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Factors affecting the pregnancy outcome of in-vitro produced day 3 embryos: a retrospective cohort study of 467 patients

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Abstract:

The purpose of this study is to evaluate several factors, such as transfer of fresh embryos & body mass index (BMI), frozen embryos, endometrium thickness, and transfer of self and donor embryos, on the pregnancy outcomes in North Indian patients. In this observational retrospective cohort study, we enrolled 500 patients who underwent infertility treatment that visited IRCC Hospital, Panchkula, Haryana, India between June 2017 to June 2020 was studied. Out of 500 patients, only 467 patients were selected (93.40%) and 33 patients were excluded (6.60%) from the study. Patients were distributed in fresh embryo transfer n=312 (66.80%) and frozen embryo transfer n = 155 (33.19%). They were divided into six groups of different age groups patients: fresh embryo transfer on day 3, correlation with grade-A and grade-B embryos; group (C-1), fresh embryo transfer on day 3, correlation with age and endometrium thickness; group (C-2), fresh embryo transfer on day 3, correlation of IVF pregnancy with age and BMI; group (C-3), frozen embryo transfer on day 3, correlation with age and endometrium thickness; group (C - 4), self-frozen embryo transfer on day 3, correlation with age and endometrium thickness; group (C-5), donor frozen embryo transfer on day 3, correlation with age and endometrium thickness; group (C-6). Of the six groups, the pregnancy rate was the lowest in the C-6 group (30.5%), while it was the highest in the C-5 group (65.2%). We also noticed that patients aged above 41 years have the lowest pregnancy rates; whereas, young patients had higher pregnancy rates. Conclusion: frozen self-embryo transfer seems to be the best choice for all maternal ages. Embryo transfers in this group might have low neonatal outcomes. In particular, frozen embryo transfers seem to benefit younger maternal age.

Key Words: In vitro fertilization, eight-celled embryo, Age & Endometrium Thickness, fresh & frozen embryo transfer, and clinical pregnancy.

1. Introduction:

Human infertility is the inability to conceive after 12 months or more of regular unprotected sexual activity [1]. Globally, infertility affects 10-15% of the human population worldwide, and 8-10 million couples fall under this category in India. Infertility affects 10-15% of the world's population, and 8-10 million Indian couples are affected by this condition [2]. It is a multi-factorial problem that mainly involved male and

female infertility or both while about 20% are for unknown reasons [3]. In order to overcome infertility, the adoption of *in-vitro* fertilization (IVF) has been increasing in recent years. Pregnancy from IVF through embryo transfer (ET) is depending on various factors among them the most critical aspects are the day of the cycle on ET, the fresh/frozen embryo transferred, and whether the embryo transferred is from a self-patient or from a donor.

2. Materials and Methods:

2.1. Materials: In this observational retrospective cohort study, we enrolled 500 patients who underwent monitoring for infertility treatment at the Institute of Reproduction and Child Cares & IRCC IVF Centre, Panchkula, Haryana, from June 2017 to June 2020. Out of 500 patients, only 467 patients were selected (93.40%) and 33 patients were excluded (6.60%) from the study. Patients were distributed in fresh embryo transfer n=312 (66.80%) and frozen embryo transfer n= 155 (33.19%).

2.2. Equipment required: Patient under treatment, OT room, ultrasound machine, trans-vaginal probe, needle aspiration assembly, oocyte collection media, thermo-control stand for collection tubes, local anaesthesia, syringes, needles, gloves, lubricants, spirit, alcohol, towels, tissue paper, soap, povidone-iodine, fully functional IVF lab, LAF with stereozoom microscope, inverted microscope with a micromanipulator, CO_2 incubator or tri gas incubator for embryo culture, functional andrology lab with LAF and phase contrast microscope, counting chamber makler for semen analysis [15], spermfuge SF800 for semen processing and use for IUI/IVF/ICSI or any other ART procedures, skilled embryologist, skilled infertility doctor for performing precise work, etc.

