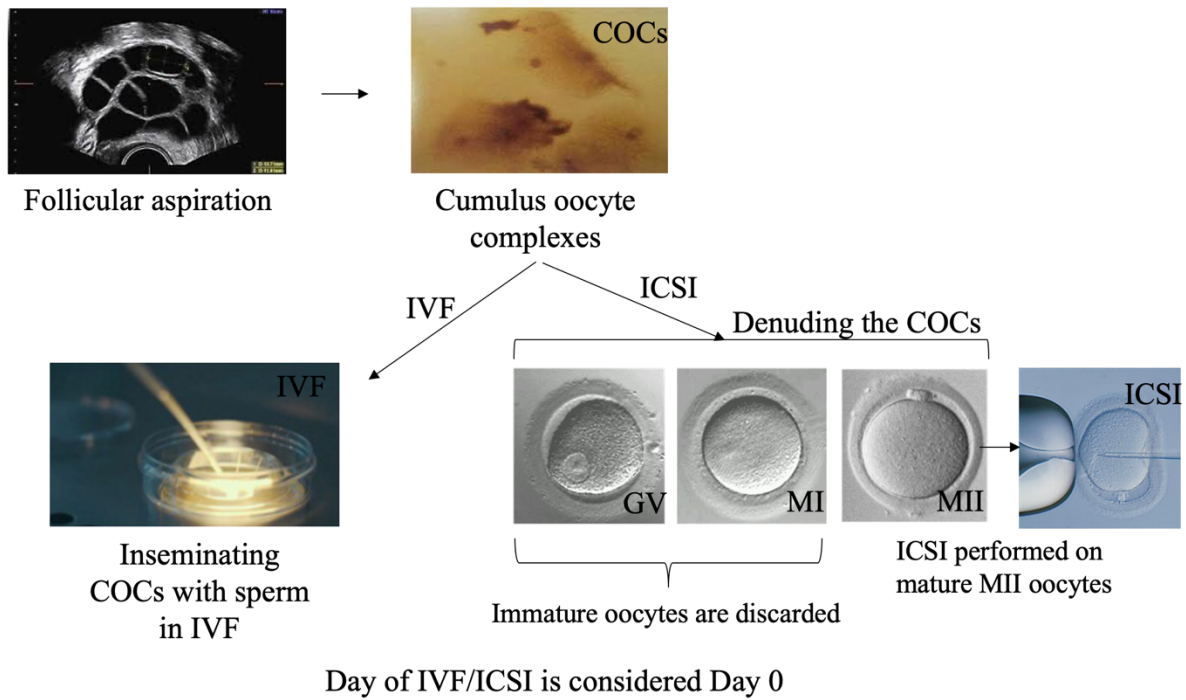
2.3 Laboratory techniques

The Cumulus Oocyte Complexes (COCs) retrieved during follicular aspiration were collected in culture media (GM501Cult with Gentamicin, Gynemed) in a culture dish (OOPW-CW05-1, Oosafe) and placed in the incubator under strict temperature, pH and clean room conditions. The partner's fresh ejaculated semen sample was parallelly processed by a standardised protocol for density gradient (GM501 Gradient 45% and 90%, Gynemed). IVF or ICSI was performed depending on the sperm characteristics. If the sperm concentration was ≥ 15 million and the progressive motility of the sperm was $\geq 32\%$, a conventional IVF (25,000 sperms were placed on each COC) was performed. In those with abnormal semen parameters, the COCs were denuded using hyaluronidase (GM501Hyaluronidase, Gynemed) and graded as immature or mature oocytes. ICSI was performed on the mature oocytes using a micromanipulator while the immature oocytes were discarded. The process in the laboratory and the development of the embryo in-vitro is summarised in figure 2.

The patients had to make 3 decisions before starting stimulation:

1. If they wanted to have a regular Day 2/3 (Cleavage stage) embryo transfer or an extended culture and a day 5 (blastocyst stage) transfer.
2. The number of embryos that would be transferred – 1, 2 or 3.
3. If they preferred culture in a time lapse incubator in which the development of the embryos could be monitored every 5 minutes by a camera system without disturbing the embryos over 5 days.

IVF/ICSI in the Lab



Development of the embryo in the ART Lab

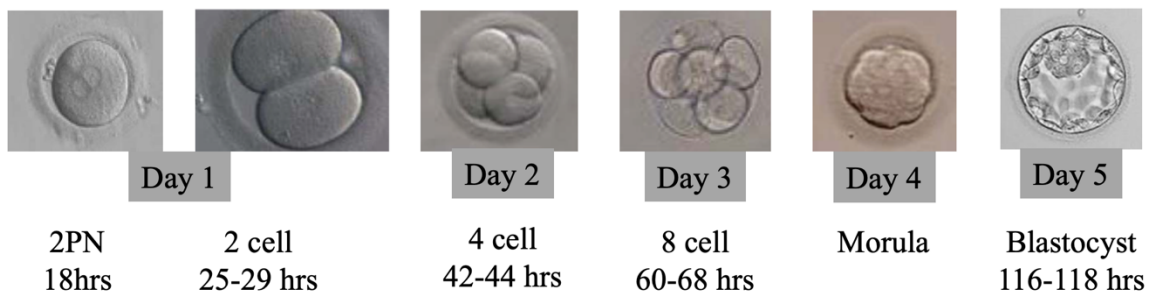


Figure 2: Overview of IVF/ICSI and embryo development in the Lab. COC: Cumulus oocyte complexes; PN: Pro-Nucleus; IVF: In-vitro fertilization; ICSI: Intra cytoplasmic sperm injection.

Considering the day of IVF or ICSI as day 0, every oocyte after IVF or ICSI was evaluated and managed as follows:

1. Day 1 - Fertilization check and freezing

After 16-18 hours of the ICSI, i.e. on Day 1 every oocyte was checked for the presence of 2 Pro-Nuclei (PN) which signifies normal fertilization. When no PN or more than 2 PN were observed, these oocytes were considered abnormal and discarded.

When the patient wanted a Day 2/3 transfer, if 2 embryos were planned to be transferred, then 2 optimal fertilized oocytes were chosen based on the zygote morphology scoring system proposed by European Society of Human Reproduction and Embryology (ESHRE) (4) and were left in culture. The rest of the 2PN stage oocytes were either frozen or discarded depending on the patient's desire. For example, if the patient wanted a single embryo transfer, then 1 fertilized oocyte was left in culture and if 3 embryos were desired, then 3 were left in culture.

When the patient wanted a day 5 transfer, depending on the age of the patient, number of embryos to be transferred and the previous ART cycles of the patient, we cultured between 2 and 6 2PN stage oocytes till Day 5 and transferred the developmentally appropriate embryos. If more than the agreed upon number of embryos to be transferred developed into blastocysts, the extra embryos were frozen.

2. Day 2 – Embryo check or embryo transfer

On Day 2, the embryos were graded according to the ESHRE consensus workshop (4) and transferred if a cleavage stage embryo transfer was planned. They were left in culture if a Day 3 or Day 5 embryo transfer was planned.

3. Day 3 – Embryo check or embryo transfer

On Day 3, the embryos were graded according to the ESHRE consensus workshop (4) and transferred if a cleavage stage embryo transfer was planned. They were left in culture if a Day 5 embryo transfer was planned.

4. Day 4 – Development of morula

On Day 4, the embryo was graded according to the ESHRE consensus workshop (4).

5. Day 5 – Development of the blastocyst stage and embryo transfer

On day 5, the embryos were graded according to the ESHRE consensus workshop (4). The development of the trophectoderm reflects the embryos ability to implant while the development of the inner cell mass reflects the ability of the foetus (5).

There is an increase in the cavity of the blastocyst during development only if the trophectoderm cells function optimally, indirectly demonstrating of embryo competence. Based on the morphological grading of the blastocyst (Figure 3), they were divided into ideal embryos and not-ideal embryos. Ideal embryos had, at least a grade 3 for the blastocoel cavity, a grade B for the inner cell mass and a grade B for the trophectoderm. Any day-5 embryos not reaching these minimum criteria was considered a non-ideal embryo. If the embryos had arrested in development and did not reach the blastocyst stage, they were discarded.

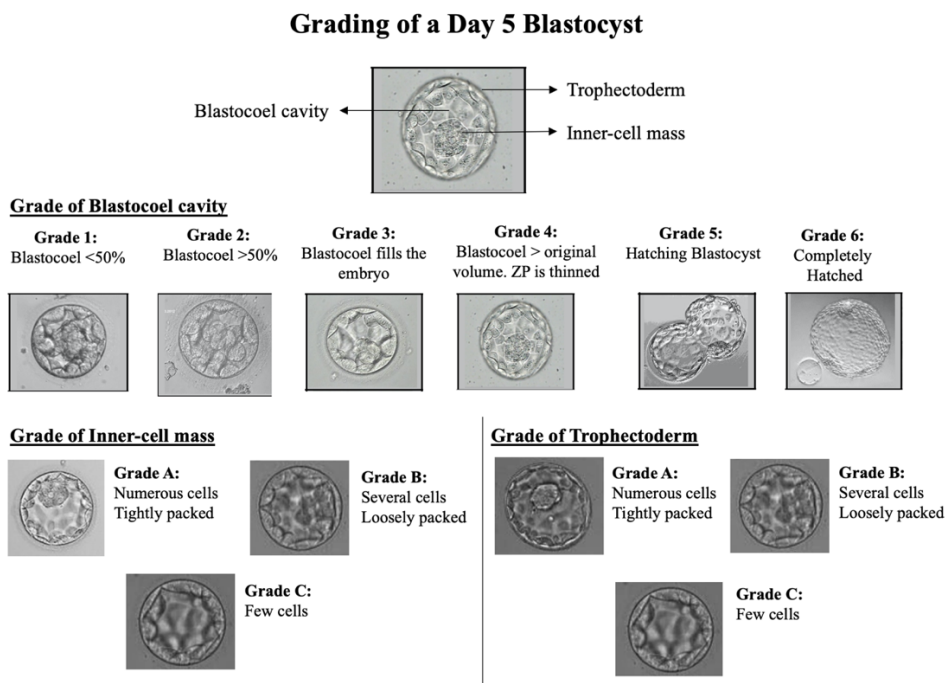


Figure 3: Grading scheme of a Day 5 embryo (Blastocyst). ZP: Zona Pelucida.

2.4 Culture Systems

An incubator (Figure 4) is a sterile chamber in which the conditions inside the uterus are simulated to foster the development of an embryo outside the body. Hence, for optimal growth of an embryo, an incubator should maintain its temperature and pH of the culture media by maintaining the CO₂ level in its chamber. When an incubator's door is opened to remove a culture dish (Figure 4d and 4e) of patient's embryos to check their progress or to change the culture media or to transfer the embryos, the environment in the incubator changes causing stress to the growing embryo. The smaller the chamber of the incubator, the faster it recovers hence, exposing the growing embryo to lesser stress.



Figure 4: Different incubators and culture dishes used in the Lab. Incubators used for culture of embryos (a,b,c); Culture dishes used for culture of embryos in the incubators (d,e)

A conventional incubator (Figure 4a) is a large incubator in which many patient's embryos in culture dishes can be simultaneously cultured. Hence, when multiple

patient's embryos are cultured simultaneously, a single opening of the incubator for evaluating the embryos of one patient affects multiple patients embryos.

