

# Cumulus coculture and cumulus-aided embryo transfer increases pregnancy rates in patients undergoing in vitro fertilization

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**Objective:** To evaluate the effect of the use of cumulus-aided embryo transfer on pregnancy rates. To study the proximity of expanded cumulus cells to the developing embryo. To document by light microscopy the anchoring of day 3 to day 4 embryos by the expanded cumulus cells. To demonstrate by transmission electron microscopy the cellular activity of the expanded cumulus cells. To evaluate the expression of growth factors (vascular endothelial growth factor, interleukin-6, insulin-like growth factor I) that are secreted by the cumulus cells.

**Design:** A comparative study of a group of women undergoing cumulus coculture and cumulus-aided embryo transfer, with those who underwent cumulus coculture but did not undergo cumulus-aided embryo transfer. The endpoint was the achievement of pregnancy.

**Setting:** Department of Infertility Management and Assisted Reproduction, Jaslok Hospital and Research Centre, Mumbai, India.

**Patient(s):** Five hundred seventeen women undergoing treatment for infertility using intracytoplasmic sperm injection and embryo transfer and fulfilling set criteria. To validate our initial results, we conducted a similar study on 208 women where randomization was performed.

**Intervention(s):** Embryos were cocultured with the patient's own cumulus cells and were transferred into the uterus with approximately 30  $\mu$ L of the expanded cumulus cells.

**Main Outcome Measure(s):** Pregnancy, implantation, and multiple gestation rates.

**Result(s):** Our study demonstrated a significant increase in the implantation rate in the study group (group A) of 25.6% versus 14.5% in the control group (group B) and a significant increase in the pregnancy rate in the study group (group A) of 47.6% versus 34% achieved in the control group (group B). Although the incidence of multiple gestation was similar (38.6% in the study group and 32.9% in the control group), the higher-order multiple gestation rate was significantly more in the study group as compared with the control group (18.1% vs. 2.4%). Similar pregnancy and implantation rates were observed in the randomized study.

**Conclusion(s):** This study demonstrates the efficacy of cumulus-aided embryo transfer, using autologous cumulus cells. It indicates a significant increase in implantation and pregnancy rates. The results suggest that cumulus cells play an important role in embryonic development, and that they may provide a mechanism to improve embryo-uterine adhesion by physical proximity, and by secreting cytokines and growth factors required to aid the implantation process. (Fertil Steril® 2006;86:839–47. ©2006 by American Society for Reproductive Medicine.)

**Key Words:** Cumulus, coculture, cotransfer, growth factors, implantation factors, enhanced pregnancy rates

Human in vitro fertilization (IVF) with coculture has been attempted in the past (1–4). The process involves growing embryos in a culture medium on top of a proliferating monolayer of cells such as those from the fallopian tube (1), endometrium (2), or cumulus cells (3, 4). Use of animal cell lines has been discontinued due to concerns about viral and bacterial transmission. It is believed that the feeder or helper cells stimulate development of morphologically sound embryos by removing toxins from the culture medium and by the addition of growth factors such as insulin-like growth

factor (IGF) I and IGF-II, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)  $\alpha$  and TGF- $\beta$ , platelet activating factor (PAF), and epidermal growth factor (EGF) (5). It has been postulated that the feeder cells metabolize the glucose present in the medium, thus allowing the embryos to be exposed to tolerable levels of glucose (6). Several studies have demonstrated faster cleavage rates, less fragmentation, and better implantation rates using a coculture system (7–10). Coculture systems reportedly increase blastulation rates to as high as 55%–70% (8, 11). Previous studies have reported that this technique is useful in women with previous multiple failed IVF cycles and those with advancing age.

An extensive literature survey enabled us to narrow down three main growth factors and cytokines—IGF-I and IGF-II

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(12–14), interleukin (IL) 1 and IL-6 (15, 16), and VEGF (17, 18)—which are expressed by cumulus cells and are known to improve embryo morphology and play a putative role in the implantation process.

