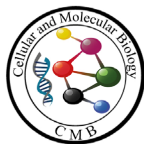

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Original Research

Morphokinetic analysis of human embryo development and its relationship to the female age: a retrospective time-lapse imaging study

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Abstract: Time-lapse imaging technique has provided embryologists with a unique chance of studying the embryo morphokinetics to select the most viable embryos for implantation in the field of IVF (In vitro fertilization). The conventional method of morphological evaluations has proved that maternal age affects the human embryo quality. This retrospective study carried out at Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan mainly focuses on the effect of female age on human embryo morphokinetics. A total number of 200 patients undergoing ICSI treatment cycles at the clinic were selected for the study and divided into five age groups (< 26, 26-30, 31-35, 36-40, and > 40 years). Embryo culture was done at 37°C, 6% CO_2 and 5% oxygen for 5-6 days. Ten time-points were selected for kinetic analysis. The number of retrieved, matured, fertilized and cleaved oocytes showed highly significant difference ($P \leq 0.0001$) when compared among different age groups. There was no significant difference in average morphokinetic time-points among young versus old women.Whereas timely cleaved embryos showed significant difference in tPNa i.e. time for pronuclear appearance ($P \leq 0.001$), t4 and t5 i.e. time for 4 and 5-cell cleavage ($P \leq 0.05$) among different age groups. The clinical pregnancy rates showed a decline with increasing age. These results indicate the effect of female age on time-lapse embryo morphokinetic parameters. In future the addition of time-lapse analysis in routine IVF can help to improve the success rate by selecting the most viable embryos for uterine transfer.

Key words: Human embryo; Time-lapse imaging; Female age; Blastocyst; Early cleavage; Morphokinetics; Implantation.

Introduction

Assisted reproduction technologies (ART) have provided a lot of help to infertile couples. Now days the latest advancements in the field of IVF have made it easy to observe the human embryonic development more closely. It is critical to identify good quality viable embryos with the highest implantation potential. Hence many criteria for selection of best quality embryos have been suggested so far emphasizing the morphological assessment of embryos. But the conventional method of cell count and morphology are not the only points to be taken into consideration while interpreting the quality of embryos and their implantation potential. Presently embryos are assessed once a day at defined hours post microinjection or insemination to avoid any undesired change in embryo culture environment i.e. temperature and pH etc. (1-4).

The dynamic nature of embryo development is known to all of us and therefore a fixed time-point evaluation of the morphological features can mislead to differentiate between their potential at different cell stages. This developmental kinetics of embryos has become more evident by use of time-lapse imaging in the clinical IVF set ups. It has been shown by the previous information that the accurate timings of particular events, for example, pronuclear arrangement, syngamy, early cleavage, cell cycle intervals, synchronicity of cell division and start of blastulation are important markers of embryonic development. A constant assessment of embryos for these markers may therefore aid in selecting best embryos for implantation. The previous data show that morphokinetic observations can help enormously in the critical evaluation of embryonic development (5-11).

There are number of different time-lapse embryo monitoring system being used in the field of IVF these days. The Primo Vision™ Time-Lapse System (VitroLife,Frolunda, Sweden) has been in wide spread practice in IVF laboratories and gained huge interest in recent years. This embryo monitoring system includes primo vision microscopes, software and culture dishes. Using this system, embryonic development can be monitored at all times without eliminating embryos from the incubator. So we can conveniently asses their developmental potential without any risks of temperature-changes, light exposure, high oxygen exposure, and pH changes in the culture medium (12, 13).

It must also be noted that the growth of embryos is not consistent and regular event and may be influenced by external factors including culture conditions as well as patient factors. These may include culture media (14), stimulation protocols (15), insemination method (16, 8), obesity (17), smoking (18) and oxygen tension (19). Among these factors one of the most important parameter is the age of the female, when blastocyst score is assessed in an IVF treatment (20, 21). In a previous study it is shown that the embryos with early cleavage

occurred more commonly in younger than older women and showed high implantation and pregnancy rates (22). Moreover maternal age does affect the stimulation regime, embryo viability, quality as well as the conception rates (23-27). Whereas another finding showed no difference in the blastocyst quality when compared among different age groups of females (28).

The patients seeking fertility treatment with age > 35 years are constantly growing in number thus it is important to evaluate the embryo quality and pregnancy out come to counsel them properly regarding the treatment plans and probability of conception with extended embryo culture. Hence main objectives of the present study included:

1) To focus on early cleavage pattern of embryos analyzed from female patients of different age groups and to assess their potential of blastocyst formation, quality of blastocyst formed and success rates using time-lapse imaging.

2) To observe any remarkable differences in morphokinetic parameters of good quality and poor quality embryos.

3) To compare the time-lapse kinetic data with previously evaluated embryo selection markers.

