The successful use of hatched blastocysts in assisted reproductive technology

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Summary

This retrospective study was undertaken to determine the value of blastocyst culture and transfer as a tool in assisted reproductive technology. Six hundred and fifty-five cycles in patients undergoing IVF treatment for infertility were involved. All patients were aged ≤ 40 years. Day-2 embryos were transferred to 427 (group 1) and day-6 embryos (blastocysts) were transferred to 228 patients (group 2). Pronucleate oocytes obtained from IVF were cultured in vitro for 2 or 6 days. One to five embryos were transferred. A total of 10,146 oocytes were retrieved, 6,105 oocytes were fertilized, 2,222 embryos were transferred and 197 clinical pregnancies were achieved in all groups. Blastocystes were transferred to almost 90% of group 2 patients. The pregnancy rate per cycle and implantation rate per transferred embryo was 42.1% and 19.4%, respectively, in the blastocyst group compared to 23.6% and 8.6%, respectively, when embryos were transferred on day 2. Even though in the blastocyst group there was an increased number of oocytes fertilized at the same time there was a significant reduction in the number of embryos being replaced (3.2 vs 3.8). This study demonstrate that transfer of blastocysts increases the success of IVF when compared with day-2 transfers and reduces the number of embryos to be transferred.

Key words: Blastocyst; Outcomes; IVF; Embryo culture; ICSI.

Introduction

The in vitro fertilization (IVF) and embryo transfer (ET) programs are generally patterned on the model established by Edward's *et al.* [1]. Embryos are usually transferred to the uterus as cleavage stage embryos on the second day after retrieval and insemination.

In nature however, the embryo enters the uterus during the later stages of preimplantation embryo development. It has therefore been argued for some time that it may be more appropriate to mimic nature and transfer in vitro fertilized human embryos to the uterine cavity at the blastocyst stage [2, 3].

Potential advantages of blastocyst culture and transfer in human IVF, therefore, include the synchronisation of embryo development with the endometrium. Blastocyst culture also facilitates the assessment of embryo viability before transfer. This can be achieved by both the identification of those embryos with little developmental potential as manifested by slow development, or degeneration in culture, and by the introduction of non-invasive tests of development potential to select the most viable blastocysts for transfer within a cohort [4-6]. Healthier embryos are thus transferred leading perhaps to increased implantation rates, thereby reducing the need for multiple embryo transfers.

Many IVF units do not culture blastocysts for two reasons: (i) pregnancies can be established when embryos are transferred to the uterus at the 4 to 6-cell stage, even though they remain in the uterus for another 90~99 hours prior to implantation and, (ii) human in vitro fertilization (IVF) embryos could be cultured to the blastocyst stage with only limited success. Recently, however, Jones *et al.* [7] described the evolution of a culture protocol utilising strict quality controlled serum-free, cell-free culture media for the development of up to 52% of all zygotes to the blastocyst stage. Using the protocol of Jones *et al.* we have applied a replica of the same culture protocol as described in the Centre for Human Reproduction and in the IVF unit at Mitera Maternity Hospital in Athens, Greece.

In this study we present our results of blastocyst development from patients undergoing IVF in the Centre for Human Reproduction and in the IVF unit at Mitera Maternity Hospital in Athens, Greece.

Material and Methods

Thi retrospective analysis involved 655 consecutive IVF cycles performed between February 1997 and June 1998 at the Centre for Human Reproduction (Athens, Greece) and at the IVF unit of Mitera Maternity Hospital (Athens, Greece). All patients gave informed consent for the extended culture period and ET on day 5 or day 6 after insemination and were left to choose on their own on the day of ET. The study was approved by the ethics comittee of the Mitera Maternity Hospital and the Hellenic Society of Obstetrics and Gynecology. All patients were aged ≤ 40 years and had undergone between 0 and 10 previous IVF cycles and had to have at least five pronucleate stage

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embryos to be considered for this study. The patients were split into two groups. The first group consisted of 427 IVF cycles performed between February 1997 and June 1998. In this group embryo transfers were performed on day 2. The second group consisted of 228 IVF cycles performed between the same period of time as group 1. In this group blastocysts were transferred on day 6. Each of the above groups were further subdivided into a): standard insemination or b): ICSI cycles in order to pick out differences from male factor contributions.