A bench-top incubator (Figure 4b) has 2-8 chambers in which 2-4 patient's embryos can be cultured in each chamber. The advantage is that due to its small chamber size, it has faster recovery and only 1-4 patient's embryos are affected with a single chamber opening. In the conventional and bench-top incubators, the embryos were cultured in 30mm dishes with 50µl drops with 1-3 oocytes per drop (Figure 4d).

A time-lapse incubator (Figure 4c) is a bench-top incubator in which a single chamber can accommodate only a single patient and it has an attached camera system which takes pictures of each embryo every 5-15 minutes. Hence, the advantage is that, with every chamber opening only one patient is affected. As the culture dish need not be removed from the incubator to morphologically grade an embryo, it prevents excessive exposure to the environment outside the incubator. A time lapse incubator can be used for 6-24 patients simultaneously as it has multiple chambers.

The Esco MIRI® Time-Lapse Incubator (TL6) incubator is the time lapse incubator that was used in this study. It has 6 individual chambers for 6 patients with an exclusively designed culture dish – “culture coin” (Figure 4e) for each patient. The culture coin has 12 microwells to enable each embryo to be separately placed and positioned in the field of camera view. The system was set to take a single picture every 5 minutes to provide continuous surveillance of all embryos in “real time” without disrupting the culture, allowing important events to be observed and assist in identifying healthy embryos.

Over the period of 5 years, our lab changed its culture technique 3 times to stay abreast with the latest technology available for embryo culture. From 2013 to 2018, all three incubators were used for patients parallelly. The decision to use a particular incubator for a particular patient was completely random. In 2014 all the embryos were cultured in a conventional incubator (Heracell™ 240i CO2 Incubator, Thermo Fischer Scientific). In 2015, a bench top incubator (IVF Cube, AD-3100, Astec) with

reduced oxygen culture was parallelly introduced. In 2017, a time-lapse incubator (Miri-TL, Esco) also with reduced oxygen culture was added.

In the conventional incubator, around 6%CO₂ was the only gas used for maintaining the pH of the culture media. 94% of the remaining gas in the incubator was atmospheric air. Hence, the oxygen concentration was that of atmosphere – 21%. In the bench-top and time lapse incubators, along with 6% CO₂, 5% O₂ form the atmosphere was maintained by adding 89% nitrogen gas to the incubator. As these incubators had 3 different gasses (CO₂, O₂ and N₂) in them, they are also called “triple gas” culture system. As the oxygen concentration was lower than the conventional incubator, they are also called “reduced oxygen culture”.

2.5 Embryo transfer and verification of pregnancy

On the day of embryo transfer, the cleavage embryos and blastocysts were graded according to the ESHRE consensus workshop (4) categorised into ideal and non-ideal embryos. The embryo transfer was performed under abdominal ultrasound guidance by an after-load technique. i.e. after the outer catheter was placed by the clinician through the cervix of the patient till the inner cervical os, the inner catheter was loaded with the embryos to be transferred and brought to the clinician by the embryologist. Following this, the clinician placed the embryos under abdominal ultrasound guidance at a distance of about 1.5cm from the fundus of the uterus. Conventional luteal support was followed.

A serum beta-HCG measurement was performed 14 days after the transfer to confirm pregnancy. A beta-HCG of more than 20 IU was considered positive for pregnancy. Some of the patients were followed up till the delivery and the week of gestation, weight, sex and condition of the baby was documented.

When the beta-HCG was positive, the patient was considered to be pregnant. When a foetal heart was documented on ultrasound between the 7th and 9th week of pregnancy, it was considered a clinical pregnancy. When the pregnancy did not continue beyond the positive beta-HCG or failed after a foetal heart was detected, it

was excluded from the evaluation and added to a common group of “abortions”. Thus, biochemical pregnancies and abortions were recorded together.

2.6 Statistical analysis

Data analysis was performed using computer software JASP(6) for descriptive statistics, independent sample t-tests, χ^2 test and Fisher's exact tests. Quantitative data was analysed using t-test and categorical data was analysed using χ^2 test or Fisher's exact test. Online Chi-Square Test calculator (www.socscistatistics.com) was used when a contingency table had more than 2 rows and 2 columns of categorical variables to determine if there were non-random associations between the variables. Fisher's Exact test was used when the frequency was less than 5. A p value of less than 0.05 was considered statistically significant.

While comparing descriptive statistics between the two groups for bias, Welch test was used if the distribution was normal and if not, the Mann-Whitney test was used.

The data of binomial logistic regression analysis was computed using JAMOV (1.2.16 current). It was entered in Excel to plot the regression analysis graphs by using the formula $P(Y = 1) = 1/(1 + \exp(-(\text{Intercept} + \text{"coefficient for year"} \times \text{year} + \text{"coefficient for method"} \times \text{method})))$. All the variables that were assessed were included in all the logistic regression analysis in order to correct any probable bias. The variables were: Age of the woman, number of IVF attempts, number of oocytes retrieved, mature oocytes, immature oocytes, fertilized oocytes, number of embryos transferred, doctor who performed the oocyte retrieval and embryo transfer, embryologist who performed the oocyte screening, ICSI/IVF and embryo transfer, culture incubator used and status of the catheter after the embryo transfer. The logistic regression analysis data was accepted only when the Nagelkerke R^2 value was >0.2 .

G*Power (7) was used to compute statistical power analyses for many different t tests, F tests and χ^2 tests. A minimum power of 80% was accepted.

3. Results

3.1 General comparison of pregnancy rates

Table 1 gives the characteristics of the patients in the study - the of age, BMI, the number of ART cycles the patient had previously (ART attempt number), number of cumulus oocyte complexes retrieved (No. of COCs), number of mature oocytes (M2), number of normal fertilized oocytes (2PN), number of embryos transferred.

	Mean	Median	Standard Deviation	Minimum	Maximum
Age	35.7	36	4.6	23	48
ART attempt number	1.8	1	1.2	1	10
No. Of COCs	9.5	9	5.7	1	31
No. Of M2	6.9	7	3.5	1	25
No. Of 2PN	4.8	5	3.4	1	25
No. Of Embryos transferred	1.9	2	0.54	1	3

Table 1: Descriptive statistics of the patients. COC: Cumulus oocyte complexes; PN: Pro-Nucleus; IVF: In-vitro fertilization; ICSI: Intra cytoplasmic sperm injection

The reproductive unit of the university hospital steadily increased the number of a blastocyst cultures from 21% in 2014 to 44% in 2018 as clearly seen in Figure 5.

Pregnancy outcomes of the different groups are shown in Table 2. The overall outcome showed a pregnancy rate between 29% – 32%.

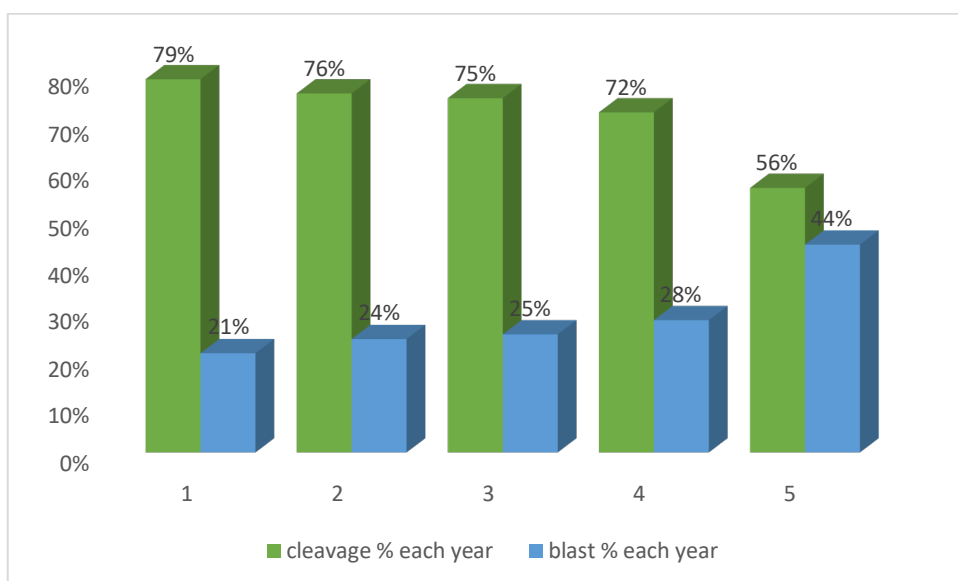


Figure 5: Percentage of blastocyst cultures compared to cleavage stage transfers each year from 2014 to 2018.

Day of transfer	Pregnant	Not pregnant	Total	Percentage
Day 2	146	350	495	29%
Day 3	84	213	296	28%
Cleavage stage (Day 2 + Day 3)	230	561	791	29%
Day 5 transfer	106	229	335	32%

Table 2: Comparison of pregnancy rates based on day of transfer of combined data from 2014 to 2018

A comparison showed that there was no statistical difference ($p=0.75$) in pregnancy rates between the Day 2 ($n=495$) and the Day 3 ($n=296$) transfer groups. When Day 2 and Day 3 transfers were combined as cleavage stage transfer group ($n=791$) and compared with the blastocyst stage, there was no significant difference ($p=0.39$) in the pregnancy rates as seen in figure 6.

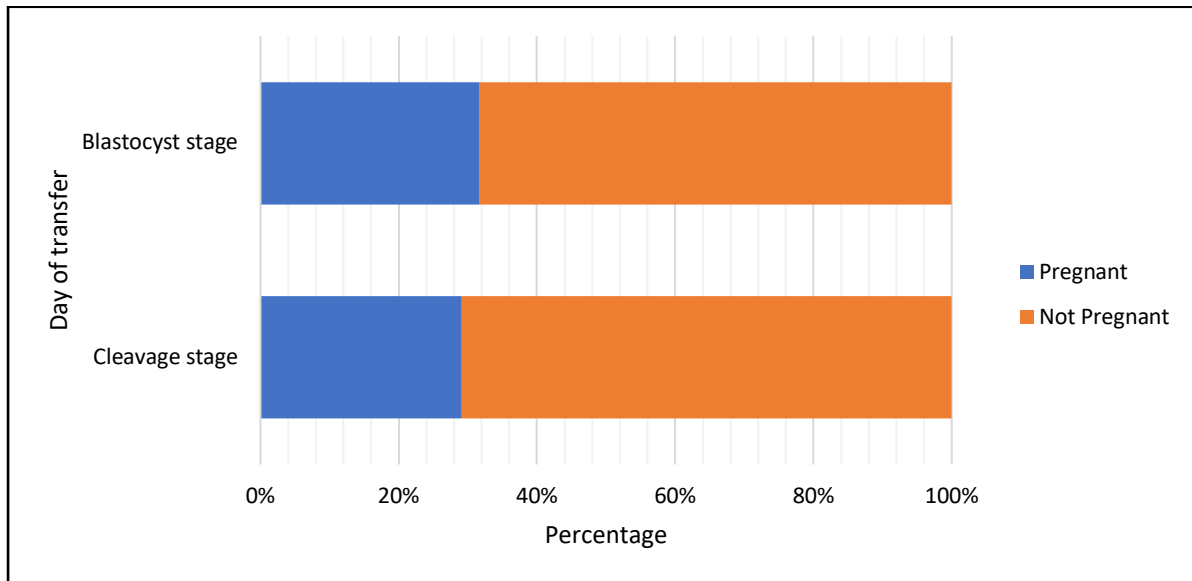


Figure 6: 100% stacked bar depiction of the pregnancy rate of total cleavage stage transfers (n=791) compared with total blastocyst transfers (n=335) from 2014 to 2018.