Materials and Methods

Ethical approval of the study

The data used in this study were obtained from Islamabad Clinic Serving Infertile Couples (ICSI), Islamabad, Pakistan from January 2013 to December 2015 after an informed consent of patients in accordance with ethical guidelines set by the clinic for IVF cycles.

Study Design

This study was a retrospective observe of prospectively obtained data of time-lapse imaging of human embryos during in vitro development. The procedures and protocols were approved and practiced by the Institution. The number of oocytes observed in this study was 4080 resulting into 2266 cleaved embryos in 200 ICSI (Intracytoplasmic sperm injection) treatment cycles. A total number of 1704 embryos were included in time-lapse monitoring due to some split cycles for conventional culturing as a quality control measure at the outset of time-lapse system in the clinic.The patients were divided into five age groups (<26, 26-30, 31-35, 36-40 and > 40 years old). Females showed a mean age of 31.8±0.3 years. Most of the embryos were obtained after fertilization by ICSI while very few patients had ICSI+IVF cycles. Embryos were evaluated for time-lapse morphokinetics by measuring precise timings of development. Ten important time-points for embryo development were compared. Embryo transfer data of both fresh and cryopreserved cycles of patients were analyzed. Implantation was confirmed by the βhCG levels in the blood sample taken after 10 days of embryo transfer and later on at an ultrasound scanning for fetal heart after 7 weeks of pregnancy.

The time-points (hours post ICSI) analyzed along with the abbreviations used to indicate the corresponding developmental stages and also the reference range used for each time-point with respect to ALPHA-ESHRE Consensus on Embryo Assessment is as follows (64):

a. Two pronuclear stage (tPNa) = 17±1 hrs.
b. 2-cell stage (t2) = 26±1 hrs.
c. 4-cell stage (t4) = 44±1 hrs.
d. 8-cell stage (t8) = 68±1 hrs.
e. Compaction (tM) = 92±2 hrs.
f. Fully Expanded blastocyst (tB) = 116±2 hrs.

In addition to these, the embryos were analyzed for four more time-points i.e. at 3-cell cleavage (t3), 5-cell cleavage (t5), start of blastulation (tSB) and at expanding blastocyst stage (tEB) in order to assess the developmental events more critically. The reference range for each of these four time-points was taken from the average of optimally growing embryos at the study set up. Thus optimal range for t3 was taken as (31-42 hrs.) for t5 (45-54 hrs.) for tSB (98-105 hrs.) and for tEB (107-114 hrs.) respectively. The abbreviation (tFEB) used in the present study indicates the cleavage time for fully expanded blastocyst stage.

The exclusions criteria for this study were as follows.

a) Unfertilized oocytes were excluded from data.

b) All embryos whose time-lapse image acquisition were started later than 20 hours and ended before 60 hours after ICSI were not included.

c) Extreme outliers were excluded that may bias the analysis (as extremes may distort the mean of the regular divisions).

The images of embryo development by time lapse analyses was re-evaluated either the lab director or one of the senior embryologists. At the end a collective consensus was made by reviewing the videos and setting the guidelines.

Down regulation and ovarian stimulation

For pituitary desensitization both long and short protocols were used. Women were given a subcutaneous administration of GnRH-agonist (gonadotropin releasing hormone-agonist) i.e. Decapeptyl 0.1 mg or Buserelin acetate (suprefact 0.1 mg) daily injections. Ovarian stimulation was carried out using daily injections of follicle stimulating hormone (FSH) by starting doses based on serum anti-Mullerian hormone levels, antral-follicle counts and previous responses to ovarian stimulation. The future doses were adjusted based on follicular monitoring by trans vaginal ultrasound (7.5 MHz probe; Aloka 500, Tokyo, Japan) three to four days after initiation of the ovarian stimulation.

Both GnRH agonist and the GnRH antagonist protocol were used for stimulation. Buserelin acetate 0.1 cc (Suprefact: Sanofi, Paris, France) was given daily from day 21 of previous menstrual cycle in the GnRH agonist group. Recombinant follicle-stimulating hormone (Follitrope; LG Life Sciences Korea or puregon;Organon, Oss, Canada) and human menopausal gonadotropin (IVF-M; LG Life Sciences, Korea) injections were given from third day of cycle till hCG (IVF-C; LG Life Sciences, Korea) administration. For GnRH antagonist group ovarian stimulation was started from third day of cycle daily along with the injection of 0.25 mg cetrorelix acetate (Cetrotide: Merck, Germany) subcutaneously till hCG administration when at least 2 leading follicle reach 18 mm in diameter. Oocyte retrieval was done at 36-37 hours post hCG.