3. Protocol for downregulation:

The first and foremost step of the IVF procedure is the down-regulation of the patient (hypothalamus & pituitary*)* i.e., ovarian stimulation by exogenous hormones to increase the number of eggs (follicles) that develop in the ovaries and control the time of ovulation [4-5]. The procedure includes oral intake of birth control pills for one or more weeks before beginning IVF to prevent the release of hormones that could stimulate natural ovulation. One week after the start of the birth control pill, intradermal injections of GnRH agonist [0.3 ml/day S.C. (till the day before of OPU) leuprolide acetate or lupron)] and Gonadotropins [(age less than 30 years, Recogon:200IU (2amp), I.M. x 5 days or Pure FSH (Gonal F): 125 IU (3 amp), I.M. x 5 days) but if age >30 years: 225 IU (3 amp) Gonal F + 2 amp Pure FSH or Pure FSH: 425 IU (5 amp)] were given to stimulate the growth of multiple follicles. If LH < 1.5 mIU then we can add 1-amp hMG more [9-10].

During stimulatory treatment, blood testing (E2 and LH) and ultrasonography were done on alternate days to study follicular growth till the follicles are 'ready' (at least two follicles reach 17 to 18 mm in size), to trigger the final maturation by injecting hCG 10,000 IU I.M. (at a specific time which was decided by the infertility team) (Decapeptyl, Ovidrel, Pregnyl, and Novarel, etc anyone decided by infertility consultant). This allows the final maturation of follicles which were aspirated within 35-38 hours of post-hCG administration.

4. Oocyte pick-up (OPU):

The patient was received in IVF OT in a precise manner at the expected time of egg rupture after hCG injection (35-38 hours). The patient was allowed to lay down in a supine position under general anaesthesia (GA). Both ovaries were scanned to determine the follicular size and number. Cleaning of the Vagina and perineal area was done with normal saline. Prepare the trans-vaginal probe assembly, insert the probe into the vagina, focus the scanning over the ovary, advance the OPU needle into the follicle applying negative pressure (ideal -90 mm Hg to -140 mm Hg), rotate the needle to aspirate all the content and observe the collapsing of the follicle. The collection line was rinsed with the human tubal fluid medium (HTF) to prevent any clogging and oocyte in the assembly. The total number of follicles aspirated was counted. The collection tube maintained at 37^0C was immediately passed through the pass box/window to the embryologist for searching the oocyte from follicular fluid, further equilibrates and culture the Oocyte in Continuous Single Culture (CSC) medium, Irvine Scientific till embryo transfer or vitrification [16-17].

4.1. Searching and Grading of Oocytes:

The aspirated follicular fluid of each tube was poured into a 100x 20 mm petri dish and searched & pick the oocytes under a stereo-zoom microscope using a 200-microliter pipette tip. All oocytes were collected in a 3001-dish having HTF medium after OPU all oocytes were washed 2-3 times in HTF and then transfer to a CSC dish for equilibration. Following the screening, we scored the oocytes based on the expansion of the cumulus [13]:

4.2. Grading of Oocyte:

	GRADING OF OOCYTE		
	I	**II**	**III**
DAY-0	The cumulus is fully expanded and has a golden-brown color. It is likely to be a mature egg	Cumulus and coronal cells that are partially expanded. It is likely to be a mature egg	There is no expanded cumulus and dark ooplasm. Probably an immature egg

4.3. Equilibration of Oocyte:

Following oocyte screening and scoring, all oocytes were transferred into a bicarbonate buffer medium or IVF medium for 2 - 4 hours of equilibration. The oocyte maturation and manipulation stress are reduced through equilibration. Once oocytes are equilibrated, they can be used for conventional IVF or ICSI (after denudation). It's important to wash the oocyte after IVF or ICSI and put it in a new bicarbonate buffer medium for culture until day 3 or blastocyst stage or ET/vitrification.

During oocyte equilibration, we prepare semen samples for conventional in-vitro fertilization (IVF) or ICSI, depending on the patient's needs. Semen parameters, as defined by the WHO Manual - 2010: 1.5 ml or

more, pH 7.2 or more, 15 million or more sperm, and sperm motility: forward (type a) + slow (b) ≥ 32%, and ≤1 million leukocytes. Approximately 100000 sperms are needed per oocyte for IVF, and one sperm is needed for ICSI [15].