Further analysis of the pregnancy rates in each year from 2014 to 2018 (Table 3) showed no statistical difference in the pregnancy rates between the years for Day 2 transfer ($p=0.96$), Day 3 transfer ($p=0.67$) and combined cleavage stage transfer ($p=0.45$). When pregnancy rates of cleavage transfers were compared with day 5 transfers for each individual year, no significance was found (p value ranging from 0.06 to 0.73). However, as Figure 7 clearly depicts the statistical difference between the pregnancy rates of blastocyst transfer between the years with a chi-square statistic of 12.97 and a p -value of 0.01 (Power or 80.6%). On individually analysing the difference between the years, it was found that the most significant ($p=0.005$) improvement in pregnancy rates were between 2017 and 2018 for blastocyst transfer.

Based on the significant findings in the comparison of individual years, a logistic regression analysis was performed without taking into account any possible bias. The Nagelkerke R^2 was 0.04 (Figure 8). The graph indicated an improvement in the pregnancy rates over 5 years with Day 5 having better pregnancy rates than cleavage stage transfer. The calculated odds ratio of 1.105 implied that per year there was an increase in the pregnancy rates by 1.1 times.

	2014	2015	2016	2017	2018	Grand total
Day 2 transfers	94	91	101	111	98	495
Pregnant	32	20	27	30	20	139
Percentage Pregnant	34%	22%	26.7%	27%	30.6%	28.1%
Day 3 transfers	42	48	69	78	59	296
Pregnant	9	10	20	23	18	80
Percentage Pregnant	21.4%	20.8%	29%	29.5%	30.5%	27%
Cleavage transfers	136	139	170	189	157	791
Pregnant	41	30	47	53	38	219
Percentage Pregnant	30%	21%	27.6%	28%	24%	26%
Day 5 transfers	37	44	56	73	125	335
Pregnant	7	13	14	16	52	102
Percentage Pregnant	18.9%	29.5%	25%	21.9%	41.6%	30.4%

Table 3: Comparison of pregnancy rates in each year from 2014 to 2018 based on day of transfer. (Cleavage transfer is day 2 and day 3 transfer together)

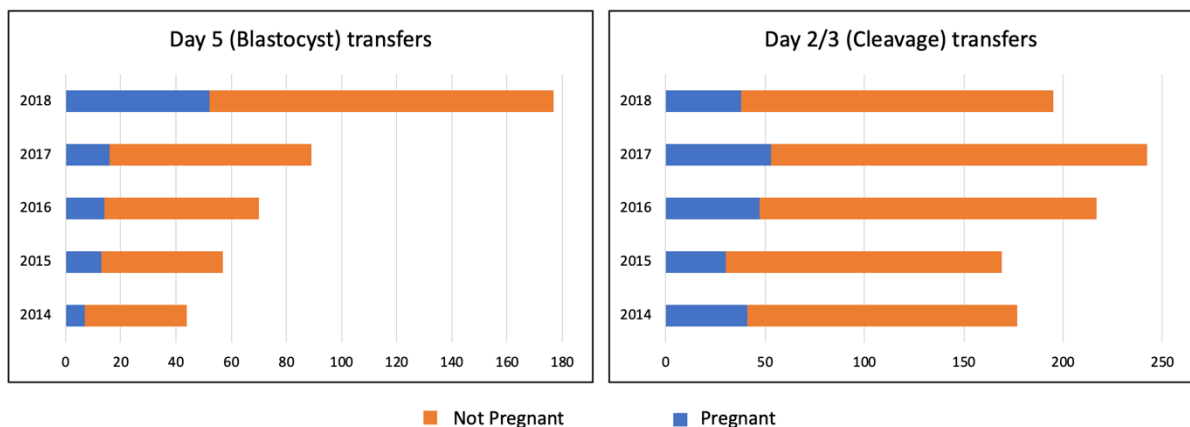


Figure 7: Stacked bar depicting for each year number of patients from 2014 to 2018 who became pregnancy after day 5 and cleavage stage transfers

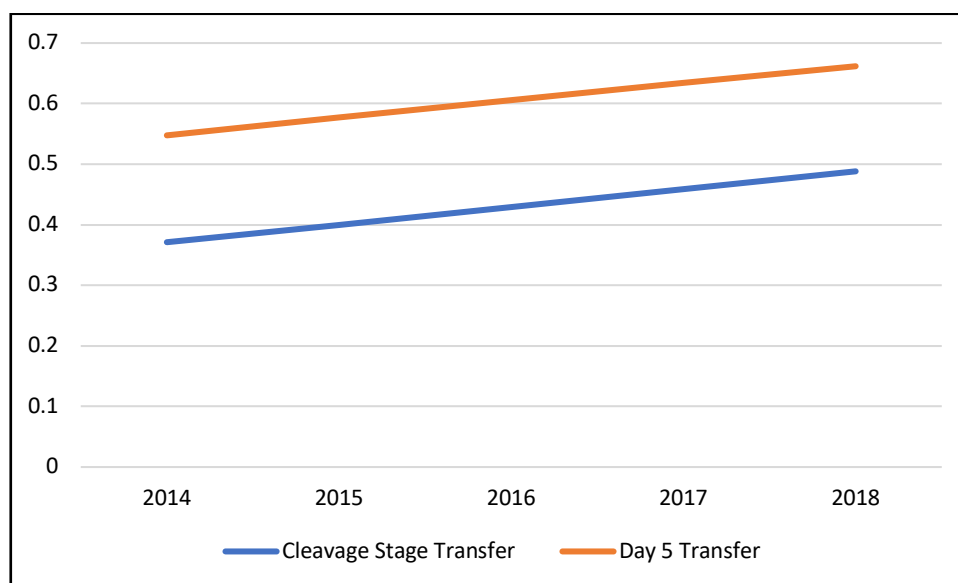


Figure 8: Graph of logistic regression curve showing pregnancy rates after cleavage transfer and blastocyst transfer from 2014 to 2018. No effect of probable bias was taken into account in this graph (Nagelkerke $R^2 = 0.04$).

		Mean	Std. Deviation	Minimum	Maximum	P value	T-Test
Age	Cleavage	35,9	4,7	23	48	0.003	Welch
	Day 5	35,0	4,3	25	44		
ART attempt number	Cleavage	1,8	1,2	1	10	0.36	Mann-Whitney
	Day 5	1,9	1,2	1	7		
No. Of COCs	Cleavage	8,6	5,6	1	32	0.3	Mann-Whitney
	Day 5	12,0	5,2	2	35		
No. Of M2	Cleavage	6,3	4,5	1	31	<0.001	Mann-Whitney
	Day 5	8,9	4,0	2	27		
No. Of 2PN	Cleavage	4,2	3,3	1	24	<0.001	Mann-Whitney
	Day 5	6,4	3,2	1	25		
No. Of Embryos transferred	Cleavage	1,8	0,5	1	3	<0.001	Welch
	Day 5	1,9	0,5	1	3		

Table 4: Comparison of basic characteristics of patients with the t-test value. $p < 0.05$ was considered significant.

An analysis comparing the baseline characteristics of the patients that could influence pregnancy rates found that age, number of oocytes retrieved, number of mature oocytes and number of oocytes fertilized showed a significant difference in the distribution between the cleavage and the blastocyst group (Table 4, Figure 9).

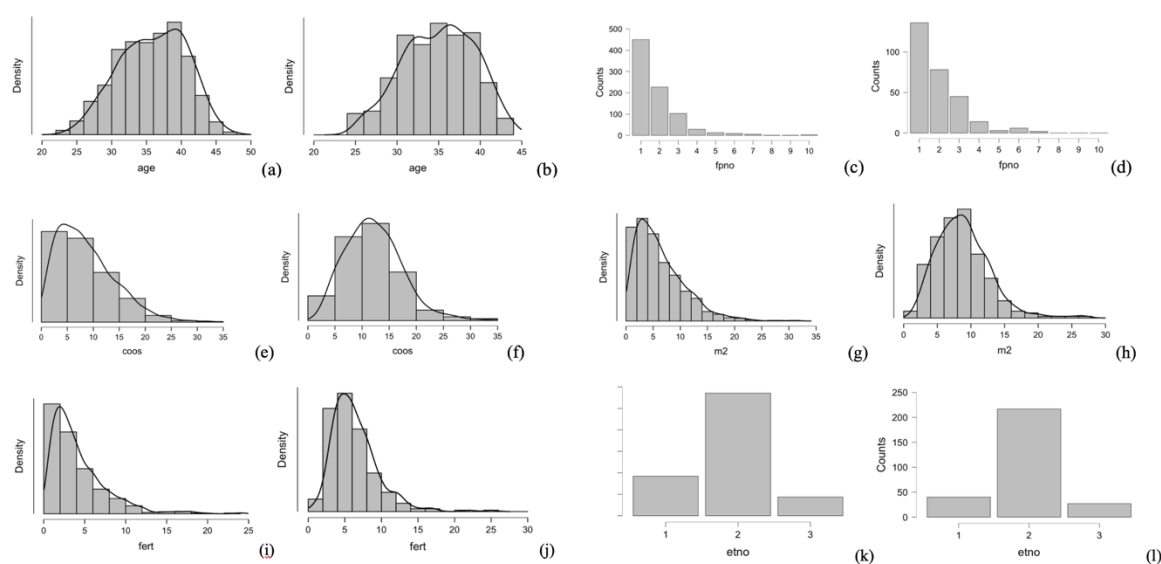


Figure 9: Distribution plots of the frequency of distribution of the characteristics of the patients in the “Blastocyst transfer” (a,c,e,g,j,k) and “Cleavage transfer” (b,d,f,h,j,l) groups. a+b: distribution plot of age of the patients; c+d: ART attempt number; e+f: number of cumulus oocyte complexes (cocs) retrieved from each patient; g+h: number of mature oocytes retrieved from each patient; i+j: number of fertilized oocytes produced by each patient; k+l: number of embryos transferred in each patient

3.2 Improvement of pregnancy rates from 2014 to 2018

Based on the significant difference between the groups, we added this information and other information as explained in “2.4 Statistical analysis” to the logistic regression model to correct the possible bias. A logistic regression analysis (Figure 10) was repeated with these variables to control their effect and it calculated the odds ratio to be 1.4, i.e. per year there was an increase in the pregnancy rates by 1.4 times.