Oocyte retrieval, ICSI and embryo culture in Time-Lapse System

Oocytes were collected 36-37 hours later by transvaginal needle aspiration of follicles under ultrasound guidance under TIVA (Total intravenous anesthesia) with Propofol used for short sedation. Oocytes were rinsed in three drops of G-IVF™ PLUS (vitolife, Frolunda, Sweden)and then placed in a single well 60 mm culture dish in G-IVF™ PLUS under an oil overlay OVOIL™ (vitrolife) pre equilibrated at 37°C with 6% $CO_2$and 5% oxygen. Dishes were incubated at 37°C with 6% CO_2 and 5% oxygen in the multi gas incubator (Sanyo, Osaka, Japan).

The denudation of oocytes was done 2-3 hours after the retrieval using hyaluronidase (Hyase 10X; vitrolife) and then transferred to the pre-equilibrated dish containing the 40-50 µl drops of G-GAMETE™ PLUS (vitrolife). ICSI was done on mature oocytes using standard protocols. Injected oocytes were transferred to pre-equilibrated 40-50µl drops of G-1™PLUS (vitrolife) under an oil overlay and incubated overnight at 37°C with 6% $CO_2$and 5% oxygen. A 9-well or 16-well primo vision™ micro well group-culture dish (vitrolife) was prepared by filling with 70-80µl of G-TL™ (vitrolife) with an oil overlay, on the day of egg collection for embryo culture after the evaluation of fertilization. It was made sure that each well is properly filled with culture media and dish was incubated overnight. The fertilization check was done 16-18 hours post ICSI. Zygotes were moved to pre-equilibrated primo vision group-culture dish keeping in mind to remove any bubbles in the wells before placing them. Dish containing zygotes was placed in the primo vision™microscope placed in the chamber of multi gas incubator (Sanyo) and connected to the primo vision™software for time-lapse monitoring and cultured for 5-6 days at 37°C with 6% CO_2 and 5% oxygen. The time-lapse video created by combined images was used to monitor the timings of specific cleavage stages as well as any associated abnormalities.

Time-lapse system and Embryo assessment

The Primo Vision Time-Lapse System consists of 1-6 Microscopes Units, an external Controlling unit that runs the installed Capture and Analyzer software, and micro well culture dishes for in vitro culture of the observed embryos. USB and power cables are also included.

A specially designed compact, airtight, digital inverted microscope (Primo Vision; Cryo-Innovation, Budapest, Hungary) was placed inside a common multi gas incubator (Sanyo, Japan). It is specially built for comfortable in-incubator use with high precision optical system. Primo vision culture dishes were placed on the dish holder of the microscope and focused for fine imaging. The culture dish enabled all the embryos to be positioned in the field of view. Illumination was provided by a reflected warm white light. The system was set to take a single picture every 10 min. Live images of the embryos were displayed on the computer screen and videos recorded for future analysis. The embryos were not moved or disturbed in any way for the whole period (5-6 days) of development, completely eliminating the sheer stress.

Daily observations of the embryo development time point were taken using the capture software. Time of micro injection was considered as (t0). The subsequent timings were taken as hour's post-insemination (hpi). Normally zygotes were placed in the time-lapse system at 17-18 hpi but some of them were also placed at 1-2 hpi for monitoring the appearance of 2pronuclei. The evaluation of time points was done according to standards set for each cleavage stage. Fragmentation, symmetry of blastomeres and vaculation was also assessed (29, 30). The quality of blastocyst was assessed on the bases of its timings, ICM (inner cell mass) development and cellular arrangement of TE(trophectoderm) (31). Blastocoel volume and development were used to gradify blastocysts as: SB = start of blastulation; EB = expanded blastocyst with more than half volume with cavitation; and FEB = fully expended blastocyst. The quality of inner cell mass was determined on the basis of either being absent or invisible, loosely arranged and well defined. Trophectoderm quality was evaluated either being organized well with high cell number, loosely arranges with low cell number and stretched with ill-defined cellular arrangement. Poor quality blastocysts show degenerative cells with low cell counts. For embryo transfer optimally growing blastocysts evalua-

Table 1. Patient demographic and cycle characteristics of five age groups.