In an effort to improve implantation rates, the search has been ongoing for a “glue” that will allow more intimate contact between the embryo and the uterus (19). We hypothesized that coculture of embryos with cumulus cells, which would serve as a source of growth factors, would contribute to increasing the adhesiveness of embryos. We therefore developed a novel technique of transferring embryos with 30  $\mu\text{L}$  of the women’s own cumulus cells to assess whether this would lead to an increase in the pregnancy rates (PRs).

## MATERIAL AND METHODS

### Patient Selection

The criteria for inclusion in this study were women undergoing the intracytoplasmic sperm injection (ICSI) procedure, in whom  $\geq 3$  metaphase II stage oocytes were injected or  $\geq 2$  embryos were available for transfer. The reason for using these criteria was to minimize variation in the results, due to inherent problems with oocytes in situations where a lower number of oocytes were retrieved. The control group (group B) comprised 250 consecutive consenting patients who had undergone ICSI, where cumulus coculture was performed, but not cumulus-aided embryo transfer, and who met the set criteria. The next 267 consecutive patients meeting these criteria were selected to form the study group (group A), who consented to undergo ICSI, along with autologous cumulus embryo coculture and cumulus-aided embryo transfer. Only a single-attempt ICSI cycle for all 517 women selected for the study was included, and repeat cycles of the same patient were not incorporated in either the study group or the control group. Appropriate written voluntary consent was obtained from both the groups. The study received internal institutional review board approval.

Keeping in mind the need for randomization, subsequent to the completion of our initial study on 517 women, the next 208 consecutive women were allocated to the study and control groups in accordance with a computer-generated randomization process, which was based on the serial numbers given to each woman. A total of 108 women who had been allocated an even serial number by the computer formed our new study group, group C. The control group, group D, comprised 100 women who had an odd serial number generated by the computer. The criteria for patient selection and the protocol followed were identical to the initial study.

### Procedure

Luteal phase suppression was followed by controlled ovarian stimulation with gonadotropins. The hCG trigger was given at follicular maturity, followed by transvaginal ultrasound-guided oocyte retrieval. Oocytes were prepared for ICSI by standard protocols and were denuded by

exposure to 80 IU of hyaluronidase (20). The dissociated cumulus cells were washed twice with Quinn’s Advantage medium with HEPES (Sage Biopharma, Bedminster, NJ). The pellet was then diluted with Quinn’s Advantage Fertilization Human Tubal Fluid (HTF) medium (Sage Biopharma) and then transferred to the center-well culture plate (3037; Falcon, Franklin Lakes, NJ) and incubated at 37°C in 5%  $\text{CO}_2$ . After culture for 24 hours in Quinn’s Advantage Fertilization medium, the medium was discarded and Quinn’s Advantage Cleavage medium (Sage Biopharma) was added. After equilibration of the medium, the pronuclear stage embryos were added to the autologous cumulus coculture. At 36 hours, the old medium was discarded and the coculture along with the cleaving embryos was exposed to fresh Quinn’s Advantage Cleavage Medium. The following day, embryos were observed for further cleavage.

In the study group (group A), comprised of 267 women, cumulus-aided embryo transfer was performed with cocultured day 3 embryos approximately 78 hours after ICSI.

In the control group (group B), comprised of 250 women, a routine embryo transfer was performed with day 3 embryos that had been cocultured on autologous cumulus cells, but were transferred without the cumulus cells approximately 78 hours after ICSI.

In both the study and control groups, the number of embryos transferred depended on the advancing age of the woman, the quality of the embryos, and the decision of the couple. While selecting embryos for transfer, those having equal blastomeres with minimal fragmentation were preferred for transfer in both groups.

