Comparison of Zona Manipulated and Zona Intact Blastocyst Transfers: A Prospective Randomized Trial

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Purpose: In this prospective randomized study the effects of enzymatic treatment of zona pellucida of blastocysts on implantation and pregnancy rates were evaluated in a group of patients who had more than five embryos on day 3.

Methods: Forty-six patients with a mean age of 29.8 ± 4.5 years and mean duration of infertility of 6.72 ± 0.63 years had blastocyst stage transfers, with a mean number of 2.9 ± 0.1 embryos replaced per patient. Patients were randomly divided into two groups. The first group consisted of patients ($n = 22$) who had zona intact blastocyst stage transfers and the second group consisted of patients ($n = 24$) who had zona manipulated (enzymatic treatment) blastocyst stage transfers. Patient and cycle characteristics were similar in both groups. A commercial cell and serum-free sequential culture system was used for all embryos.

Results: Overall blastocyst formation rate was 50.3%. Transfer could be done in all patients. The positive β -human chorionic gonadotropin rate in the zona intact group was 50% (11/22) and in the zona-manipulated group was 70.8% (17/24). Clinical pregnancy, ongoing pregnancy, and implantation rates in zona intact and manipulated groups were 45.5%, 27.3%, and 19%, and 62.5%, 45.8%, and 24% respectively. Although implantation and pregnancy rates in the zona-manipulated group were higher, there were no statistically significant differences in terms of these variables between two groups. No triplet pregnancy was obtained in either group, and the twin pregnancy rate was 20% (2/10) in the zona intact group and 13.3% (2/15) in the zona-manipulated group.

Conclusions: With further improvements in the embryo culture systems it will become possible in the near future to achieve high implantation rates even with single blastocyst transfers. Enzymatic treatment of the zona pellucida seems that it does not alter the pregnancy and implantation rates, but further studies with larger group of patients are needed

to clarify the real effect of this zona manipulation on pregnancy outcome.

KEY WORDS: Blastocyst; embryo transfer; intracytoplasmic sperm injection; zona manipulation.

INTRODUCTION

Despite the significant advances in assisted reproductive technologies, implantation rates in human in vitro fertilization (IVF) remain unsatisfactory in routine practice. On the other hand, increases in the knowledge of mammalian embryo physiology (1) and subsequent developments in the human embryo culture media (2) allowed researchers to test the value of delayed embryo transfers in the improvement of implantation and pregnancy rates in human IVF. As a result of this development it has been shown recently that day 5 or day 6 blastocyst transfers using serum-free culture systems improve the implantation rates in IVF programs (3–5), and further improvements in the implantation and pregnancy rates by enzymatic treatment of zona pellucida of blastocysts have been suggested by some of these researchers (5,6). The aim of this study is to evaluate the effect of enzymatic treatment of zona pellucida of day 5 or day 6 blastocysts on implantation and pregnancy rates and to compare these figures with the zona intact blastocyst transfers in a prospective randomized trial.

MATERIALS AND METHODS

This study was performed in a private hospital IVF unit between April 1, 1998 and October 30, 1998. The study was approved by the Human Investigation Committee of the hospital and an informed consent was taken from all participants. Patients who had more than five day-3 cleavage stage embryos were enrolled to the study.

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Patients were randomized by using a random table and two groups were formed. The first group included zona intact blastocyst transfer patients (group A, $n = 22$) and the second group included zona free blastocyst transfer patients (group B, $n = 24$). All the cycles in the study were down-regulated by using a long protocol gonadotropin-releasing hormone agonist (GnRHa)—800 μg nafarelin (Synarel, Abdi Ibrahim, Turkey) nasal spray—or 1 mg leuprolide acetate (Lucrin, Abbot, Turkey) subcutaneous injections started in the luteal phase of the preceding cycle day 21. The dose was halved when the pituitary suppression was documented by E2 levels < 50 pg/ml and absence of an ovarian cyst on transvaginal ultrasound examination. Then ovarian stimulation was achieved by step-down administration of pure follicle-stimulating hormone (FSH) (Metrodin, Serono, Istanbul, or Follegon Organon, Istanbul, Turkey); the starting dose ranged between 150 and 600 IU daily. To trigger the final follicular maturation before oocyte collection, 5000–10000 IU of human chorionic gonadotropin (hCG) (Pregnyl, Organon, Turkey) was administered to each patient when there were at least three follicles > 15 mm in diameter. Oocytes were recovered transvaginally with ultrasound guidance 35–36 hr after the hCG injection under intravenous total anesthesia with Propofol (Zeneca, Istanbul, Turkey). Luteal phase support was provided by 400 mg micronized progesterone transvaginal (Utrogestane, Laboratory, Besins Iscovesco, France) daily. In all cycles intracytoplasmic injection of ejaculated spermatozoa was used as there was a male component in the etiology of infertility in all couples.