Patient's characteristics	<26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	> 40 yrs.
No. of patients (n)	**24**	**62**	**68**	**34**	**12**
Age (yrs.) (mean±SEM)	23.6±0.2	28.2±0.2	33.0±0.2	37.9±0.2	42.7±0.4
BMI (Kg/m^2) (mean±SEM)	25.0±0.8	24.9±0.4	26.2±0.4	27.3±0.5	26.3±1.2*
Primary infertility (%)	23/24**(95.8%)**	50/62**(80.6%)**	48/68 **(70.5%)**	23/34 **(67.6%)**	5/12 **(41.6%)**
Secondary infertility (%)	1/24**(4.1%)**	12/62**(19.3%)**	20/68 **(29.4%)**	11/34 **(32.3%)**	7/12 **(58.3%)**
1st icsi attempt (%)	21/24**(87.5%)**	54/62**(87.0%)**	55/68 **(80.8%)**	26/34 **(76.4%)**	9/12**(75%)**
2nd icsi attempt (%)	3/24**(12.5%)**	8/62 **(12.9%)**	13/68 **(19.1%)**	8/34 **(23.5%)**	3/12**(25%)**
ICSI cycles (n)	23	55	64	33	10
ICSI+IVF cycles (n)	1	7	4	1	2
No. of oocytes retrieved(mean±SEM)	26.5±2.0	25.5±1.4	17.0±1.3	15.9±1.5	13.1±2.4***
No. of mature oocytes(mean±SEM)	21.6±1.9	19.3±1.2	12.9±0.9	12.7±1.3	8.5±1.5***
No. of oocytes fertilized(mean±SEM)	15.5±1.7	14.3±1.1	9.8±0.9	9.4±1.1	5.5±0.8***
No. of oocytes cleaved(mean±SEM)	15.1±1.6	14.0±1.1	9.6±0.9	9.2±1.1	5.4±0.8***

*P≤0.05 , ***P≤0.001 (ANOVA followed by Tuckey's test)

Table 2. Embryo morphokinetic parameters of five age groups from day-1 to day-5 of development.

Morphokinetic parameters	< 26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	> 40 yrs.
tPNa (mean±SEM)	18.9±4.9	13.4±0.4	14.9±1.4	13.8±1.8	13.9±1.1
t2(mean±SEM)	28.9±0.6	28.8±0.5	30.3±1.1	28.9±0.6	30.5±1.4
t3(mean±SEM)	37.3±0.8	36.9±0.7	37.1±0.6	36.8±0.7	38.1±1.5
t4(mean±SEM)	42.4±0.8	42.2±0.6	42.6±0.6	42.6±1.0	43.4±1.7
t5(mean±SEM)	52.0±1.3	51.3±0.9	51.2±1.0	52.6±1.4	53.3±1.8
t8(mean±SEM)	67.5±1.3	68.8±0.9	69.0±1.1	71.2±1.6	71.5±1.8
tM(mean±SEM)	100.4±1.2	99.4±1.2	100.4±1.1	100.6±1.7	99.9±2.3
tSB(mean±SEM)	108.4±1.2	108.0±0.7	108.6±1.2	110.0±1.9	109.0±1.4
tEB(mean±SEM)	112±0.9	112.9±0.8	112.1±1.0	112.0±1.6	112.8±2.3
tFEB(mean±SEM)	114.1±0.8	115.5±0.3	115.4±0.9	115.5±0.6	116.8±1.5

ted by morphological assessment through conventional method as well as by time-lapse imaging were selected at 114-116 hpi. For cryopreservation morulae with start of cavitation and blastocysts with good quality ICM and TE were selected.

Embryo transfer was done on day 5 transvaginaly using Sure-View catheter (Wallace, US). For luteal support patients use progesterone either intravaginaly (Cyclogest; Actavis, UK) or Gestone injections when needed. After 10 days of embryo transfer beta-hCG levels were measured. Clinical pregnancy was confirmed on ultrasound after 7 weeks of embryo transfer.

Results

The demographic and cycle characteristics of five female age groups are shown in table 1. The patients were divided into five age groups (<26, 26-30, 31-35, 36-40 and > 40 years old). Mean BMI was statistically significant when compared among different age groups (P≤0.05). The rate of primary infertility was higher than secondary infertility in all age groups except the females aged above 40 years.The rate of 1st attempt of the cycle was also higher than 2nd attempt in all age groups. Similarly the number of ICSI cycles was higher than ICSI+IVF cycles in all groups studied. The number of retrieved, matured, fertilized and cleaved oocytes showed highly significant difference (P≤ 0.0001) when compared among different age groups.

Time-lapse analysis (table 2) showed that mean values of all kinetic parameters were found to have no significant difference among all age groups when compared by ANOVA (Analysis of variance). The timely and untimely cleaved embryos of each age group were segregated and ANOVA was performed among timely cleaved embryos for all morphokinetic parameters to check the significance of differences. Hence the mean time for fertilization (tPNa) was found to be significantly higher in < 26 years when compared to 26-30 years (P≤0.05) and 36-40 years age group (P≤0.0001). The tPNa was also significantly higher in 31-35 years age group as compared to 36-40 years (P≤0.05)(Fig1). There was no significant difference in time for 2-cell (t2) and 3-cell (t3) cleavages among all age groups but the time for 4-cell cleavage (t4) turned out to be significantly higher in > 40 years female as compared to 26-30 years age group (P≤0.05) (Fig 2). Similarly time for 5-cell cleavage (t5) also found to be significantly higher (P≤0.05) in > 40 years females when compared to 26-30, 31-35 and 36-40 years age groups (Fig 3).Time for 8-cell cleavage was found to have no significant difference among the study groups. All events for compaction and start of cavitation i.e. tM, tSB, tEB, tFEB, were found to have no significant difference when compared among timely cleaved embryos of all age groups.