### Light Microscopy Evaluation of the Cumulus Cells from Day 0 to Day 10 of Oocyte Retrieval

Individual patient cumulus cells of both the study group and the control group were cultured from day 0–day 10 because autologous cumulus coculture was performed for both groups of patients. Serial observations were made involving the daily visualization of the expanded cumulus cells using an inverted microscope at 4 $\times$ , 10 $\times$ , and 40 $\times$  magnification. Images were grabbed using the Image Grabber Software—Avanttec Image Plus<sup>TM</sup> Version 8.0a (Avanttec Medical Systems (P) Ltd, Chennai, India).

### Electron Microscopy Studies

On completion of cumulus-aided embryo transfer with cocultured day 3 embryos in the study group, the expanded cumulus cells in the Petri dish, which originally contained the embryos, were further allowed to grow in the laboratory up to day 10 of culture. Electron microscopy studies were performed on these expanded cumulus cells on day 4 and day 5 of oocyte retrieval. Similar studies were also conducted on cultured day 4 and day 5 expanded cumulus cells obtained from women in the control group. The 1–2-mm

sized specimen was fixed in 3% cold glutaraldehyde solution for 3 hours. The specimen was then transferred to 0.1 M of cold cacodylate buffer for 30 minutes. This procedure was repeated three times. The tissue was then postfixed with osmium tetroxide for 2 hours, followed by dehydration, infiltration, and embedding. Semithin and ultrathin sections were cut with the help of Leica Ultracut R (Leica Mikrosysteme, Wien, Austria). The glass knives used were prepared on Leica EMKM R2 (Leica Microsystems). The grids were observed on a JEOL 1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan). Photographic documentation was performed at  $\times 8,000$ ,  $\times 25,000$ , and  $\times 30,000$  magnification.

### Quantitative Determination of VEGF, IL-6, and IGF-I in the Cumulus Cells from Day 0 to Day 10 of Oocyte Retrieval

This was performed on a daily basis from day 0 to day 10 in 5 women using the quantitative sandwich enzyme immunoassay technique (Quantikine; R&D Systems, Minneapolis, MN). The Petri dish containing the embryo coculture was isolated after the embryo was either transferred into a fresh Petri dish with expanded cumulus cells (days 1 and 2) or after cumulus-aided transfer (day 3). For the purpose of evaluation of growth factors that were secreted by the cumulus cells, the same procedure was performed on expanded cumulus cells containing surplus day 4–5 embryos that had not been transferred. Further evaluation of growth factors from day 6 to day 10 occurred in the absence of embryos. The attached cumulus cells were mechanically detached from the bottom of the Petri dish with a transfer pipette (7575; Falcon). These cells, along with 1.5 mL of the medium that was already present in the Petri dish at the time of coculture, were added to Eppendorf tubes. The test involved the use of three microplates, each precoated with a monoclonal antibody specific for VEGF, IL-6, and IGF-I. Centrifugation of the cumulus cells in the 1.5-mL medium was performed at 1,000 rpm for 5 minutes, and 200  $\mu$ L of the supernatant was pipetted into each precoated well. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF, IL-6, and IGF-I was added to the respective wells. This was followed by a wash to remove any unbound antibody-enzyme reagent. A substrate solution was added to the wells, and color developed in proportion to the amount of VEGF, IL-6, and IGF-I that was bound in the initial step. The color development was stopped, and the intensity of the color was measured.

### Description of the Embryo Loading Procedure

In preparation for transfer, the embryo was removed from the Petri dish containing the expanded cumulus cells and placed in a Petri dish containing the embryo transfer medium: Quinn's Advantage Cleavage medium with 30 mg/mL of human serum albumin (Sage Biopharma). A cluster of approximately 30  $\mu$ L of cumulus cells that were growing in

close proximity to the embryo was placed in the transfer medium. These cells were mechanically lifted from the Petri dish with the use of denuding pipettes. The collected cells were deposited around the embryo. The embryos and the cumulus cells were loaded together into the embryo transfer catheter (Labotect 320200, Gottingen, Germany). Approximately 2,300–2,700 cells were present in 30  $\mu$ L of the cumulus cell suspension. The contents of the catheter were released into the uterus over a period of 8–10 seconds, and the catheter was held in position *in vivo* for about 15 seconds.