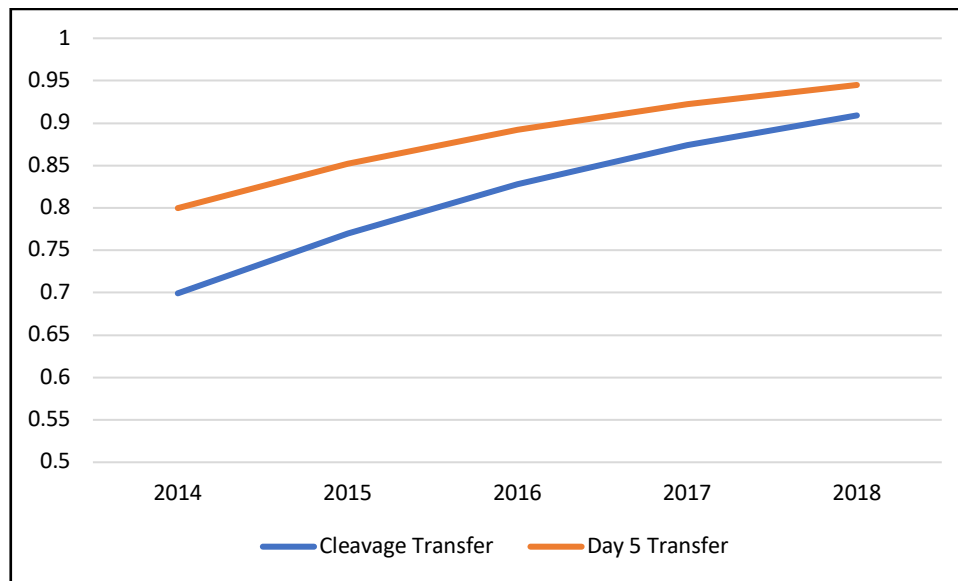


Figure 10: Graph of logistic regression showing pregnancy rates after cleavage transfer and blastocyst transfer from 2014 to 2018 after influence of various variables was taken into account. (Nagelkerke $R^2 = 0.2$)

Based on the fact that the pregnancy rates improved over the years, we broke down the data to identify the factors that changed over 5 years. We methodically examined the changes made in the clinics and in the lab separately. We explored the possibilities of change in patient characteristics, clinical treatment protocols, doctors, lab protocols, lab personnel and lab equipment over the years.

There was no variation in the basic patient characteristics and no significant changes in the stimulation protocol, type of gonadotrophin used and, timing and medication used for final oocyte maturation. There was no change in the method or timing of the oocyte retrieval or embryo transfer procedure. There was no change in the luteal phase support provided. In the clinics and the lab, the same documented standard operating procedures were followed from 2014 to 2018. A statistical analysis of the pregnancy rates between both the doctors based on who performed the oocyte aspiration ($p=0.07$) and embryo transfer ($p=0.38$) was done and no significant difference was found.

However, on scrutinizing the quality of embryos transferred (Figure 11), it was found that the number of ideal embryos for transfer had increased steady over the years

($p < 0.001$). On further inspection, the number of ideal cleavage embryos did not show a significant increase ($p = 0.1$) but, the increase in the number of ideal blastocysts was significant ($p < 0.001$).

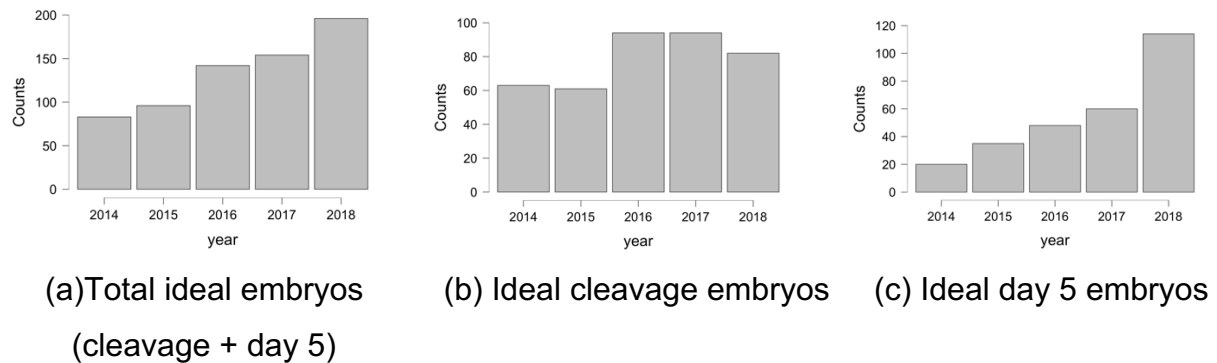


Figure 11: Histograms depicting the number of ideal embryos transferred each year. (a) number of total ideal embryos (cleavage + day 5) transferred (b) number of ideal cleavage embryos (c) number of ideal day 5 embryos transferred

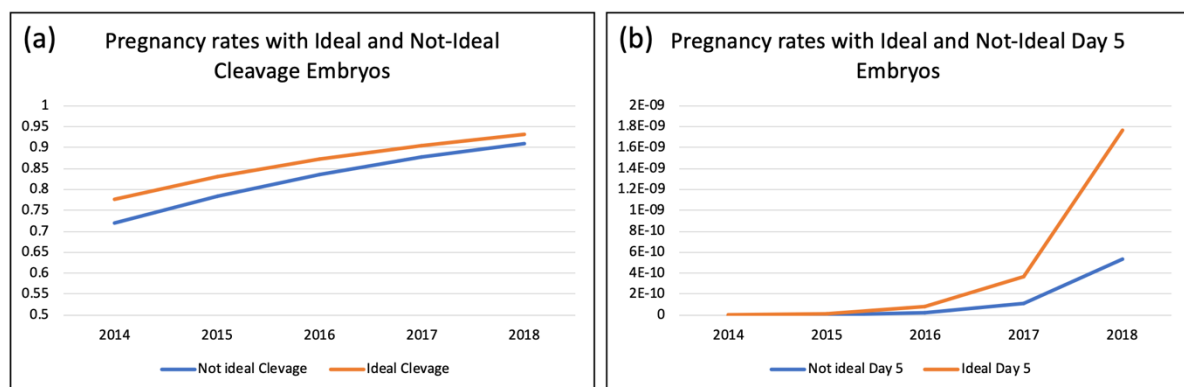


Figure 12: Graph of logistic regression showing pregnancy rates after transfer of ideal and not ideal cleavage and blastocyst stage embryos from 2014 to 2018. Nagelkerke $R^2 = 0.46$

Ideal cleavage stage embryos had significantly ($p = 0.001$) higher pregnancy rates than not-ideal cleavage stage embryos and logistic regression analysis (Figure 12a) showed an increase of pregnancy rates by 1.4 times each year. On the other hand, there was no statistically significant improvement in the number of ideal cleavage stage embryos over the years.

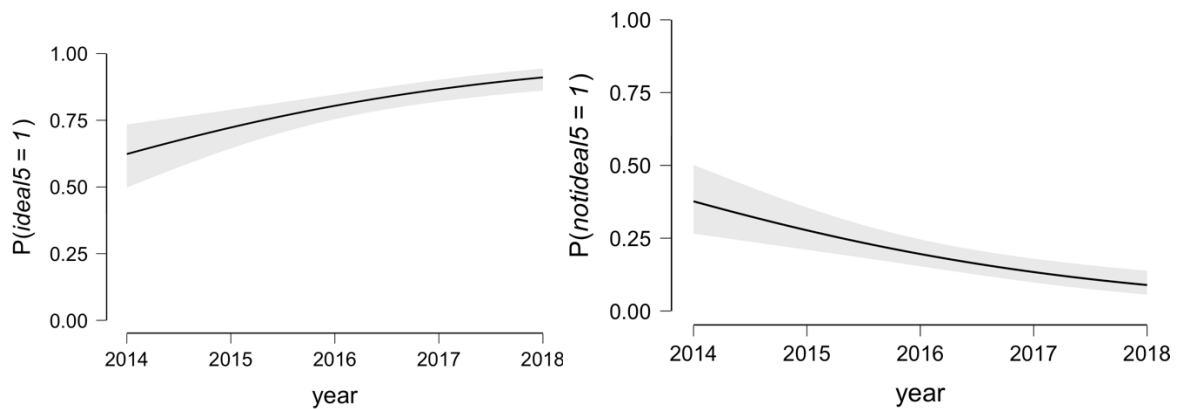


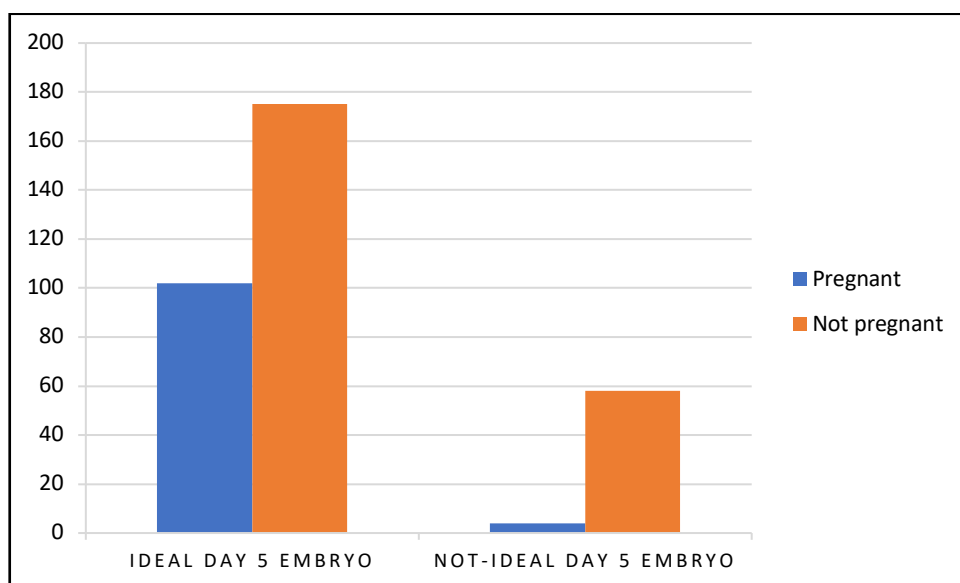
Figure 13: Logistic regression graph depicting the increase in number of day-5 ideal embryos and the decrease in the day 5 not-ideal embryos over 5 years

The pregnancy rates improved also significantly (Figure 12b) with the transfer of an ideal day 5 embryos compared to a not-ideal day 5 embryos ($p=0.001$). The pregnancy rates increased by 4.8 times each year with the transfer of ideal day 5 embryos.

A logistic regression analysis (Figure 13) of the number of ideal day 5 embryos produced per year shows an increase by 1.5 times per year in the number of ideal day 5 embryos from 2014 to 2018. There was 4 times increase in numbers after the bench-top incubator was introduced and 8 times after the time-lapse incubator was introduced. While there was a rise in the number of ideal day 5 embryos, there was a simultaneous fall by 0.64 times in the number of not-ideal day 5 embryos per year.

Extreme significance (Figure 14, $p=0.0001$, power 99%) was noticed when the pregnancy rates were compared between those patients who received a transfer of an ideal day 5 embryo and those that received a transfer of a not-ideal day 5 embryo.

While exploring the reason for increase in the number of ideal day 5 embryos leading to an improvement in the pregnancy rates, it was observed that there was no significant difference based on the embryologist who performed oocyte screening ($p=0.37$), the ICSI ($p=0.68$) or the embryo transfer ($p=0.34$).



	Pregnant	Not pregnant	Total
Ideal Day 5 Embryo	102	175	277
Not-ideal Day 5 Embryo	4	58	58
	106	229	335

Figure 14: Clustered column showing patients who got pregnant compared to those who did not get pregnant after transfer of an ideal or not-ideal day 5 embryo

There was no significant change in the operating protocols in the lab. As the only significant change was addition of new culture incubators in the lab from 2014 to 2018, on further investigation, a significant difference (Figure 15, $p=0.01$) was found in the number of ideal embryos that resulted from the culture in each incubator.

45% of cleavage stage embryos were cultured in the conventional incubator while 47% were cultured in the time-lapse incubator. 31% of the total day 5 embryos were cultured in the conventional incubator, 43% in the bench-top and 26% in the time-lapse incubator. No significant change in the pregnancy rates was seen between cleavage and day 5 transfers in each incubator individually and between the incubators.