For all the steps of gamete and embryo handling and culture, a spectrum of commercial media obtained from Scandinavian IVF Science Company, Sweden, was used and no serum was added in any step. All oocytes were incubated singly, before and after intracytoplasmic sperm injection (ICSI), in 50- μl droplets of IVF-50, under Ovoil-150. The ICSI procedure was performed 2–3 hr after oocyte recovery. Before the ICSI procedure oocytes were denuded by using hyaluronidase solution prepared from Hyase-10X. After ICSI, oocytes were incubated at 37°C and a humidified atmosphere of 5% CO_2 in air in Heraus incubators (6220 and BB16 models, Germany).

Fertilization was assessed 16–19 hr after ICSI procedure and culture was continued in IV-50 medium droplets under Ovoil-150 until day 3. In two cases (one in group A and one in group B) (PN) stage freezing was performed. On day 2, embryos were graded at the 2- to 4-cell stage and in four cases (three in group A and one in group B) day 2 freezing of some of the embryos

was utilized. In both stages propanediol-based freezing kit was used (freeze kit 1). On day 3, the medium was changed to S2 medium, and until the transfer S2 medium was used. Medium was freshened every 24 hr. Embryos were assessed for blastocyst development in the morning of day 5 post-ICSI. Blastocoel formation, expansion, zona thinning, hatching, and cell structure and distribution of trophoctoderm were documented. Full expansion, zona thinning, hatching, and well-defined borders and homogeneous distribution of trophoctoderm cells were accepted as favorable signs.

In two patients blastocyst transfer was done on day 5 (PM) and in the remaining patients ($n = 44$) transfer was done on day 6 (AM). Up to four (in cases with previous multiple implantation failures) but usually two or three blastocysts were transferred. Excess blastocysts were cryopreserved by using a glycerol-based freezing kit (freeze kit 2) in 15 patients. All the transfers were done by using S2 medium and Frydman transfer catheter (CCD, ref. No. 1307000, France). In group B, blastocysts were exposed briefly (30 sec to 3 min) to 0.2% pronase (Sigma Chemical Co. P 8811) in Gamete-100 medium droplets under Ovoil-150. During this time period embryos were kept in an humidified atmosphere of 5% CO_2 in air on the heated table of a K system laminar airflow hood (L-26, Knudsen, Denmark) and were checked every 20–30 sec with a stereozoom microscope (Olympus SZ 1610); zona softening and the beginning of the disappearance of zona were recorded. Just before the complete disappearance of zona, embryos were transferred gently and washed twice in the Gamete-100 droplets under Ovoil-150 located in the same dish. Following the zona manipulation embryos were incubated in S2 medium for an extra 30–60 min before the transfer.

Two days after the embryo transfer, E2 level was measured in all patients; if the E2 level was below 2500 pmole/liter, 2000–5000 IU single-dose HCG administration was performed. More than 25 mIU/ml β -hCG serum level was considered a positive pregnancy test on day 11 or later after the transfer. Presence of gestational sac with embryonal pole with or without fetal heartbeat was considered as a clinical pregnancy. Abortion before clinical stage was considered to be a biochemical abortion and after this stage as a clinical abortion; viable pregnancies were those that reached beyond 12 weeks of gestation and continuing on were considered to be ongoing pregnancies. Statistical analysis was achieved by using SPSS for MS Windows program and independent samples t test or χ^2 test were used where appropriate. P value < 0.05 was considered to be statistically significant.