### Statistical Analysis

A comparison in the outcome of pregnancy, implantation, multiple gestation, and higher-order multiple gestation rates in both the study and control groups was performed using the  $\chi^2$  test. A value of  $P < .05$  was considered statistically significant. Data was presented as mean  $\pm$  SD wherever necessary.

## RESULTS

The average age in group A (study group) was  $31.7 \pm 4.92$  years (range 21–42 years) and group B (control group) was  $32.0 \pm 4.42$  years (range 24–40 years). No differences were found in the mean ages and the indications for assisted reproductive technology in the two groups. In group A, 1,489 oocytes were retrieved (5.6 oocytes/cycle). Of those, 1,266 (85%) were at metaphase II stage. Embryo transfer (2 to 4 embryos) was performed on day 3. In group B, 1,533 oocytes were retrieved (6.1 oocytes/cycle). Of these, 1,225 (79.9%) were at metaphase II stage. Fertilization and cleavage rates in group A were 73.1% and 100%, and in group B were 71.8% and 100%, respectively. The average number of embryos transferred in group A and group B was 3.1 and 3.2, respectively (Table 1). Pregnancy rates and implantation rates in group A were 47.6% and 25.6%, and in group B were 34% and 14.5%, respectively. Our study thus demonstrated a significant increase in the implantation rates (25.6% vs. 14.5%,  $P < .001$ ) and also a significant increase in the PRs (47.6% vs. 34%,  $P < .01$ ). In addition, multiple gestation rates were comparable in group A ( $n = 49$ , 38.6%) and in group B ( $n = 28$ , 32.9%). The higher-order multiple gestation rate was significant in group A as compared with group B (18.1% vs. 2.4%;  $P < .01$ ) (Table 2).

### Light Microscopy Evaluation of the Cumulus Cells from Day 0 to Day 10 of Oocyte Retrieval

Within 24 hours of being in culture, the cumulus cells demonstrated dendrite-like processes. On further culture, these processes appeared to have an affinity for the embryo. Increased colonization with cumulus cells in close proximity of the embryo (Fig. 1A) and a paucity of these cumulus cells away from the embryo were noticed (Fig. 1B).

Once the oocyte was denuded, the cumulus cells appeared in small groups. After culture for 24 hours in Quinn's Ad-

**TABLE 1****Results for demography and procedure variables.**

Parameters	Study group (group A) (n = 267)	Control group (group B) (n = 250)	P value <sup>a</sup>
Average age (y)	31.7 ± 4.92	32.0 ± 4.42	NS
No. of oocytes	5.6 ± 2.0	6.1 ± 2.2	NS
No. of MII stage oocytes	4.7 ± 1.6	4.9 ± 1.7	NS
Fertilization rate (%)	926/1,266 (73.1)	880/1,225 (71.8)	NS
Cleavage rate (%)	926/926 (100)	880/880 (100)	NS
No. of embryos transferred	3.1 ± 0.7	3.2 ± 0.8	NS

Note: Values are mean ± SD. NS = not significant.  
<sup>a</sup> Statistical analysis with the  $\chi^2$  test.

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vantage Fertilization medium, expansion of these cells into fibroblast-like cells was observed, and a vast expanse of dendrite-like processes consisting of cytoplasmic extensions of the cumulus cells was noticed. These processes stretched across the Petri dish, establishing contact with the dendritic processes of neighboring cumulus cells. At 36 hours, colonies coalescing together were noticed. On further coculture, between 36 and 72 hours of oocyte retrieval, the entire Petri dish was studded with colonies of cumulus cells, which had button-like dense appearances with proliferating dendritic processes forming interlacing fibers. The cumulus cells expanded both below and above the embryo, engulfing it from all sides. On several occasions, embryos from the 4–10-cell stage onward stopped floating in the Petri dish and appeared to be completely immobilized because the cumulus complex adhered strongly to the embryo (Fig. 2). The dendritic processes of the cumulus cells were also observed extending toward the cleaving embryo (Fig. 3). This effect was observed in approximately 8%–10% of embryos. In the 96–120-hour period, the number of colonies increased marginally, but further expansion continued so that sheets of cumulus cells were observed beneath colonies that merged together. Apoptosis set in by day 9.5–day 10 of oocyte

retrieval, when the cells were no longer adherent to the Petri dish, the dendritic processes had a shrunken appearance, and the colonies lost their plumpness (Fig. 4).