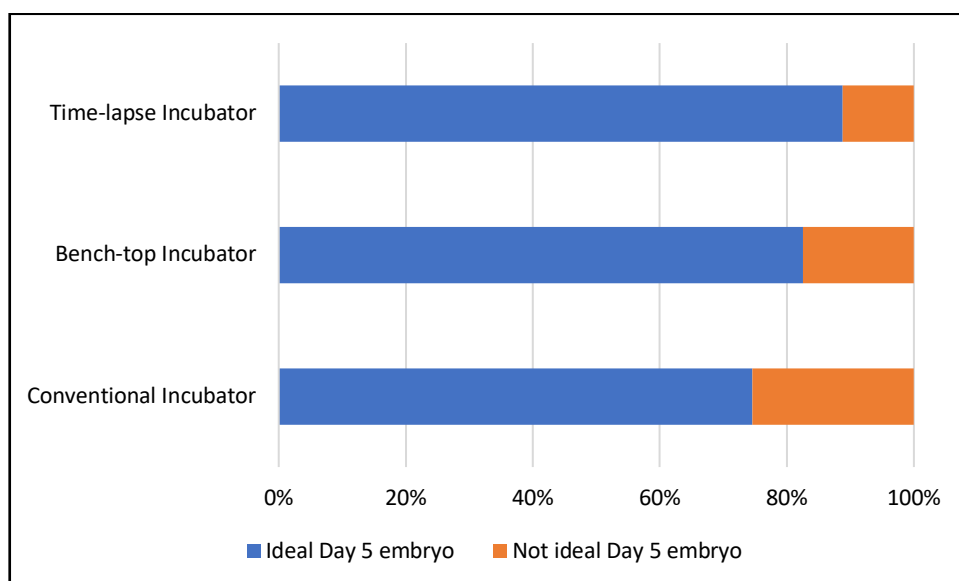


Figure 15: Percentage of Day 5 embryos that were “ideal” and “not-ideal” when cultured in different incubators. 89% of day 5 embryos in the time-lapse incubator, 83% in the bench-top incubator and 75% in the conventional incubator were “ideal”.

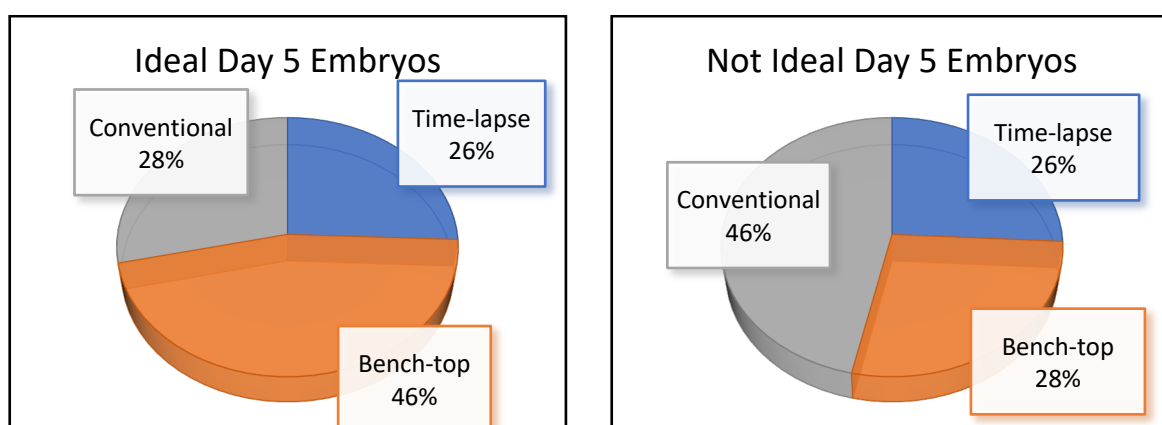


Figure 16: Pie diagrams showing the percentage of ideal and not ideal day 5 embryos from each incubator.

As depicted in Figure 16 and Table 5, the most ideal day 5 embryos were produced in the Bench-top / time-lapse incubators and the most not-ideal day 5 embryos were produced in the conventional incubator. Additionally, the comparison of the incubators with each other revealed there was no difference (Table 8, $p=0.23$) in the production on ideal embryos between the bench-top and the time-lapse incubators and the most significant difference (Table , $p=0.004$, power 84%) in the culture of ideal embryos was seen between the conventional and the time-lapse incubators.

	Ideal Day 5 embryo	Not ideal Day 5 embryo	Total
Conventional Incubator	79 (75%)	27 (25%)	106
Bench-top Incubator	71 (83%)	15 (17%)	86
Time-lapse Incubator	127 (89%)	16 (11%)	143
	227	58	335 (Grand total)

Table 5: Percentages and absolute numbers of ideal embryos that developed in each culture incubator.

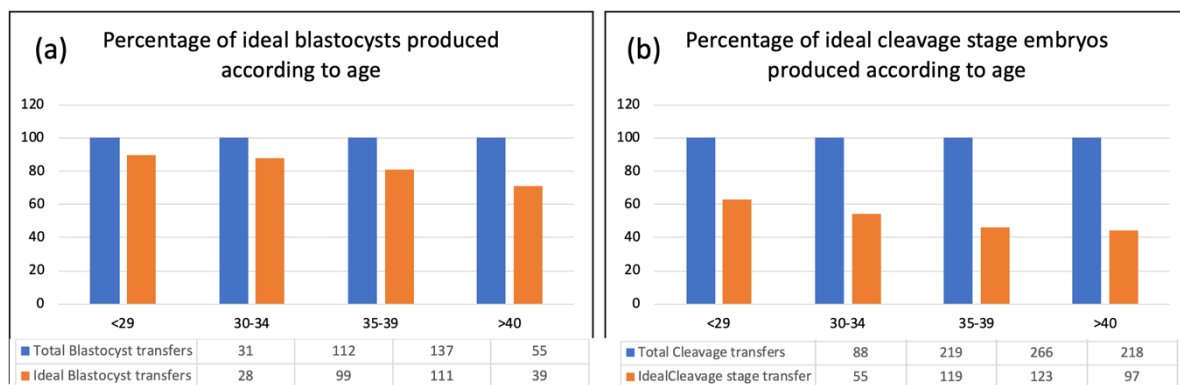


Figure 17: The relation between age of the patient and the percentage of (a) ideal blastocysts developed and (b) ideal cleavage stage embryos developed

Figure 17 depicts that as the age of the woman increased, the percentage chance of development of an ideal blastocyst decreased from 90% for a woman below 30 years to 71% for women above 40 years of age. For those between 30 and 39 years it was anywhere from 81 – 88%. Hence, the chance of a woman having an ideal blastocyst when a day 5 transfer was planned was above 70%. In contrast, in the cleavage stage transfer group, the percentage of ideal cleavage stage embryos developed were always below 64% irrespective of age.

Evaluation of the abortion rates (Figure 18b) brought to light that there was no significant difference ($p=0.75$) in the abortion rates between day-2, day-3, cleavage stage and blastocyst transfers. When individual years were compared (Figure 18a), there was also no significant difference ($p=0.33$) between the abortion rates. When an ideal day 5 embryo was transferred, there was no change ($p=0.07$) in the abortion rates compared to a not-ideal day 5 embryo. But, when an ideal and not-ideal cleavage stage embryo was transferred, it had a significant ($p=0.008$) influence on the abortion rates (Figure 19).

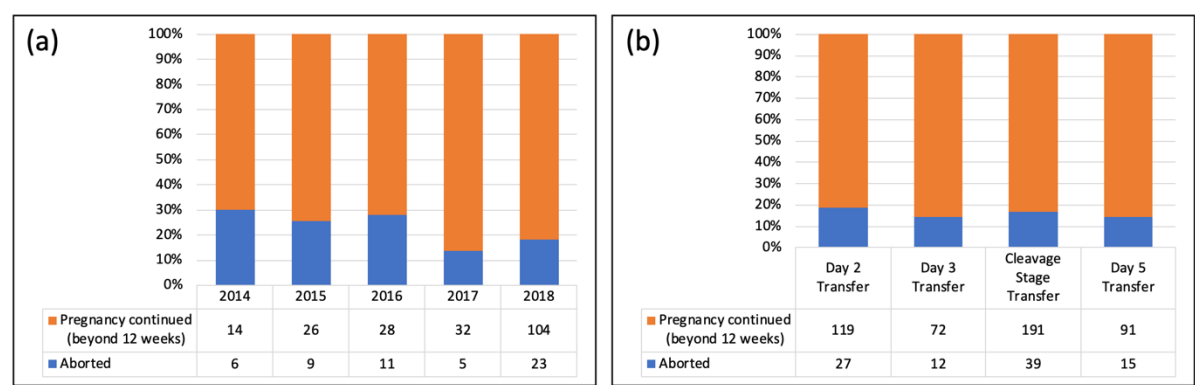


Figure 18: 100% stacked column depicting abortion rates according to year (a) and day of transfer (b)

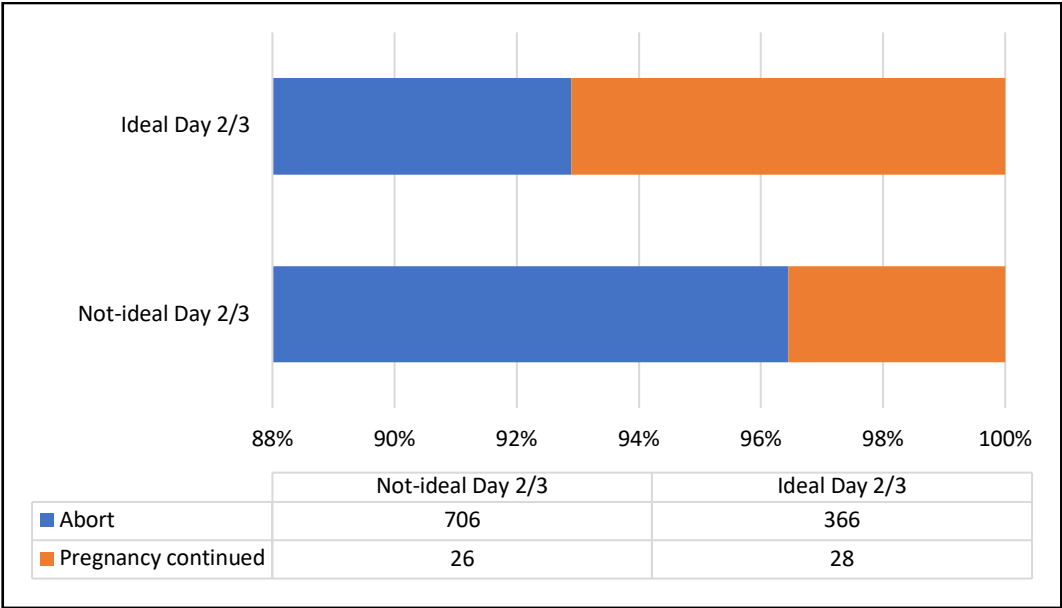


Figure 19: 100% stacked bar depiction of the abortion rate with transfer of an ideal and not-ideal cleavage embryo.

Out of 282 pregnant patients that continued beyond 12 weeks of pregnancy, 197 patients were followed up to live birth (power 19-31%). 143 had a cleavage stage transfer and 54 had a day 5 transfer. Out of the 34 twin births, 26 were from cleavage stage transfers and 8 from day 5 transfers. No statistical difference (Figure 20) was found between birth method, preterm delivery rate, sex, weight, length and health of the baby between the day 5 transfer and the cleavage transfer group. There was no increased rate of twinning when 2 blastocysts were transferred compared to 2 cleavage stage embryos.