Clinical IVF indications were tubal disease, endometriosis, polycystic ovarian disease, immunological and cervical infertility, male subfertility, idiopathic infertility, combined pathologies and long-standing infertility with failure of other medical and surgial treatments. Male subfertility was defined by a spermiogram showing one or more abnormal parameters including number, motility and morphology of spermatozoa. ICSI indications included fertilization failure or less than $0.8x10^{a}6$ spermatozoa after preparation, or sperm morphology < 5% normal or antisperm antibodies. In the ICSI group, systematic karyotypes of all men were performed. The ovarian stimulation protocol was conducted using long-protocol down-regulation with GnRH analogue (Arvekap Ipsen 0.1 mg/d) subcutaneous injection for ten days commencing on day 20 or 21 of an idealised 28-day cycle. A satisfactory suppression was checked by assaying estradiol (E2) serum levels (E_2<50pg/ml). Once suppression had been achieved, ovarian stimulation was commenced with a predetermined (200-300 IU/d) individualised dose of subcutaneous recombinant FSH (follitropin beta, Puregon, Organon, Oss the Netherlands). Follicular growth was monitored using ultrasonography (US) and measurement of estradiol concentrations. Human chorionic gonadotropin (hCG - Pregnyl; Organon) was administered at a 10,000 IU dose when there were more than three follicles ≥ 17 mm in diameter and estradiol concentration was ≥ 2,000 pmol/l. Transvaginal oocyte retrieval was performed 36 h post hCG under sedation using a 17-gauge single lumen needle.

Oocytes were identified in the laboratory and briefly rinsed of follicular fluid and freed from blood in handling medium (Gamete-100, Scandinavian IVF Science AB, Gothenburg, Sweden) before being placed in 1 ml pre-equilibrated culture medium (IVF-S1, Scandinavian IVF Science AB Gothenburg, Sweden) with one to two oocytes per culture tube (Falcon #2003, Becton Dickinson Labware, Franklin Lakes, NS, USA).

For the IVF-standard insemination groups, after oocyte pick-up, the oocytes were inseminated with 50,000-100,000 normal motile 4-6 hours after collection and after an hour's exposure were rinsed briefly in handling medium before being placed in fresh pre-equilibrated culture medium. Preparations for ICSI followed procedures similar to those described by both Tucker *et al.* [8] and Palermo *et al.* [9]. The culture protocol used for growth to blastocysts is based on the protocol of Jones, as has been described previously [7]. Briefly, the day after insemination the oocytes where checked for fertilization and then were cultured in groups of 2 to 3 in microdrops of Scandinavian IVF Science (SIS) IVF-50 medium under SIS ovoil-150 for two days. Embryos were then regrouped according to their similarity in cell stage and were cultured in groups of 2 to 3 in microdrops of SIS G2 medium under oil from days 3 to 5. Embryos were then transferred to fresh SIS S2 and cultured for a further day. The zona of all blastocysts were removed enzymatically with 0.2% pronase before embryo transfer.

Blastocysts were selected for transfer according to availability. One to four blastocysts were transferred on day 6. If the patient was > 35 years of age or if the patient had failed to achieve a pregnancy after three previous IVF cycles then consideration was given to transferring three or occasionally four blastocysts rather than two blastocysts, which would be the usual recommedation to a patient of a younger age or with a limited IVF history.

All patients received luteal phase support in the form of hCG 1500 IU IM on days 4, 7 and 10 after oocyte retrieval. In cycles with ovarian hyperstimulation the luteal phase was supported with vaginal progesterone only supplementation (Crinone; Wyeth) until the day of ß-hCG measurement.

Pregnancies were recorded by serun hCG assay (≥ 251U/L) ≥ 17 days after ovum pick-up and were confirmed by the demonstration of gestational sac by US scan four weeks after ET. Pregnancy was defined by the presence of a fetal heartbeat past the 7th week of gestation. The implantation rate was calculated as the number of embryos that were implanted (fetal heart rate) divided by the number of blastocysts that were transferred, multiplied by 100.

Statistical analysis

Statistical analysis included x^2, t-test and comparison of proportions.