#### Electron Microscopy Studies of the Expanded Cumulus Cells on Day 4 and Day 5 of Oocyte Retrieval

The expanded cumulus cells displayed increased mitotic activity, cytoplasmic processes, cytoplasmic vacuoles, and many gap junctions. These are suggestive of enhanced cellular activity (Fig. 5A, Fig. 5B).

#### Quantitative Determination of VEGF, IL-6, and IGF-I in the Cumulus Cells from Day 0 to Day 10 of Oocyte Retrieval

In our study, cumulus cells were tested for the expression of IL-6, IGF-I, and VEGF, and these were present in the assay until day 10 of culture. Apoptosis occurred on day 10 of coculture, at which time the secretion of these factors decreased to a minimum. A mean value of the daily estimation of the individual growth factor was reported for the five patients in the study. Daily assessment of VEGF exhibited a gradual rise until day 4. This was followed by a peaking of

**TABLE 2****Results for pregnancy outcome.**

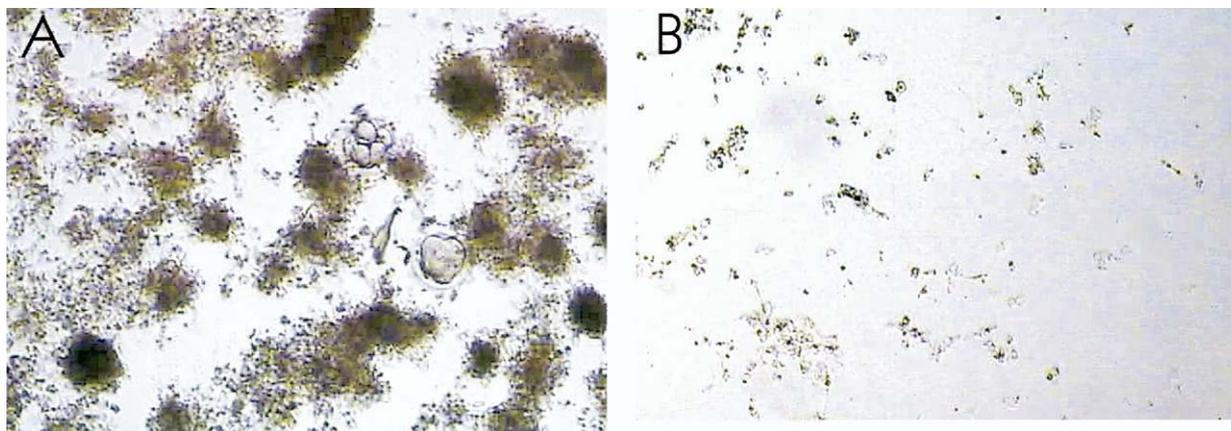
Parameters	Study group (group A) (n = 267)	Control group (group B) (n = 250)	P value <sup>a</sup>
Pregnancy rate (%)	127/267 (47.6)	85/250 (34.0)	<.01
Implantation rate (%)	213/833 (25.6)	117/806 (14.5)	<.001
Multiple gestation (%)	49/127 (38.6)	28/85 (32.9)	NS
Higher-order multiple gestation (%)	23/127 (18.1)	2/85 (2.4)	<.01

Note: NS = not significant.  
<sup>a</sup> Statistical analysis with the  $\chi^2$  test.