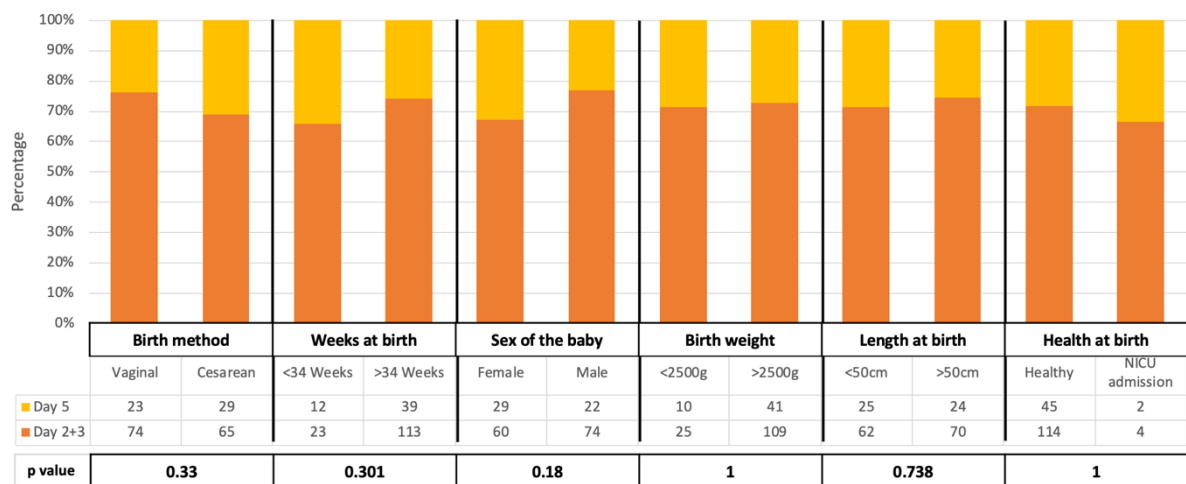


Figure 20: 100% stacked column with chi square analysis of the obstetric outcomes after cleavage stage and blastocyst stage transfer.

4. Discussion of Results

Today, more than 40 years after the first in-vitro fertilized baby was born, ART is available as a successful treatment option for infertile couples almost all over the world. Over 2.5 million cycles are performed per year resulting in 500,000 deliveries annually. Although more than 8 million children have been born, many challenges remain unanswered.

Over the past decade, there appears to be a movement of the world ART community towards blastocyst culture, elective Single Embryo Transfers (eSET) and “Freeze all” strategy to improve the singleton live birth rates. Initially, Ovarian Hyperstimulation Syndrome (OHSS) was the most feared complication of ovarian stimulation. After the improved survival of embryos after vitrification, OHSS could be avoided by performing a “Freeze all” cycle where after development of the embryos, the best embryos are frozen and transferred in another cycle. Now, the complication of multiple pregnancy could be curtailed through eSET. The success of eSET and the “Freeze all” strategy to an extent depend on successful blastocyst culture. It has been identified that for 1-2 viable blastocysts to be produced, in 85% of the ART cycles, up to 6 fertilized oocytes have to be left in culture (3). Younger compared to older women would require fewer fertilized oocytes to be left in culture as the chances of developing a blastocyst mainly depends on the age and is a highly individualised decision.

The success of an ART cycle depends on multiple clinical and lab factors. The couple's medical characteristics, their clinical evaluation and the treatment given play important roles. In the lab, the number of oocytes retrieved, the different techniques used in the lab, the development of the patient's embryos and the embryo transfer procedure are crucial to the success of an ART cycle. It is known that the age of the woman plays the most significant role in her chance of pregnancy in an ART cycle (8). The presence of uterine fibroids, polyps, adenomyosis, uterine septum, Polycystic Ovarian Syndrome (PCOS), dermoid cysts, etc. also affects the outcome. In the male partner, the presence of reduced sperm counts (oligozoospermia), sperm motility (asthenozoospermia), sperm morphology (teratozoospermia) or a combination of the three affect the outcome of an ART cycle. The presence of a

varicocele, hydrocoele, diabetes, and sperm antibodies can certainly also affect the outcome (9).

The initial days of embryonic development are driven by the maternal genome and from day 3 onwards, the embryonic genome is activated. Hence, prolonged culture enables self-selection of the embryos with higher implantation potential and accurate assessment. When many cleavage stage embryos are left in culture, in an average lab only 40% will develop into blastocysts and 60% will convert in an excellent lab (10). In-vivo, while the cleavage embryos develop in the fallopian tube, the blastocyst is the first stage of the embryo to be exposed to the uterine environment. Hence, the transfer of the embryo into the uterus at the blastocyst stage provides better physiological synchronization between the embryo and the endometrium (11) and minimizes the risk of expulsion due to exposure to increased uterine contractility which is present during the earlier days (12). It is known that blastocysts have, better cryo-survival (99%) and eSETs can be planned without decreasing the pregnancy rates while minimizing multiple gestation (13). In view of the “Embryo protection act”, if elective freezing of embryos was allowed, unnecessary freezing of fertilized oocytes with poor developmental potential could be avoided.

When a blastocyst (day 5) transfer was planned, the morphology of 4 to 6 embryos were compared to choose 1, 2 or 3 embryos for transfer. Whereas, in the cleavage stage (day 2/3) transfer, the morphology of the 2PN stage was compared to choose 1, 2, or 3 embryos for transfer. It is known that a good quality and poor quality grading of a 2PN oocyte had similar pregnancy rates (14) and embryo grading after the 2PN stage are more predictive of implantation than 2PN scoring (15). Therefore, there is a higher possibility of transferring good quality embryos in the patients opting for a blastocyst transfer (day 5) than those undergoing a cleavage stage transfer.

While several studies including the 2016 Cochrane Systematic Review concluded that live birth rate was higher in the blastocyst transfer group compared to cleavage transfer group, there have been concerns raised about the genetic and epigenetic changes produced due to prolonged culture. While the cryo-survival of blastocysts are higher, prolonged culture is associated with lower embryos to freeze and increased risk of cancelation of transfer. It has been suggested that if 1% of women have no embryos to transfer at the cleavage stage, 2%-4% will have no embryos to

transfer if a blastocyst transfer is planned (16). Exposing the embryos for longer periods to sub-optimal lab culture conditions might stress the embryos causing a fall in their implantation potential (17) while increasing the costs for no advantage. Blastocyst transfer has also been associated poor perinatal outcome (18), altered male to female ratio and increased risk on monozygotic twinning (19). While some studies stated that there was no increased risk of birth defects (20), some studies claimed an increase (21). The HEFA (Human Fertilisation and Embryology Authority) concluded that there might be an apparent reduction in risk of congenital anomalies with blastocyst transfer.

There are several meta-analyses that find no superiority of blastocyst compared with cleavage-stage embryo transfer in clinical practice (22) and numerous that support the culture till day 5. Keeping in mind the German “Embryo Protection Law”, it had been the protocol in the lab, like many labs in Germany, to perform principally day 2/3 transfers and rarely day 5 transfers.

The primary aim of this study was to critically analyse if a predominant shift to blastocyst culture and transfer improved the chances of a patient getting pregnant when they were undergoing an ART cycle in the Reproductive Unit of the University Hospital, Kiel, Germany. The critical analysis included all the possible benefits and disadvantages of shifting from day 2/3 to day 5 culture and the changes made to have an optimal blastocyst rate. In the course of the evaluation, we realised that if the ideal blastocyst development rate was >60% (10), it was an excellent evaluation of the culture system in the lab and provided early detection of negative outcomes due to changes in the conditions in the lab (23) .

It is known that as the number of oocytes retrieved increase, there is an increase in the number of mature oocytes, fertilized oocytes and a significant improvement in the possibility of pregnancy (24). In patients who have undergone several ART cycles, it has been interpreted that while the chances of pregnancy increase for every additional cycle till the 4th cycle, they decreased significantly afterwards (25). As the number of embryos transferred increased, there was a parallel increase in the pregnancy rates (26) while blood found outside the catheter after an embryo transfer was associated with low implantation rates (27). Culture conditions in the lab have

been without question been proven to affect the pregnancy outcomes but, there have been mixed opinions regarding the effect of personal (doctors and embryologists) in the presence of a fixed protocol on the pregnancy rates.

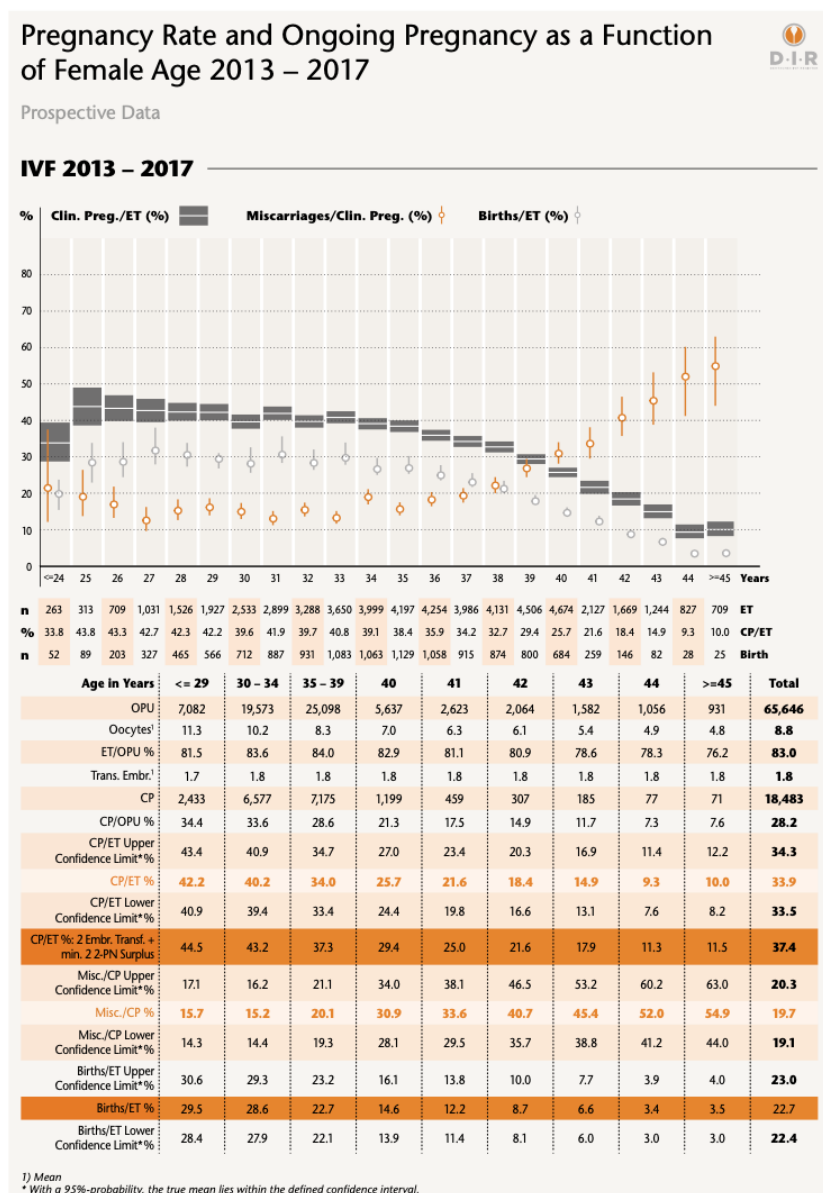


Figure 21: Pregnancy rate and ongoing pregnancy as a function of female age (2013-2017). Adapted from the German IVF-Registry (D.I.R) 2019, Journal of Reproductive Medicine and Endocrinology

The average age of a patient in this study was 36 years and when compared to the DIR, the pregnancy rates over the examined 5 years reveal that they were within the range of results in Germany (Figure 21). Initial analysis of the 1126 cycles comparing the total cleavage transfers with the total blastocyst transfers revealed no significant improvement of the pregnancy rates with blastocyst transfer. Day 2 transfers and Day 3 transfer pregnancy rates were also compared and no difference was found. It was only after comparing the cleavage vs blastocyst transfer pregnancy rates individually for each year where a significant rise in the blastocyst transfer pregnancy rate between 2017 and 2018 was found. A simple logistic regression graph depicted an increase in the pregnancy rates by 1.1 times each year for both the groups between 2014 and 2018.