5. Semen processing:

We analyzed all semen samples after liquefaction or IVF procedures using double-density gradients (DDG) or single-density gradients (SDG) depending on the semen parameters. All preparations were done with Pure Sperm Grade II media (Cryolab International). We gently layered liquefied semen over a double-density or single-density gradient for 12 minutes at 1500 RPM. We aspirated the supernatant and transferred the pellet into a new centrifuge tube with 4 ml washing media (HTF). Remove the supernatant and overlay the pellet with IVF culture medium and let it swim up in 6% CO2 for at least 15-20 minutes for a complete swim-up. Counting chamber Maklers (Sefi Medical Instruments, Israel) were used to determine sperm concentration by pipetting an aliquot of swim-up sample onto them, then counting with 20x objectives under a phase contrast microscope [15].

6. *In-vitro* fertilization (IVF):

Semen samples were collected by masturbation after a gap of 2–7 days of sexual abstinence. Freshly collected and processed semen from the husband or donor aged 25-55 years during 2017-2020 was used for in vitro fertilization. *In-vitro* capacitation was induced by ingredients of the IVF medium. The semen sample upon liquefaction was examined for concentration, motility, and abnormality. Samples with leukocytopenia ($>1\times10^6$/ml) white blood cells/mL were excluded from the study. Sample (n=392) meeting the criteria for concentration, motility, and morphology (WHO, 2010) were included in this study and processed for IVF as per the procedure described under sperm processing. Oocytes in micro drops or groups were co-incubated with 1 X 10^6/ml processed sperm per oocyte for 5 hrs in 6 % CO_2 at 37^0C in the incubator.

Semen samples with normal parameters were used for conventional IVF. Incubation and fertilization were done in microdrop cultures (21 microliters to 50 microliters) or well cultures (500 microliters) with a continuous single culture medium (Irvine Scientific). The incubation period depends on the embryologist's experience and varies from lab to lab. A short protocol of IVF involves denuding the oocyte after five hours of insemination, with sperm count and motility very good, and a swim-up of one lakh sperms per oocyte. In conventional IVF, we used good quality normal motile sperm for insemination (overnight or not more than 18 hours) for long incubation (around sixty thousand to one lakh sperm per oocyte). After denudation, if some oocytes don't show 2 PB or PN, rescue ICSI can be done (optional) [11]. The drop size of the culture medium (50 µl) is enough for 1 to 3 oocytes/embryos in micro-culture, and 500 µl is enough for 1 to 10 oocytes/embryos in well culture. It varies from lab to lab and embryologist to embryologist.

6.1. Denudation after conventional IVF:

When the cumulus is separated after 5 hours or overnight of insemination, we denuded the oocyte. There were different sizes of denuding pipettes used on different days; on day 0, 145 to 175 micrometers, on day 2 or 3, 175 micrometers, and on day 4 or 5, 270 to 300 micrometers. During this study, we were denuded after 5 hours of insemination (IVF); 99 % of the eggs were fertilized after a short period of incubation.

Additionally, we performed Intracytoplasmic Sperm Injections (ICSI) only on patients with poor sperm quality, poor sperm morphology, oligoasthenozoospermia, TESA/MESA/PESA samples, failed IVF, poor sperm survival, or a higher DFI. The oocyte must be denuded prior to ICSI, and only MII oocytes were used in the procedure [12].