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## FIGURE 1

(A) Increased colonization with cumulus cells in close proximity of the embryo. (B) Paucity of cumulus cells away from the embryo. Both the photographs are from the same Petri dish.



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VEGF values on days 8 and 9, followed by a fall on day 10. No secretion of IL-6 by the cumulus cells occurred until day 5. The amount of IL-6 secretion was reduced from day 8 onward, and IGF-I secretion was erratic. The assay for IGF-I was not possible on days 1, 3, and 4 due to a technical difficulty. Evaluation was not possible on days 6 and 7 due to nonavailability of the samples. Estimation of these factors in the test control, water, and saline revealed no secretion. These factors were not present in Quinn's Advantage medium with HEPES and Quinn's Advantage Fertilization (HTF) medium. The IL-6 and VEGF were absent in Quinn's Advantage Cleavage medium and Quinn's Advantage Blas-

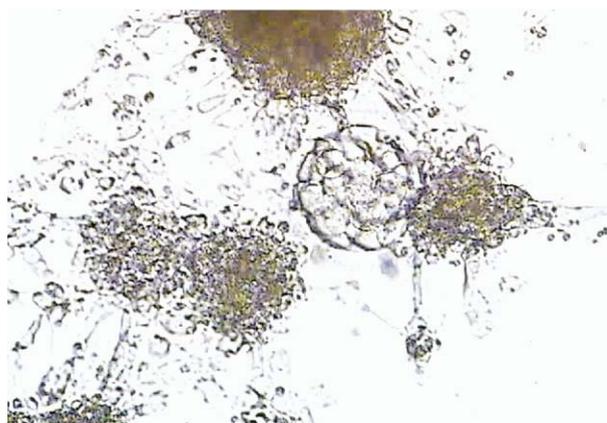
tocyst medium. However, Quinn's Advantage Cleavage medium and Quinn's Advantage Blastocyst medium indicated traces of IGF-I (Table 3).

### Results of the Randomized Study

Our initial findings in a recent study in terms of pregnancy and implantation rates are strongly suggestive of the efficacy of the procedure of cumulus-aided embryo transfer. A significantly higher PR was observed in the study group (group C) compared with the control group (group D) (47.2% vs. 33%;  $P < .05$ ). A significantly higher implantation rate was

## FIGURE 2

A day 3.5 embryo completely immobilized by the encroaching expanding cumulus cell colonies. Interlacing dendritic processes are pictured.



Parikh. Cumulus-aided embryo transfer. *Fertil Steril* 2006.

## FIGURE 3

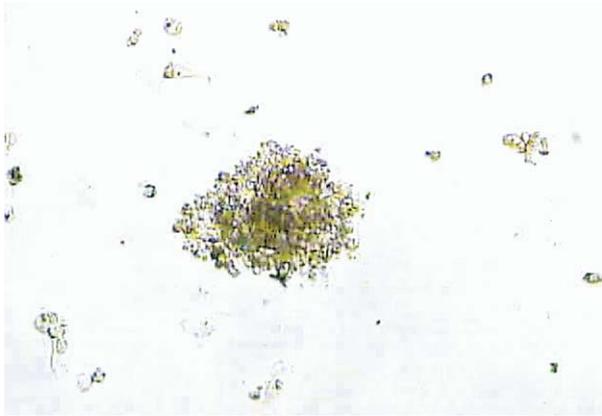
Visualization of a single dendritic process (indicated by an arrow) of the cumulus cell extending toward the cleaving embryo.



Parikh. Cumulus-aided embryo transfer. *Fertil Steril* 2006.

## FIGURE 4

Apoptosis of cumulus cells observed on day 10 of culture.



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also observed in the study group (group C) compared with the control group (group D) (27% vs. 15%;  $P < .001$ ). In both the studies, we observed spontaneous in utero twinning, suggesting that this procedure perhaps has an impact on embryonic growth.