A logistic regression graph was then created with an attempt to correct the possible bias in the outcome due to the variables explained previously. The new graph showed a 1.4 time increase in the pregnancy rates per year from 2014 to 2018 for both groups. Taking into account all the possible reasons for improvement in the pregnancy rates from 2014 to 2018, it was concluded that over the years, there was no change in the age or medical characteristics of the patients seeking treatment and there no alteration in the stimulation protocols used, the method of oocyte retrieval or embryo transfer.

When pregnancy rates for cleavage stage transfers were compared with day 5 transfers for each individual year, no significance was found but, the logistic regression curve showed an improvement in the pregnancy rates from 2014 to 2018 gradually over the years in both cleavage and blastocyst transfers. As there was improvement in pregnancy rates in both the cleavage stage and blastocyst stage, it could be assumed that the conditions in the lab that influenced cleavage pregnancy rates also influenced Day 5 pregnancy rates.

On evaluation of lab parameters that could have contributed the improvement in pregnancy rates, it was observed that, pregnancy rates were significantly higher when an ideal blastocyst was transferred compared to a not-ideal blastocyst. But, when transfer of ideal and not-ideal cleavage stage embryos were compared, there was no significant effect on the pregnancy rates.

It has been observed that when the blastocyst morphology is “ideal”, i.e. a higher expansion grade, top quality inner cell mass and trophectoderm, it was found to be genetically euploid (28). An in-vitro study of the interaction between the endometrium and a good or bad quality embryo demonstrated that decidual cells are programmed to select embryos that are perceived to be competent to prevent investing energy in growth and development of a less viable embryo (29). Hence, a higher pregnancy rate in ideal day 5 embryo transfer group could be explained by increased chances of implantation due to apparent euploidy and the active involvement of the endometrium as a bio-sensor in helping the ideal blastocysts implant. A year wise analysis from 2014 to 2018 showed that the number of ideal day 5 embryos increased by 1.5 times every year while the number of non-ideal embryos decreased by 0.64 times each year.

As it was clearly seen that rise of the number of ideal day 5 embryos transferred caused the overall improvement in the pregnancy rates over the years and culture of a fragile human blastocyst requires sustained stable lab conditions that support the growth of the embryo with changing needs, we analysed the changes made in the lab that were responsible for this effect. Over 5 years, the 21% oxygen tension in the incubator was changed to 5% while shifting from a conventional culture incubator to a bench top and then to a time lapse incubator.

In-vivo, a human embryo develops at a constant temperature of $37^{\circ}\text{C} \pm 2$. The constant movement of the embryo through the fallopian tube into the uterus helps in the constant supply of fresh substrates to that particular stage of the embryo for its metabolism and removal of toxins or wastes the embryo produces over 6 days. The requirements of the embryo change as it develops and the fallopian tubes and endometrium have mechanisms in place to protect and nourish the growing embryo. In the human body, the embryo has a low oxygen (5%) environment and a dynamic system around it always maintaining the right pH for its further development (30).

It is known that a change in the temperature by 2 degrees causes non-reversible damage in the spindle of the oocyte causing abnormal cleavage (31). A change in the temperature could cause a change in the pH of the media which accounts for fragmentation in embryos (32). It has been extensively studied that a low oxygen tension (5%) in incubators gives increased blastocyst rates and higher pregnancy

rates (33). Hence, when a culture dish with embryos is taken out of an incubator, it is exposed to lower room temperature, a change in pH and higher O₂ tension than normal culminating as a stressful event for the embryo. Embryo assessment during its culture in the ART lab is performed by removing it from a conventional/ bench-top incubator daily for quality assessment under a microscope by an embryologist.

While it is universally agreed upon to maintain a narrow range of temperature and pH in the incubator to facilitate the optimal growth of embryos, the concentration of O₂ in the incubator is still a matter of debate in various labs. The physiological concentration of oxygen in the fallopian tube and uterus which is the physiological home of the embryo is between 2% to 5%. A reduced oxygen tension is one of the most potent regulators of embryo function as it plays a critical role in gene expression, epigenetics and metabolism of the embryo (34). While blastocyst culture under atmospheric oxygen might not only decrease the number of ideal embryos and pregnancy rates, it might also have an adverse effect on children born (35). In this study, a comparison of the number of ideal embryos produced by each culture system showed that the time lapse and the bench top incubators which had 5% oxygen culture had a higher ideal day 5 embryo rate and pregnancy rate than the conventional incubator which had atmospheric oxygen tension for culture.

Existing reports indicate although that benchtop incubators have smaller chambers and direct heat to try to speed environmental recovery following incubator openings/closings providing faster recovery of environmental variables compared to conventional incubators, there is no clear advantage of any particular incubator based on clinical outcomes (36). The advantages of a time-lapse incubator are that embryo development can be continuously monitored without physically removing them from the incubator with the advantage of maintaining a stable culture environment and limiting the exposure of embryos to changes in gas composition, temperature, and movement. Despite evidence to show higher proportion of developmentally appropriate day 2 and day 3 embryos in time-lapse compared to bench-top incubators (37), no clear consensus has been reached on the benefits of uninterrupted embryo culture (38). While some meta-analysis claim significantly higher ongoing pregnancy rates and lower early pregnancy losses (39), the Cochrane Review of 2019 states that the quality of evidence at present is insufficient

to state that time-lapse incubators with uninterrupted culture are better than a conventional incubator (40).

When the 3 different types of incubators were individually compared to each other, there was a significant increase in the number of ideal day 5 embryos in the bench-top and time-lapse incubators compared to the conventional incubator. While, no difference in the percentage of ideal day 5 embryo production was found in between the bench-top and time-lapse incubators, the most significant difference was seen between the time-lapse and the conventional incubator.

Although in our study, there was an improvement in pregnancy rates as a result of increase in the number of ideal blastocysts cultured in the lab due to improvement in the lab conditions, the idea of Blastocyst culture for all should be viewed extremely critically. As it is known, the lesser the number of embryos developed in a cycle and the older the patient, the lower the possibility of a euploid blastocyst developing (41). In many countries Preimplantation Genetic Testing -Aneuploidy (PGT-A) is performed as a screening method to identify the euploid embryo before transfer by trophoblast biopsy of the blastocyst to improve pregnancy chances of the patient and prevent transfer of aneuploid embryos. In Germany, only polar body biopsy is allowed as a screening method and trophoblast biopsy is allowed only for detection of a known genetic disease in the family. The results from the largest randomised trial on the chromosomal screening of oocytes before embryo transfer showed failure to improve the chance of having a baby (42). Hence, in patients with lesser number of oocytes where PGT-A is not possible, it is a matter of debate if cleavage and not a blastocyst transfer should be offered as there is no role of selection in these patients.

An increase in the pregnancy rates was seen both in cleavage and blastocyst transfer groups with improvement in the culture systems in the lab. However, the effect of the improvement was more significant in the blastocyst culture compared to cleavage stage culture. It could be speculated that the blastocyst stage is more susceptible to changes in the culture environment than cleavage stage. Hence, it could be logical in situations where there are sub-optimal conditions in the lab that a cleavage stage transfer should be opted for over a blastocyst transfer.

Regardless of age, when a day 5 culture was planned, in more than 70 % of the patients, an ideal embryo was available for transfer. Whereas when a cleavage stage transfer was planned an ideal embryo was available for transfer in under 63% of patients. The possible explanation for this difference could be that in the blastocyst transfer group, 4 or more embryos were left in culture to identify the one with the best development potential for transfer whereas in the cleavage group only 1, 2 or 3 embryos were left in culture which were then transferred irrespective of their development potential.

There was no association between abortion rates and day of transfer and there was no change in the abortion rates over the years. On comparing the abortion rates in the day 5 transfer group, no difference was noticed if an ideal or not-ideal day 5 embryo was transferred. However, the abortion rate if an ideal cleavage stage embryo was transferred was significantly lower than if a not-ideal cleavage stage embryo was transferred. Although the numbers are low, it can be hypothesised that the day 2/3 morphology can predict the chances of abortion in an ART cycle.

Some studies argue that compared to cleavage stage transfer, blastocyst transfer has been associated with a skewed sex ratio in favour of males (19), higher risk of preterm delivery (18) and large for gestational age babies (43) while some assert that there is no increased risk for these factors (44). The follow up of 70% of the pregnant patients (n=197), it was found that there was no difference in the mode of delivery (vaginal or caesarean section), week of birth (pre-term or term), sex of the baby (male or female), birth weight or health of the baby at birth.

The HEFA report of all the ART cycles performed in UK clearly shows that the number of blastocyst transfers in the UK had increased from an average of 7% in 2006, 12% in 2008, 25% in 2010 to over 40% in 2012/3 with a steady upward trend. Similar to HEFA, our centre increased the blastocyst transfer rate over 5 years with 21% of the transfers being day 5 transfers in 2014 to 44% in 2018. In the UK, the 35-37-year-old population with day 5 transfer had a pregnancy rate of 46.8%. The higher pregnancy rates compared to this study could be due to the law in UK which unlike the German law allows the culture of all the fertilized oocytes and gives the ART Lab the liberty to choose the best embryos for transfer and freeze or discard extra embryos. In most countries (USA, UK, Spain, Australia and most South

American and Asian countries) that report higher pregnancy rates than Germany, there are no laws to curb the number of embryos cultured and hinder embryo selection.

The limitation of this study was that the sample size of blastocyst group was relatively small and a larger randomized controlled study should be carried out in the future to further confirm these findings. There are multiple factors that can affect the pregnancy chances in a couple in an ART cycle. While Some have been proven, most factors do not have enough strong evidence to be taken into account. In this study, effort was made to correct the bias due to a limited number of factors. No all proven and un-proven confounding factors were taken into account.

5. Conclusion

The world is moving towards extended embryo culture (Day 5 - Blastocyst Culture) and transfer of human embryos in IVF/ICSI cycles instead of the traditional day 2/3 (cleavage stage) transfer to improve pregnancy rates in patients seeking treatment for infertility. Due to the Embryo protection law preventing embryo selection, it is standard in Germany to perform day 2/3 (Cleavage) transfers and seldom, the German Middle Way is adopted to perform blastocyst transfers. Although the outcome of an ART cycle depends on a multitude of clinical and laboratory factors, this study pursued to critically explore the various advantages and disadvantages of changing the protocol in a German lab to international standards where blastocyst culture is the norm.