RESULTS

A total of 46 patients were enrolled to the study. Patient age ranged between 20 and 39 years and infertility durations varied between 2 and 17 years. Patient and cycle characteristics are presented in Table I. No significant differences were found between group A and group B in terms of the female age, infertility duration, day-3 FSH of previous cycle, number of the retrieved oocytes, number of the previous attempts, fertilization rate with ICSI, number of the embryos obtained, and number of the embryos transferred. Blastocyst formation rate in group A and in group B were 53.8% and 47.2%, respectively (in group A, 134 blastocysts out of 249 day-3 embryos, and in group B, 129 blastocysts out of 273 day-3 embryos), and overall blastocyst formation rate was 50.3% (263 out of 522). Full expansion and thinning of the zona were found in 64.9% and 61.2% of day-5 and day-6 blastocysts in groups A and B, respectively. Blastocyst stage transfer could be done in all patients. Eight patients in group A (36.4%) and seven patients in group B (29.2%) had at least one previous failed IVF attempt. Transfer was done on day 6 (AM) in 44 patients, whereas in 2 patients transfer was performed on day 5 (PM). Although pregnancy, implantation, and ongoing pregnancy rates are higher in group B (zona-manipulated group) there was no statistically significant difference in terms of these variables between the two groups (Table II). No triplet pregnancy was obtained in either group, and four twin

Table II. Comparison of Implantation and Pregnancy Rates in Two Groups^a

	Group A (zona intact)	Group B (zona manipulated)	P value
Number of patients	22	24	NS ^b
+ β -hCG	11 (50)	17 (70.8)	NS
Clinical pregnancy rate	10 (45.8)	15 (62.5)	NS
Ongoing pregnancy rate	6 (27.3)	11 (45.8)	NS
Implantation rate	12/63 (19)	17/71 (24)	NS
Number of singletons	8 (80)	13 (86.7)	NS
Number of twins	2 (20)	2 (13.3)	NS
Number of triplets	0	0	NS

^a Values in the parenthesis are percentages.

^b NS, not significant.

pregnancies (two in group A, two in group B) were detected. In group B, there were no high-quality (no full expansion, zona thinning, or hatching) blastocysts (only cavitating morula stage or small blastocoel-formed embryos were transferred) in four patients, and no pregnancy was obtained in this group. Another way of saying this is that in 20 out of 24 patients in group B there was at least one good quality, fully expanded, zona-thinned embryo and 17 of these (85%) became pregnant. On the other hand, in group A in all patients there was at least one good quality blastocyst available for the transfer. In 15 patients (32.6%) (nine in group A, six in group B) there were excess blastocysts for cryopreservation.

Table I. Comparison of Patient and Cycle Characteristics in Two Groups

	Group A ^a (zona intact)	Group B (zona manipulated)	P value
Number of patients	22	24	NS ^c
Female age	29.1 $\pm$ 3.6	30.5 $\pm$ 5.2	NS
Infertility duration	6.8 $\pm$ 3.9	6.6 $\pm$ 4.5	NS
Day-3 FSH ^b	6.1 $\pm$ 3.0	5.5 $\pm$ 1.4	NS
Previous attempts	1.3 $\pm$ 0.7	1.3 $\pm$ 0.5	NS
No. of collected oocytes	18.5 $\pm$ 6.8	17.0 $\pm$ 7.0	NS
Fertilization rate (%)	83.7	77.1	NS
No. of day-3 embryos	12.8 $\pm$ 4.6	12.0 $\pm$ 5.2	NS
Blastocyst formation rate (%)	53.8	47.2	NS
Expanded blastocyst (%)	64.9	61.2	NS
No. of transferred blastocysts	2.86 $\pm$ 0.8	2.95 $\pm$ 0.9	NS

^a Values are mean $\pm$ standard deviation.

^b Day 3 of previous cycle.

^c NS, not significant.

DISCUSSION

In human IVF the delivery rates are around 14–15% for a mean number of three cleavage-stage embryos transferred on the second or third day after insemination for IVF and ICSI (7), and these values have remained lower than those routinely achieved for animal IVF, where mainly the blastocyst stage embryo transfer is utilized (1,8,9). Therefore, it can be postulated that if blastocyst transfers can be achieved in human IVF routinely, a dramatic increase in both pregnancy and implantation rates can be expected. In support of this when in vivo developed human blastocysts were flushed from the uterus and subsequently transferred singly to recipient women, a 60% implantation and pregnancy rate was achieved (10).