6.2. Denudation of oocyte for ICSI:

This was carried out using hyaluronidase enzyme (that is, 200 microliter pipette tips were first used, followed by 140 micrometer of denuding pipette within 40 seconds in concentrated hyaluronidase and 80 seconds in 1:1 diluted hyaluronidase). The denuded oocyte is first washed in HTF medium three times, followed by three further washes in continuous single culture medium three times. Once the oocyte has been transferred to a bicarbonate buffer medium micro-culture dish, the dish is placed in an incubator at 370C with 6% CO2 to balance and reduce stress. Prior to ICSI, we recorded the quality of oocytes and the number of mature (MII) and immature (MI) oocytes. The micromanipulator machine was aligned during oocyte equilibration, the air was removed from the oil injector and the oil was filled. Set the injection needle as well as holding the pipette by using a lower objective lens (4X or 10 X). Keep the oil injector under positive pressure for a few minutes after fitting the needle. We make an ICSI dish and add less than 1l sperm in a line of polyvinylpyrrolidone (PVP) [12]. The semen sample had already been prepared and the swim-up had been done after OPU, during oocyte calibration. As a good fertilization result, we found 2PN after 16 to 18 hours of incubation [14].

6.3. *In-vitro* culture (IVC): After 3-6 hr of IVF these presumptive zygotes were washed 3-5 times in an *in-vitro* culture medium (CSC, Irvine scientific) and were placed in pre-equilibrated IVC drops. These zygotes were *in vitro* cultured for 3 days and the quality of developing embryos (cleavage embryos) was examined at 20 X objective of an inverted microscope [7].

7. Embryo Transfer (ET): Transfer of OPU-IVF-produced embryo:

Embryo transfer — two or three or in some cases more than three embryos of grade A were transferred in the uterus using a thin flexible ET catheter (Sydney IVF Embryo Transfer Set Cook, Ref # K-JETS-7019-SIVF) [6]. In some difficult ETs, we used Gynetics Stellet ET Catheter (REF # 4219 set) under transabdominal ultrasonographic guidance with a filled urinary bladder. Non-surgical ET was performed by passing a catheter through the cervix on the 17th day of a menstrual cycle for Day 3 embryos. It's important

to pass the catheter as gently as possible to avoid trauma or injury to the uterus. ET was performed without anaesthesia, but the patient was advised to rest at home for a few hours afterward.

Table 1: Fresh embryo transfer on day 3, correlation with grade A and grade B embryos; group (C-1):

Embryo stage		`Grade			
		Grade-A		Grade-B	
	No. of Patients	Pregnant	Non-pregnant	Pregnant	Non-pregnant
8-celled	144	47 (32.63 %)	44 (30.55 %)	11 (7.63 %)	42 (29.16%)
Early compaction	168	61 (36.30 %)	38 (22.61 %)	25 (14.88 %)	44 (26.19 %)
Total Patients	312	108 (34.61%)	82 (26.28 %)	36 (11.53 %)	86 (27.56 %)
Percentage of conception		**34.61 %**	26.28 %	**11.53 %**	27.56 %

Table 2: Fresh embryo transfer on day 3, correlation with age and endometrium thickness; group (C-2):

Age of patient (yrs)	No. of patient	EMT (mm) on day of ET	Pregnant	Non-pregnant
21-25	15	10.00 ± 0.99	12 (80.00 %)	3 (20.00 %)
26-30	67	10.16 ± 0.84	36 (53.73 %)	31 (46.26 %)
31-35	170	10.00 ± 2.99	79 (46.47 %)	91 (53.52 %)
36-40	36	9.82 ± 2.18	11 (30.55 %)	25 (69.44 %)
41 and above	24	8.88 ± 1.82	6 (25.00 %)	18 (75.00 %)
Total patients	**312**		**144 (46.15 %)**	**168 (53.84 %)**

Table 3: Fresh embryo transfer on day 3, a correlation between age and body mass index (BMI); group (C-3):

Age of patient (yrs)	BMI of patient	Pregnant
21-25	21.29± 9.11	9 (100.00 %)
26-30	19.02 ± 9.99	41 (65.07 %)
31-35	27.17 ±4.02	48 (59.25 %)
36-40	25.84 ±4.15	42 (36.52 %)
41 and above	16.50 ± 6.80	4 (9.09 %)
Total patients	**312**	**144 (46.15 %)**

Table 4: Frozen embryo transfer on day 3, correlation with age and endometrium thickness; group (C - 4):