Results

A total of 281 IVF-standard inseminations and 374 ICSI cycle procedures were performed in our ART unit during the study period. In all 10,146 oocytes were retrieved, 6,105 oocytes were fertilized, 2,222 embryos were transferred and 197 clinical pregnancies were achieved in all groups. In group 2 when blastocysts were transferred, almost 90% of patients underwent embryo transfer. Blastization rate was 46% in the IVF-standard insemination group versus 37% in the ICSI group. Two different groups were identified. Group 1 included 427 cycles in women ≤ 40 years of age having a day-2 ET. Group 2 comprised 228 cycles in women ≤ 40 years of age having a day-6 ET.

Table 1 presents details concerning the mean number of oocytes retrieved, mean number of oocytes fertilized, mean number of embryos transferred and number of clinical pregnancies in all groups. There was no significant difference in mean age between the two groups. There was a significant difference in mean number of oocytes retrieved, oocytes fertilized, number of embryos transferred and clinical pregnancies between group 1 and group 2. When embryos were transferred on day 6 there was an increased yield of retrieved oocytes and number of oocytes fertilized. The pregnancy rate was significantly increased while at the same time the number of embryos transferred was reduced.

Table 2 presents the same details of the two groups when IVF with standard insemination was performed. In this table there was a significant difference between group 2 and group 1 in the mean number of oocytes retrieved, oocytes fertilized, embryos transferred and in the pregnancy rate. There was no significant difference in mean age between the two groups, even though in group 2 there was an increased number of embryos being replaced (3.2 vs 4.1).

Table 3 presents the same details of the two groups when ICSI was performed. In this table there was a signi-

Table 1. — *Effects of day of transfer on pregnancy outcome in assisted reproductive technology.*

Variable	Day 2	Day 6
No. of cycles	427	228
No. (%) of embryo transfers	416	206
Mean age (±SD) in years	33.18±4.5	33.2±4.3
Mean No. (±SD) of oocytes retrieved	12.92±11.3	20±8.5*
Mean No. (±SD) of oocytes fertilized	7.63±5.2	13.6±6.8*
Mean No. (±SD) of embryos transferred	3.8±1.2	3.2±1.2*
Clinical pregnancies	101	96
Percentage of pregnancies per/cycle	23.65	42.1*
Implantation rate	8.62%	19.4%*
Sacs	135	125
No. of abortions	19	12
Multiple pregnancies	40	31
Multiple pregnancy rate (%)	39.6	32.2
Cancelation rate (%)	2.6	9.6*

$p < 0.05$ (versus day 2)

Table 2. — *Effects of day of transfer on pregnancy outcome in IVF-standard insemination group*

Variable	Day 2	Day 6
No. of cycles	186	95
No. (%) of embryo transfers	182	86
Mean age (±SD) in years	33.4±4.2	33.8±4.5
Mean No. (±SD) of oocytes retrieved	12.5±10.7	19.51±9.57*
Mean No. (±SD) of oocytes fertilized	8±5.9	14.23±6.15*
Mean No. (±SD) of embryos transferred	3.9±1.4	3.2±1.2*
Clinical pregnancies	46	40*
Percentage of pregnancies per/cycle	24.7	42.1*
Implantation rate#	8.5%	22.1%*

No. of embryos that implanted / No. of embryos that transferred

* $p < 0.05$ (versus day 2)

Table 3. — *Effects of day of transfer on pregnancy outcome in ICSI group*

Variable	Day 2	Day 6
No. of cycles	241	133
No. (%) of embryo transfers	234	120
Mean age (±SD) in years	33.3±4.6	32.7±4.2
Mean No. (±SD) of oocytes retrieved	13.07±10.16	20.5±7.5*
Mean No. (±SD) of oocytes fertilized	7.15±4.7	13.1±5.2*
Mean No. (±SD) of embryos transferred	3.8±1.3	3.3±1.1*
Clinical pregnancies	55	56
Percentage of pregnancies per/cycle	22.8	42.1*
Implantation rate#	8.7%	17.6%†

No. of embryos that implanted / No. of embryos that transferred

* $p < .005$ (versus day 2)

† $p < .05$ (versus day 2)

Table 4. — *Data on blastocyst development in IVF-standard insemination group and ICSI group*

Variable	IVF-STANDARD INSEMINATION GROUP	ICSI GROUP
No. of cycles	95	133
No. (%) of embryo transfers	86 (90)	120 (90)
No. of pronucleates	993	1415
No. (%) of blastocysts (total)	456 (46)	524 (37)*
No. of blastocysts transferred	253	390
No. of blastocysts frozen	203	134
No. of blastocysts per embryo transfer.	3.2	3.3
No. of pregnancies	40	56
Percentage of pregnancies per/cycle	42.1	42.1
No. of sacs	56	69
Implantation rate#	22.1	17.6
No. of Abortions	5	7

No. of embryos that implanted / No. of blastocysts transferred.