So why is the human embryo not routinely transferred at blastocyst stage in human IVF? According to Gardner and Lane (11), the reasons for this inability to maintain the viability of the human embryo are

twofold. First, most culture media that are used are too simple and lack some of the most important regulators of mammalian embryo development such as amino acids. Second, attempts to optimize culture conditions have focused on the formulation of a single medium for the entire preimplantation period. Such an approach does not take into account the changing physiology and energy metabolism of the embryo as it develops from the zygote to the blastocyst stage, and that the requirements of each stage differ markedly from each other. The development of two sequential serum-free media formulated for the culture of human zygotes to the blastocyst stage made a considerable contribution to human IVF. On the other hand, there also were other efforts to improve the implantation rates achieved by the blastocyst stage transfers, one of which is enzymatic treatment of zona of blastocysts. The first pregnancy that was obtained by the transfer of blastocysts with zona completely removed with pronase was reported by Fong *et al.* in 1997 (12). After this report two series were reported (5,6) where zona-manipulated blastocysts were transferred and high implantation rates were achieved.

When we looked at the data retrospectively in our IVF program, implantation rate obtained in the patients who had more than five embryos available and had been transferred on day 3 was found to be 14% (unpublished data). In our study in group A, implantation rate was 19% and in group B it was 24% when we consider the two groups together the implantation rate was 21.6%. These values can be considered more optimistic than the value (14%) that we have obtained before. However, as these values cannot be comparable to document the presence of any statistically significant difference between day 3 and days 5 or 6 transfers, a prospective randomized study is currently being performed in our center. In the study of Jones *et al.* (5) and Scholtes and Zeilmaker (3) very similar implantation and pregnancy rates to our study were reported with day-5 and day-6 embryo transfers. In the study of Scholtes and Zeilmaker (3) embryos were transferred as zona intact on day 5, whereas in the study of Jones *et al.* (5), only zona-manipulated blastocysts both on days 5 or 6 were transferred. Again, in a very recently published study, Fong *et al.* (6) reported 33% implantation and 53% clinical pregnancy rates, and they used a very similar embryo culture protocol to us. However, they performed only zona-manipulated transfers in a selected group of patients. The success rates obtained in the Fong *et al.* (6) study also are comparable with group B in our study. When we looked at the figures in group A, we found a high rate of early pregnancy wastage; this may be related with

zona, hatching, and subsequent implantation problems. On the other hand, in a recent study by Gardner *et al.* (13) very high implantation and clinical pregnancy rates were achieved in a selected group of patients with the use of sequential modified culture media formulations (G 1.2 and G 2.2). Another interesting aspect of this study is the presence of the very high clinical pregnancy rate in the day-3 transfer group (66%), which may be another indicator of improved culture conditions. In this study the authors did not use any enzymatic manipulation of blastocyst zona but they performed selective assisted hatching of day-3 embryos. With day 5 transfer they reported 50% implantation and 71% clinical pregnancy rate. Although these figures are astonishing, it is still possible to speculate that enzymatic zona treatment may further increase the implantation rate even up to 60%, which was achieved previously in an in vivo study (10). Supporting this speculation, in the study of Fong *et al.* (6), the in vitro implantation of zona-free blastocysts on a variety of feeder monolayers was found to be 92%. In our study in group B when there was at least one good quality blastocyst for the transfer the pregnancy rate was found to be 85% (17/20), clinical pregnancy rate was 75% (15/20), and ongoing pregnancy rate was 55% (11/20). These figures also support the importance of availability of high-quality blastocysts for the transfer, and of course this is mostly related to the improved culture conditions.

It can be concluded that blastocyst stage transfer, at least in a selected group of patients (good responders) can increase implantation and pregnancy rates; and even with only a single good quality blastocyst-stage embryo transfer very high pregnancy rates can be achieved in the near future without the risk of multiple pregnancy. Enzymatic treatment of zona pellicula appears to not alter the pregnancy and implantation rates, but further studies with larger group of patients are necessary to clarify the real effect of this zona manipulation on pregnancy outcome. Improvements in the culture systems will most probably bring the blastocyst stage transfers to routine use in all patients in the near future; we also can expect important developments in preimplantation genetic diagnoses with the availability of more blastocyst-stage embryos and an extended study period of diagnostic procedures after the biopsies.

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