In table 3 the timely cleaved and untimely cleaved embryos were separated for each age group. The number of timely cleaved embryos was greater than

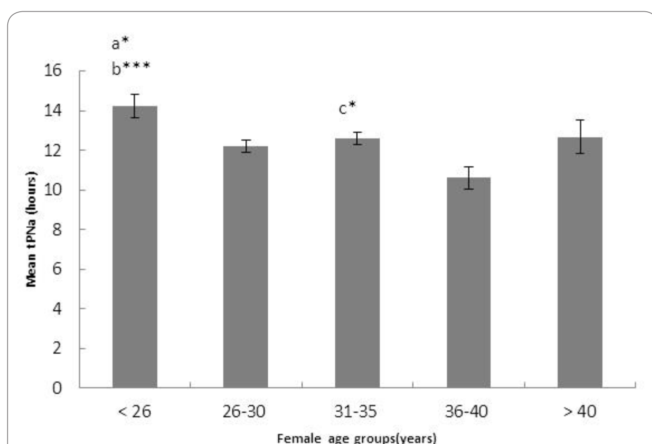

Figure 1. A comparison of timely pronuclear appearance (tPNa) among five study groups. a* P≤0.05 vs. 26-30 yrs. age group (ANOVA followed by Tukey's test) b*** P≤0.001 vs. 36-40 yrs. age group (ANOVA followed by Tukey's test) c* P≤0.05 vs. 36-40 yrs. age group (ANOVA followed by Tukey's test).

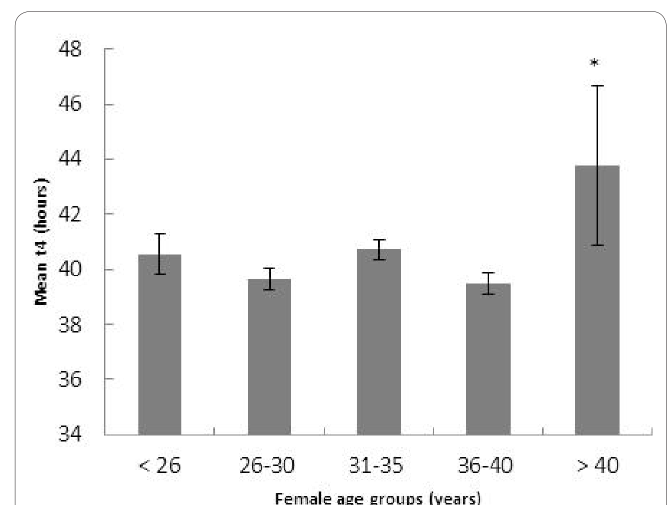

Figure 2. A comparison of timely 4-cell cleavage (t4) among five study groups.* P≤0.05 vs. 26-30 yrs. age group (ANOVA followed by Tukey's test).

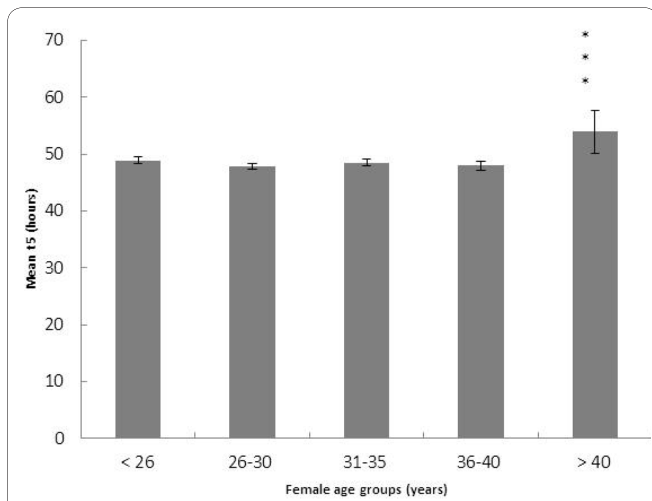

Figure 3. A comparison of timely 5-cell cleavage (t5) among five study groups.* P≤0.05 vs. 26-30, 31-35 & 36-40 yrs. age groups (ANOVA followed by Tukey's test)

untimely cleaved in all age groups except the above 40 years group. For 2-cell cleavage the rate of timely cleaving embryos was higher than untimely dividing in all age groups except the above 40 years in which more 2-cell cleavage was out of timeline. Among other early cleavage parameters i.e. for 4 and 5-cell stage the untimely cleaving embryos showed a higher rate than timely cleaving in all age groups whereas for 8-cell cleavage the rate of embryos dividing untimely was higher than timely cleavage for all other age groups except 36-40 years in which timely dividing 8-cell embryo rate was higher than untimely dividing but with a minor difference in rate. On the other hand the rate of embryos cleaving timely for start of blastulation (tSB) was far lower than those cleaving untimely in all age groups.