* $p < .005$ (versus IVF-standard insemination group).

ficant difference between group 2 and group 1 in the mean number of oocytes retrieved, oocytes fertilized, embryos transferred and in the pregnancy rate. Even though in group 2 there was an increased number of oocytes being fertilized at the same time there was a significant reduction in the number of embryos being replaced (3.3 vs 3.8). There was no significant difference in mean age between the two groups.

Results for the transfer of blastocysts only are detailed in Table 4. In the IVF-standard insemination group from a total of 993 pronucleate stage embryos, 456 developed to the blastocyst stage (45.9%). In the ICSI group from a total of 1,415 pronucleate stage embryos, 524 developed to the blastocyst stage (37%), significantly different from the IVF-standard insemination group ($p < 0.005$). In the IVF standard insemination group 253 blastocysts were transferred resulting in a pregnancy rate and implantation rate of 42.1% and 22.1%, respectively. In the ICSI group 390 blastocysts were transferred resulting in a pregnancy rate and implantantion rate of 42.1% and 17.6%, respectively.

In this study we identified two groups of patients with different clinical success rates following IVF-standard insemination and ICSI treatment. Patients for whom IVF or ICSI was performed and had embryos transferred on day 2 had significantly lower pregnancy rates (24.7% and 23.6%, respectively) than patients treated with IVF and ICSI respectively, who had embryos transferred on day 6 (42.1% and 42.1%, respectively).

In the blastocyst group the multiple pregnancy rate was 32.2% while in the day-2 group it was 39.6%. The cancelation rate in the blastocyst group was 9.6% while in the day-2 group it was 2.6% and the abortion rate in the blastocyst group was 12.5% while in the day-2 group it was 18.8%.

Discussion

It is now widely accepted that human embryos resulting from IVF have different development capacities which are related to their intrinsic quality [10]. Embryos graded as morphologically better and those that develop into blastocysts may have a better metabolic and genetic capacity to implant. The transfer of blastocysts appears to

be extremely important in enabling the selection of those embryos with the highest potential to implant and thus making possible the reduction of the number of embryos to be transferred, limiting the possibility of multiple implantations [11].

In our study, 46% of zygotes developed to the blastocyst stage in the IVF-standard insemination group. This compares favourably with a previous report of 52% of zygotes developing to blastocysts [7]. In the ICSI group 37% of zygotes developed to the blastocyst stage. This is statistically significantly different ($p<0.005$). These findings may suggest that the sperm provides a major contribution to, and has a significant influence on the fate of the developing embryo. In our study we noticed that in five women with severe male factor who had originally agreed to donte surplus eggs to other couples, blastocycts developed only with the normal sperm of the other couples while with their own sperm they failed to develop any blastocysts. It may be that sperm factor plays a significant role in the development of blastocysts. In a recent publication [12] a similar fertilization rate was demonstrated for "ideal" oocytes and those containing a refractive body, a defect that is invariably associated with fertilization failure in standard IVF. It was said that "eventually all oocytes can be fertilized by ICSI" implying that ICSI may "force" the production of some embryos. This however does not mean that all embryos will develop as easily to blastocysts. The results of our study are in agreement with this concept, namely fewer blastocysts available for transfer.

We did not however, find a statistically significant difference in pregnancy rate between IVF and ICSI in the blastocyst groups. In the literature [13] a high pregnancy rate (PR) was achieved using ICSI in a group of patients with total fertilisation failure after conventional IVF. It is important to note the semen characteristics in all patients with total fertilisation failure after conventional IVF. In one other study [14] a higher clinical PR was achieved using IVF embryos than with ICSI embryos. This result was detected despite the significantly older age of the female partner and the initially fewer oocytes in the IVF group. In this study there was no significant difference in pregnancy rate and implantation rate between the IVF-standard insemination group and the ICSI group.

Twenty-two women (9.6%) did not complete successful development of embryos to the blastocyst stage in vitro. In these women embryo transfers were not performed. Zygotes may be endowed with an inherent capacity to progress to later stages of embryonic development and this capacity may be reflected in the sibling oocyte undergoing in vitro culture [15]. These women were perhaps spared an embryo transfer at an earlier stage which would have been an inevitable failure.