Age of patient (yrs)	No. of patient	EMT (mm) on the day of ET	Pregnant	Non-pregnant
21 to 25	0	0	0	0
26 to 30	18	8.90-11.00	15 (83.33 %)	3 (16.66 %)
31 to 35	31	7.5 – 13.00	24 (77.31 %)	7 (22.58 %)
36 to 40	74	7.5-12.00	37 (50.00 %)	37 (50.00 %)
41 and above	32	7.5-10.7	5 (15.62 %)	27 (84.37 %)
Total patients	**155**		**81(52.25 %)**	**74 (47.74 %)**

Table 5: Self-frozen embryo transfer (S-FET) on day 3, correlation with age and endometrium thickness; group (C-5):

Age of patient (yrs)	No. of patient	EMT (mm)	Pregnant	Non-pregnant
21 to 25	0	0	0	0
26 to 30	18	8.9-11	15 (83.33 %)	3 (16.66 %)
31 to 35	30	7.5-13	23 (76.66 %)	7 (23.33 %)
36 to 40	40	8.5-11	24 (60.00 %)	16 (40.00 %)
41 and above	8	7.5-10.5	1 (12.50 %)	7 (87.50 %)
Total	**96**		**63 (65.62 %)**	**33 (34.37 %)**

Table 6: Donor frozen embryo transfer (D-FET) on day 3, correlation with age and endometrium thickness; group (C-6):

Age of patient (yrs.)	No. of patient	EMT (mm)	Pregnant	Non-pregnant
21 - 25	0	-	-	-
26 - 30	0	-	-	-
31 - 35	1	10.00 ± 2.99	1 (100 %)	0
36 - 40	34	9.82 ± 2.18	13 (38.23%)	21 (61.76 %)
41 and above	24	8.88 ± 1.82	4 (16.66 %)	20 (83.33 %)
Total	**59**		**18 (30.50 %)**	**41 (69.49%)**

8. Pregnancy diagnosis:

Two weeks after the ET, a urine pregnancy test was done and same time blood sample of the patient was tested for beta hCG concentration to assess the early pregnancy. A beta hCG level of >25 mIU/ml with an increasing trend on subsequent analysis from 48 hrs onwards signifies the pregnancy. A confirmed

diagnosis of pregnancy was done by pelvic ultrasonographic examination of a fluid-filled sac gestational containing the embryo/sac. Later heartbeat visibility was examined by 6 to 6.5 weeks of pregnancy (4 to 4.5 weeks after the embryo transfer). The heartbeat visibility was checked by 6 to 6.5 weeks of pregnancy (4 to 4.5 weeks after embryo transfer) [8].

9. Conclusions:

In Table 1: Fresh embryo transfer on day 3 correlated with grade-A and grade-B embryos; group (C-1) showed a 46.14% correlation with grade-A and grade-B embryos. Table 2: Fresh embryo transfer on day 3, correlation with age and endometrium thickness; group (C-2) also showed similar results with grade A and grade B embryos at 46.15 percent. A correlation of IVF pregnancy with age and BMI was shown in Table 3: fresh embryo transfer on day 3; group (C-3) also showed similar results with grade A and grade B embryos at 46.15 percent. In **Table 4:** frozen embryo transfer on day 3, correlation with age and endometrium thickness; group (C - 4) in this study showed similar results with grade A and Grade B embryos was 52.25%. **In Table 5:** Self-frozen embryo transfer on day 3, correlation with age and endometrium thickness; group (C-5), **in** this showing similar results with grade A and Grade B embryos was 65.62%. **In Table 6, the correlation between age and endometrium thickness for donor frozen embryo transfer on day 3 was determined for the group (C-6). Results were shown to be 30.5% for grade-A and grade-B embryos.**

The lowest pregnancy rate was in the C-6 group (30.5%), and the highest was in the C-5 group (65.2%). The lowest pregnancy rates were observed in patients over the age of 41 years; in contrast, the highest pregnancy rates were found in young patients. In conclusion, frozen self-embryo transfers are the best option for women of all ages. A significant reduction in adverse neonatal outcomes is possible in this group of embryo transfers. Frozen embryo transfer appears to be particularly beneficial for patients who are younger than 35 years of age.

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