As far as the developmental potential and qualitative parameters of embryonic development are concerned (table 4) the rate of embryo cleavage did not vary much among all age groups. The rate of blastocysts formation showed a decline with increasing age of females. The rate of good quality blastocysts was higher than poor quality in all age groups but both did not show any remarkable change with increasing age of females as shown in the table. The rate of embryos with slow early cleavage was higher than abnormally cleaving embryos in all age groups. The percentage of embryos failed to form blastocyst showed an increase with increasing age of females.

Table 5 shows the pregnancy outcome of patients. The mean number of embryo transferred in fresh cycle had no significant difference among all age groups while the mean number of frozen-thawed embryos transferred showed significant differences when compared among the age groups (P≤0.01). The percentage of frozen-thawed embryo transfers was higher than fresh cycle transfers in all age groups but each of them did not vary much when compared individually with increasing age of females however the rate of frozen transfers was comparatively low in younger (<26 years) patients. The clinical pregnancy rate of both fresh and frozen transfer showed a decline with increasing age of females. The implantation rates were higher than abortion rates in both fresh and frozen-thawed cycles but did not vary much with increasing age. The single pregnancy rates came out to be higher than multiple pregnancy rates both for fresh and frozen transfers as shown in the table.

Discussion

Infertility is a prevalent condition participated both by the male and female partner or it can be unexplained

Table 3. Rates of timely and untimely cleaved embryos for important kinetics parameters in five study groups.

Morphokinetic markers	Timely cleaved embryos in different age groups					Untimely cleaved embryos in different age groups				
	< 26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	> 40 yrs.	< 26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	> 40 yrs.
t2	133/245 **(54.3%)**	332/617 **(53.8%)**	270/530 **(50.9%)**	135/251 **(53.8%)**	24/61 **(39.3%)**	112/245 **(45.7%)**	285/617 **(46.1%)**	260/530 **(49.0%)**	116/251 **(46.2%)**	37/61 **(60.6%)**
t4	120/245 **(48.9%)**	278/617 **(45.0%)**	222/530 **(41.8%)**	116/251 **(46.2%)**	21/61 **(34.4%)**	125/245 **(51.0%)**	339/617 **(54.9%)**	308/530 **(58.1%)**	135/251 **(53.7%)**	40/61 **(65.5%)**
t5	92/245 **(37.5%)**	216/617 **(35.0%)**	171/530 **(32.2%)**	**97/251** **(38.6%)**	18/61 **(29.5%)**	153/245 **(62.4)**	401/617 **(64.9%)**	359/530 **(67.7%)**	154/251 **(61.6%)**	43/61 **(70.5%)**
t8	121/245 **(49.4%)**	306/617 **(49.6%)**	240/530 **(45.3%)**	126/251 **(50.1%)**	21/61 **(34.4%)**	124/245 **(50.6%)**	311/617 **(50.4%)**	290/530 **(54.7%)**	125/251 **(49.8%)**	40/61 **(65.5%)**
tSB	73/245 **(29.8%)**	239/617 **(38.7%)**	182/530 **(34.3%)**	88/251 **(35.0%)**	15/61 **(24.9%)**	172/245 **(70.2%)**	378/617 **(61.2%)**	348/530 **(65.6%)**	163/251 **(64.9%)**	46/61 **(75.4%)**

Table 4. A comparison of developmental potential and embryos quality in five age groups.

Embryonic development	< 26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	> 40 yrs.
Total embryos analyzed(n)	372	890	670	319	67
Cleavage rate (%)	364/372 **(97.8%)**	869/890 **(97.6%)**	655/670 **(97.7%)**	313/319 **(98.1%)**	65/67 **(97%)**
Blastocyst formed (%)	215/364 **(59%)**	475/869 **(54.6%)**	310/655 **(47.3%)**	137/313 **(43.7%)**	32/65 **(49.2%)**
Good quality blastocysts (%)	163/215 **(75.8%)**	367/475 **(77.2%)**	238/310 **(76.7%)**	96/137 **(70%)**	23/32 **(71.8%)**
Poor quality blastocysts (%)	52/215 **(24.1%)**	108/475 **(22.7%)**	72/310 **(23.2%)**	41/137 **(29.9%)**	9/32 **(28.1%)**
Blastocyst not formed (%)	149/364 **(40.9%)**	394/869 **(45.3%)**	345/655 **(52.6%)**	176/313 **(56.2%)**	33/65 **(50.7%)**
Slow early cleavage (%)	128/364 **(35.1%)**	293/869 **(33.7%)**	249/655 **(38%)**	131/313 **(41.8%)**	28/65 **(43%)**
Abnormal cleavage (%)	21/364 **(5.7%)**	101/869 **(11.6%)**	96/655 **(14.6%)**	45/313 **(14.3%)**	5/65 **(7.6%)**
Not cleaved (%)	8/372 **(2.1%)**	21/890 **(2.3%)**	15/670 **(2.2%)**	6/319 **(1.8%)**	2/67 **(2.9%)**

Table 5. Clinical data outcome in five age groups after culture in Time-Lapse System.