Analysis of 1126 ART cycles that were performed from 2014 to 2018 in the University Reproductive Medical Unit of UKSH, Kiel showed that there was an improvement in pregnancy rates from 2014 to 2018 in both cleavage stage transfer and blastocyst transfer with a 1.4 time increase every year. There was a significant rise in the pregnancy rates with blastocyst transfer compared to cleavage stage transfer as a direct result of increase in the number of morphologically ideal blastocysts cultured in the lab due to improvement in the lab culture conditions by shifting from conventional incubators which had atmospheric oxygen tension (21%) to the sustained stable culture conditions (temperature and pH) of bench-top and time-lapse incubators which had 5% oxygen. The improvement in culture conditions had a higher impact on day 5 rather than day 2/3 culture as it could be speculated that the blastocyst stage is more susceptible to changes in the culture environment than cleavage stage. Hence, it would be logical in situations where there are sub-optimal conditions in the lab that a cleavage stage transfer should be opted for over a blastocyst transfer.

Regardless of age, significantly more patients had an ideal day 5 embryos compared to a day 2/3 embryos possibly because in the blastocyst transfer group, 4 or more embryos were left in culture to identify the one with the best development potential for transfer whereas in the cleavage group only 1, 2 or 3 embryos were left in culture which were then transferred irrespective of their development potential. While ideal

day 5 embryos were an excellent tool to assess the culture system in the lab and were associated with higher pregnancy rates, not-ideal cleavage stage embryos were associated with higher abortion rates.

Although we had significant improvement in our pregnancy rates with changeover to blastocyst transfers, they were not as high as those in the UK. Perhaps because unlike the German law, culture of all the fertilized oocytes gave the ART Lab the liberty to choose the best embryos for transfer and freeze or discard extra embryos. The limitation of this study was that the sample size of blastocyst group was relatively small and a larger randomized controlled study should be carried out in the future to further confirm these findings.

In conclusion, if an IVF Unit would want to improve its pregnancy rates by shifting to blastocyst culture, it would be advisable to monitor and to improve its culture systems till the ideal blastocyst rate is >60% before its wider application. Furthermore, a change in the German Law to support wider application of Blastocyst culture would definitely have a greater impact on overall pregnancy rates.

6. Bibliography

1. Blumenauer V, Czeromin U, Fehr D, Fiedler K, Gnoth C, Krüssel J, et al. D.I.R-Annual 2018. Journal of Reproductive Medicine and Endocrinology. 2019;16:272-311.
2. G.M.Jones, A.O.Trounson, D.K.Gardner, A.Kausche, N.Lolatgis, C.Wood. Evolution of a culture protocol for successful blastocyst development and pregnancy. Human Reproduction. 1998;13:169-77.
3. Kliebisch TK, Bielfeld AP, Krüssel JS, Baston-Bust DM. The German Middleway as Precursor for Single Embryo Transfer. A Retrospective Data-analysis of the Dusseldorf University Hospital's Interdisciplinary Fertility Centre - UniKiD. Geburtshilfe Frauenheilkd. 2016;76(6):690-8.
4. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Hum Reprod. 2011;26(6):1270-83.
5. Kovacic B, Vlaisavljevic V, Reljic M, Cizek-Sajko M. Developmental capacity of different morphological types of day 5 human morulae and blastocysts. Reprod Biomed Online. 2004;8(6):687-94.
6. Team J. JASP (Version 0.12)[Computer software]. 2020.
7. Faul F, Erdfelder E, Lang A-G, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behavior Research Methods. 2007(39):175-91.
8. Bhattacharya S, Maheshwari A, Mollison J. Factors associated with failed treatment: an analysis of 121,744 women embarking on their first IVF cycles. PLoS One. 2013;8(12):e82249.
9. Fainberg J, Kashanian JA. Recent advances in understanding and managing male infertility. F1000Res. 2019;8.

10. The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators. *Reprod Biomed Online*. 2017;35(5):494-510.
11. Blastocyst culture and transfer in clinically assisted reproduction: a committee opinion. *Fertil Steril*. 2018;110(7):1246-52.
12. Fanchin R. Assessing uterine receptivity in 2001: Ultrasonographic glances at the new millennium. *Annals of the New York Academy of Sciences*. 2001;943:185-202.
13. Mehta VP, Patel JA, Gupta RH, Shah SI, Banker MR. One Plus One Is Better Than Two: Cumulative Reproductive Outcomes Are Better after Two Elective Single Blastocyst Embryo Transfers Compared to One Double Blastocyst Embryo Transfer. *J Hum Reprod Sci*. 2018;11(2):161-8.
14. Payne JF, Raburn DJ, Couchman GM, Price TM, Jamison MG, Walmer DK. Relationship between pre-embryo pronuclear morphology (zygote score) and standard day 2 or 3 embryo morphology with regard to assisted reproductive technique outcomes. *Fertil Steril*. 2005;84(4):900-9.
15. Weitzman VN, Schnee-Riesz J, Benadiva C, Nulsen J, Siano L, Maier D. Predictive value of embryo grading for embryos with known outcomes. *Fertil Steril*. 2010;93(2):658-62.
16. Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev*. 2016(6):Cd002118.
17. Gardner DK, Schoolcraft WB. Human embryo viability: what determines developmental potential, and can it be assessed? *Journal of assisted reproduction and genetics*. 1998;15(8):455-8.

18. Maheshwari A, Kalampokas T, Davidson J, Bhattacharya S. Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer of blastocyst-stage versus cleavage-stage embryos generated through in vitro fertilization treatment: a systematic review and meta-analysis. *Fertil Steril*. 2013;100(6):1615-21.e1-10.

19. Chang HJ, Lee JR, Jee BC, Suh CS, Kim SH. Impact of blastocyst transfer on offspring sex ratio and the monozygotic twinning rate: a systematic review and meta-analysis. *Fertil Steril*. 2009;91(6):2381-90.

20. Ginstrom Ernstad E, Bergh C, Khatibi A, Kallen KB, Westlander G, Nilsson S, et al. Neonatal and maternal outcome after blastocyst transfer: a population-based registry study. *Am J Obstet Gynecol*. 2016;214(3):378.e1-.e10.

21. Dar S, Lazer T, Shah PS, Librach CL. Neonatal outcomes among singleton births after blastocyst versus cleavage stage embryo transfer: a systematic review and meta-analysis. *Hum Reprod Update*. 2014;20(3):439-48.

22. Martins WP, Nastri CO, Rienzi L, van der Poel SZ, Gracia C, Racowsky C. Blastocyst vs cleavage-stage embryo transfer: systematic review and meta-analysis of reproductive outcomes. *Ultrasound Obstet Gynecol*. 2017;49(5):583-91.

23. Hammond ER, Morbeck DE. Tracking quality: can embryology key performance indicators be used to identify clinically relevant shifts in pregnancy rate? *Hum Reprod*. 2019;34(1):37-43.

24. Hariton E, Kim K, Mumford SL, Palmor M, Bortoletto P, Cardozo ER, et al. Total number of oocytes and zygotes are predictive of live birth pregnancy in fresh donor oocyte in vitro fertilization cycles. *Fertil Steril*. 2017;108(2):262-8.

25. Meldrum DR, Silverberg KM, Bustillo M, Stokes L. Success Rate with Repeated Cycles of In Vitro Fertilization–Embryo Transfer. *Fertility and Sterility*. 1998;69(6):1005-9.

26. Cetin MT, Kumtepe Y, Kiran H, Seydaoglu G. Factors affecting pregnancy in IVF: age and duration of embryo transfer. *Reprod Biomed Online*. 2010;20(3):380-6.
27. Goudas VT, Hammitt DG, Damario MA, Session DR, Singh AP, Dumesic DA. Blood on the embryo transfer catheter is associated with decreased rates of embryo implantation and clinical pregnancy with the use of in vitro fertilization-embryo transfer. *Fertil Steril*. 1998;70(5):878-82.
28. Minasi MG, Colasante A, Riccio T, Ruberti A, Casciani V, Scarselli F, et al. Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: a consecutive case series study. *Hum Reprod*. 2016;31(10):2245-54.
29. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo quality. *Biol Reprod*. 2014;91(4):98.
30. Sciorio R, Smith GD. Embryo culture at a reduced oxygen concentration of 5%: a mini review. *Zygote*. 2019;27(6):355-61.
31. Wang W-H, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling–rewarming observed using polarized light microscopy. *Human Reproduction*. 2001;16(11):2374-8.
32. Elder K, Van den Bergh M, Woodward B. *Troubleshooting and Problem-Solving in the IVF Laboratory*. Cambridge: Cambridge University Press; 2015.
33. Gelo N, Kirinec G, Baldani DP, Vrcic H, Jezek D, Milosevic M, et al. Influence of human embryo cultivation in a classic CO₂ incubator with 20% oxygen versus benchtop incubator with 5% oxygen on live births: the randomized prospective trial. *Zygote*. 2019;27(3):131-6.
34. Kasterstein E, Strassburger D, Komarovskiy D, Bern O, Komsky A, Raziel A, et al. The effect of two distinct levels of oxygen concentration on embryo development in a sibling oocyte study. *J Assist Reprod Genet*. 2013;30(8):1073-9.

35. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update*. 2016;22(1):2-22.
36. Swain JE. Decisions for the IVF laboratory: comparative analysis of embryo culture incubators. *Reprod Biomed Online*. 2014;28(5):535-47.
37. Kalleas D, McEvoy K, Horne G, Roberts SA, Brison DR. Live birth rate following undisturbed embryo culture at low oxygen in a time-lapse incubator compared to a high-quality benchtop incubator. *Hum Fertil (Camb)*. 2020:1-7.
38. Swain JE. Controversies in ART: considerations and risks for uninterrupted embryo culture. *Reprod Biomed Online*. 2019;39(1):19-26.
39. Pribenszky C, Nilselid AM, Montag M. Time-lapse culture with morphokinetic embryo selection improves pregnancy and live birth chances and reduces early pregnancy loss: a meta-analysis. *Reprod Biomed Online*. 2017;35(5):511-20.
40. Armstrong S, Bhide P, Jordan V, Pacey A, Marjoribanks J, Farquhar C. Time-lapse systems for embryo incubation and assessment in assisted reproduction. *Cochrane Database Syst Rev*. 2019;5:Cd011320.
41. Esteves SC, Carvalho JF, Bento FC, Santos J. A Novel Predictive Model to Estimate the Number of Mature Oocytes Required for Obtaining at Least One Euploid Blastocyst for Transfer in Couples Undergoing in vitro Fertilization/Intracytoplasmic Sperm Injection: The ART Calculator. *Front Endocrinol (Lausanne)*. 2019;10:99.
42. Verpoest W, Staessen C, Bossuyt PM, Goossens V, Altarescu G, Bonduelle M, et al. Preimplantation genetic testing for aneuploidy by microarray analysis of polar bodies in advanced maternal age: a randomized clinical trial. *Hum Reprod*. 2018;33(9):1767-76.

43. Wang X, Du M, Guan Y, Wang B, Zhang J, Liu Z. Comparative neonatal outcomes in singleton births from blastocyst transfers or cleavage-stage embryo transfers: a systematic review and meta-analysis. *Reprod Biol Endocrinol.* 2017;15(1):36.
44. Marconi N, Raja EA, Bhattacharya S, Maheshwari A. Perinatal outcomes in singleton live births after fresh blastocyst-stage embryo transfer: a retrospective analysis of 67 147 IVF/ICSI cycles. *Hum Reprod.* 2019;34(9):1716-25.

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