Since November 1997 in our clinics we have offered blastocyst culture. At the beginning we used a cut-off point in the number of oocytes fertilized. More than five oocytes had to be fertilized before blastocyst culture could be offered. Now, in our opinion, blastocyst culture could be comfortably offered to all patients. No blastocyst development in vitro may be, according to us, equivalent to a negative pregnancy test several days before. Those embryos that fail to reach the blastocyst stage in vitro may perhaps not be able to give rise to offspring if transferred earlier. Further, for women of 40 years and over who do not produce any blastocysts, they may be more easily convinced of the value of donor eggs [16, 17].

In our study, 30 women with previous repeated failure got pregnant when blastocysts were transferred. In a previous study Olivennes *et al.* [2] reported that the transfer of blastocysts was beneficial in women with previous repeated failures of implantation. These findings also may suggest that the premature exposure of cleavage stage embryos to the uterus after day-2 transfers may compromise embryo development and viability [18]. It may, however, also be a zona-hardening factor which is removed with the zona removal before blastocyst ET.

In the present study eight women with huge hydrosalpinges got pregnant with blastocysts. These women had two or more previously failed IVF attempts. It has been reported recently that the presence of a hydrosalpinx has a negative impact on IVF/ET because of suspected embryotoxicity of the hydrosalpingeal fluid [19, 20]. Other studies [21], demonstrate the absence of a detrimental effect of the active hydrosalpinx fluid and suggest that constant passage of fluid into the uterine cavity could possibly introduce some mechanical interference that may result in implantation failure. In contrast to this publication, from our study it seems that hydrosalpinx fluid may not have a negative effect in blastocyst transfer. This will have to be examined further.

Multiple pregnancy is one of the most important and preventable complications in IVF and embryo transfer. Multiple-embryo transfer carries the risk of multiple gestation. In recent puplications [22] it has been proposed that new technologic procedures such as embryo biopsy for aneuploid screening is one means of reducing the embryo numbers transferred without decreasing the overall pregnancy rate, while another study [23] suggests that the number of embryos transferred should be limited to a maximum of three regardless of the age of patients in order to reduce the high frequency of multiple gestations in an IVF program. However Austin *et al.* [24], in a recent publication demonstrated that the risk of multiple pregnancy cannot be eliminated without decreasing the pregnancy rate. In our study we demonstrate that we can comfortably reduce the mean number of embryos to be transferred and at the same time increase the pregnancy rate by the use of blastocysts. In view of these findings the transfer of blastocysts appears to be extremely important in enabling the selection of those embryos with the highest potential to implant and thus limit the possibility of multiple implantation by reducing the number of embryos to be transferred. Our findings are in agreement with a previous publication by Plachot *et al.* [11].

The success rate of the blastocyst group is confounded by the embryos having assisted hatching and the patients of this group having a greater number of oocytes as compared to the day-1 transfers. In the literature there are several

studies to refute the contention that the difference in pregnancy rates between these groups is due to differences in response to ovulation induction or assisted hatching.

Clinical pregnancy has been mostly significantly influenced positively by the numbers of embryos transferred [22, 25].

Lashen *et al.* reported that poor responders (≤ 5 follicles) had a clinical pregnancy rate/cycle of 25.5% and this was comparable to their overall rate in their study period [26]. Hanoch *et al.* in another study demonstrates that young low-responders were protected from the untoward effects of reduced ovarian response [27] while Simon *et al.* suggests that an excessive response may eventially interfere with successful implantation [28].

In our study the blastocyst group had a statistically lower number of embryos transferred per ET and thus may not have had as great a chance of pregnancy to begin with in comparison to the day-2 group.

Furthermore, in the literature there are studies to confirm that assisted hatching through partial zona dissection prior to embryo transfer does not actually improve pregnancy and embryo implantantion rates in unselected patients undergoing IVF or ICSI [29].

In conclusion, this study though retrospective, demonstrates that the transfer of blastocysts does increase the success of IVF when compared with day-2 transfers, increases the success of ICSI when compared with day-2 transfers, and more importantly reduces the number of embryos to be transferred. Consequently the use of balstocysts in assisted reproductive technology offers long awaited encouraging improvements. More prospective studies are, however, needed to confirm our results.

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