Clinical outcome	< 26 yrs.	26-30 yrs.	31-35 yrs.	36-40 yrs.	> 40 yrs.
No. of embryos transferred Fresh cycle (mean±SEM)	2±0.2	1.8±0.1	2.1±0.1	2.0±0.2	1.6±0.2
No. of embryos transferred Frozen-thawed cycle (mean±SEM)	2.3±0.1	2.1±0.1	1.8±0.1	1.8±0.1	2.4±0.3**
Fresh cycle transfers (%)	11/24**(45.8%)**	19/62**(30.6%)**	31/68 **(45.5%)**	14/34**(41.1%)**	5/12 **(41.6%)**
Clinical pregnancies Fresh cycle (%)	5/11 **(45.4%)**	7/19 **(36.8%)**	11/31 **(35.4%)**	4/14**(28.5%)**	1/5**(20%)**
Implantations Fresh cycle (%)	4/5**(80%)**	6/7 **(85.7%)**	9/11 **(81.8%)**	3/4**(75%)**	1/1**(100%)**
Abortions Fresh cycle (%)	1/5**(20%)**	1/7 **(14.2%)**	2/11**(18.1%)**	1/4**(25%)**	Nil
Singleton pregnancies Fresh cycle (%)	3/5**(60%)**	5/7 **(71.4%)**	10/11**(90.9%)**	3/4**(75%)**	1/1**(100%)**
Multiple pregnancies Fresh cycle (%)	2/5**(40%)**	2/7 **(28.5%)**	1/11 **(9.0%)**	1/4**(25%)**	Nil
Frozen-thawed transfers (%)	18/24 **(54.1%)**	50/62 **(80.6%)**	46/68 **(67.6%)**	26/34**(76.4%)**	9/12**(75%)**
Clinical pregnancies Frozen-thawed (%)	14/18 **(77.7%)**	41/50**(82%)**	25/46 **(54.3%)**	13/26**(50%)**	2/9 **(22.2%)**
Implantations Frozen-thawed (%)	14/14**(100%)**	37/41 **(90.2%)**	23/25**(92%)**	12/13**(92.3%)**	2/2**(100%)**
Abortions Frozen-thawed (%)	Nil	4/41 **(9.7%)**	2/25**(8%)**	1/13**(7.6%)**	Nil
Singleton pregnancies Frozen-thawed (%)	10/14 **(71.4%)**	38/41 **(92.6%)**	24/25**(96%)**	13/13**(100%)**	1/2**(50%)**
Multiple pregnancies Frozen-thawed (%)	4/14 **(28.5%)**	3/41 **(7.3%)**	1/25**(4%)**	Nil	1/2**(50%)**

**P≤0.01.

and can result into an emotional stress for the couple. The female factor infertility is almost 30-40% of all infertility cases and mainly includes the cervix, uterus, endometrium, ovarian function, fallopian tubes, peritoneum and the hormonal imbalance as major causes for difficulty in conception (32). One of the most important factors influencing pregnancy success is maternal age and female fertility is known well to decrease as age increases (33, 34). Many reports have demonstrated the effects of aging on ovarian response to stimulation and fertilization rate. Aging has also been shown to contribute to decreased pregnancy and birth rates in assisted reproduction programs (35). Female with advanced age showed increased spontaneous miscarriage rate and congenital anomalies (36) and also the decreased numbers of retrieved oocytes and reduced fertilization and implantation rates (37, 38). In addition to the anatomical and endocrine factors the iatrogenic disorders (complications resulting from chemo-radiotherapy or surgical treatments) also play an important role in female infertility. The post-surgical adhesions result into pelvic inflammatory disease (PID) and endometriosis thus affecting the female fertility (39).

The objective of this study was to investigate the effect of female age on developmental kinetics of embryos cultured in multi gas incubator equipped with time-lapse microscopes. Time lapse is a popular photographic technique used to show events that usually happen at very slow speeds, hard to sense for the human eye. By capturing multiple fixed pictures or images at predefined intervals of time and the subsequent superposition with a specific speed, the time-lapse effect is achieved. In the last few years, time-lapse image capture applied to IVF has been used to obtain a deeper and more accurate understanding about the biological competence and potential of the embryo.

While female fertility peaks between the ages of 22 to 26 years, the age-related decrease in fertility first becomes prominent at the age of 35 years. This age is a discrete time point after which women exhibit significantly increased risks of adverse reproductive outcomes (40-44). A number of previous studies have compared IVF outcomes in women aged 40 years and above versus those younger than 40 years. Women over 37 years of age showed reduced fertility and in the age of 40 years, the likelihood of success for ART declines sharply with a low chance of a successful pregnancy in women aged ≥41 years. Therefore, in our study we stratified patients into five age groups (< 26 years, 26–30 years, 31–35 years, 36–40 years and > 40 years) for a clear comparison of young and old ages for embryonic development (45-48).

Female-related parameters, such as age and number of retrieved oocytes, may also have a significant contribution to the success of pregnancy (49). From this perspective it was reported in a study (50) that the female partner's age, number of matured oocytes, oocyte status, and embryos transferred all affected the IVF outcomes. The observed decreases in ovarian reserve and uterine receptivity are probable reasons for the reduced fertility associated with aging (40). This decline becomes more obvious at the age of 35 years. These effects can be explained partly by the age-related decrease in ovarian follicular reserve and the increased chromosomal anomaly rate in the oocytes (51) which is probably associated with the aging cytoplasm and increased aneu-

ploidy rates during meiosis (52). In our study too the embryo quality parameters assessed primarily i.e. the number of oocytes retrieved, matured, fertilized, cleaved and the blastocyst formation rate showed a decline with increasing age and BMI. In previous studies, significantly worse pregnancy results were achieved in subjects aged above 40 than those with age below 40 years (53, 54). Consistent with this, our study also showed low conception rates in embryo transferred in fresh and frozen-thawed cycles in above 40 years age group but implantation and abortion rates did not show any marked difference with increasing age.Contrary to this, it was reported previously that maternal aging has been associated with increased spontaneous miscarriage rate, pregnancy complications, congenital anomalies, and higher perinatal mortality (34).

In our findings two younger groups showed normal BMI while three older groups were overweight but not obese. According to another study the prevalence of overweight and obesity was lowest among women <20 years of age. Women with obesity before pregnancy were more likely to be older (40–54 years of age) (55). Obesity is associated with impaired fertility. An increasing body of literature indicates that the oocyte and embryo are adversely affected by maternal overweight and obesity. Blastocyst formation rate has also been shown to be reduced in overweight females compared with normal-weight women (56).

The aneuploidy rate and genetic abnormalities in oocytes of aged patients affects the IVF outcome badly (57-60). Maternal age is associated with genetic impairment of oocytes due to aging mitochondrial apparatuses and fragmented DNA (61). It has been documented that the average values of morphokinetic parameters from oocytes of females with advanced age were found to be in normal range as of younger women. The present study also found that the average time-points of all kinetic parameters of embryo development were non-significantly different among the study groups.

The comparison between timely and untimely cleaving embryos for important kinetic parameters indicated that time for 2pronuclei appearance and early cleavage time-points (t4 and t5) showed significant difference among the five age groups whereas late kinetic parameters did not show any significant difference among different ages of female. In another study data were analyzed in younger versus older subjects (20-30, 30-40, and >40). The early morphokinetic parameters i.e. the times from insemination to tPnf, t2, t3, t4 were significantly shorter in the younger females than older ones ($p<0.05$) (62). In our findings the time for pronuclear appearance found to be significantly delayed in < 26 years as compared to other study groups whereas the t4 and t5 were found to be delayed in > 40 years as compare to young patients. In above mentioned study the percentages of optimal embryos, according to t5 did not differ statistically between the groups while we found that rate of timely cleaving embryos for t5 cleavage were far lower than untimely dividing among all age groups showing the lowest rate in patients aged above 40 years. Similar to our findings another study stated that abnormally and untimely cleaving embryo are predominant than timely cleavages (63). The embryos rate for t5 found to be closely associated with the percentage of embryos starting blastocoel cavity. This is in accordance with the findings of the study (64) which showed that t5 and t8 cleavages were more predictive of blastulation as compare to t2, t3, t4. Moreover same results declared by another finding have shown that t5 as one of the most important variables predicting implantation (10). Future work regarding the effect of female age on embryo morphokinetics can be more refined by selecting the prospective study design with limited number of embryos focusing on their pregnancy out comes.

The present study illustrates the effect of female age on embryo morphokinetics and the importance of time-lapse imaging to study the embryonic developmental pattern more precisely. More over the study emphasizes the early cleavage as being the most crucial factor for viable embryo development.

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Declaration of interest

The authors have no conflicts of interest relevant to this article.

Authors' contributions

Noureen Akhter was involved in designing, acquisition, analysis of data and drafting manuscript and Dr. Muhammad Shahab played a role in the analysis and interpretation of data, drafting of the manuscript and final approval.

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