## DISCUSSION

This study has demonstrated an increase in pregnancy and implantation rates and higher-order multiple gestation rates using the cumulus-aided embryo transfer technique. Some speculative explanations can be considered for the increased efficacy of the cumulus-aided embryo transfer technique. The continuation of the secretion of growth factors by the cumulus cells in utero appears to be plausible because we have demonstrated the secretion of three growth factors in an in vitro culture system. Besides their cytokine action, the fact that these expanded cumulus cells may continue to contribute to the increase in adhesiveness between the embryo and the uterus in utero was demonstrated in our in vitro system. When the Petri dish was shaken in a horizontal direction over the microscope stage, it was noted that some of the embryos did not float and were caught up in the dendritic processes of the cumulus cells on which they were being cocultured.

We have demonstrated apoptosis of the cumulus cells around day 10 of oocyte retrieval, as depicted by shrinkage, darkening, deformation, and fragmentation of the cells along with shrinkage and discontinuity of the dendritic processes. The cumulus cells deposited into the uterus would thus continue to be viable beyond the implantation stage of the embryo in utero.

Cumulus cells have been previously used as a feeder layer in a coculture system (3, 4), and several modulators of embryonic cell development have been identified in these

cells. The rationale of using the combined technique of cumulus coculture and cumulus-aided embryo transfer was to generate better-quality embryos and to improve the potential for implantation by utilizing growth factors and cytokines secreted by the cumulus cells.

Cytokines, growth factors, and their receptors have been detected in preimplantation and periimplantation embryos, the fallopian tube, and uterine endometrium (21–24). Moreover, their role in embryo development, endometrial preparation, and the implantation process is now well documented (21, 25, 26).

The role of VEGF in embryonic implantation and development has been studied. The VEGF and its receptors have been identified in several reproductive tissues, including the human endometrium (27), placenta (28), fallopian tube, and ovary (29). Recently, VEGF messenger RNA (mRNA) and protein were detected in the human endometrium throughout the menstrual cycle, with maximal expression in the secretory endometrium during the luteal phase, and the protein was localized in the glandular epithelial cells (30).

The IGF system plays a key role in the monthly development of the endometrium and in the process of implantation (31). The presence of IGFs at the maternal–fetal interface has been documented both in humans and a variety of animal species (32). Further, evidence exists that fetal growth is dependent on IGF in humans (32).

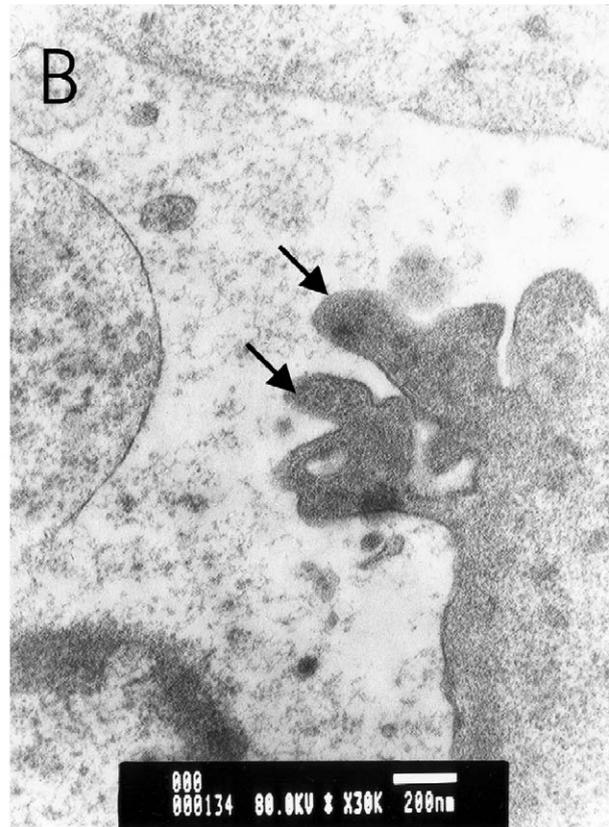
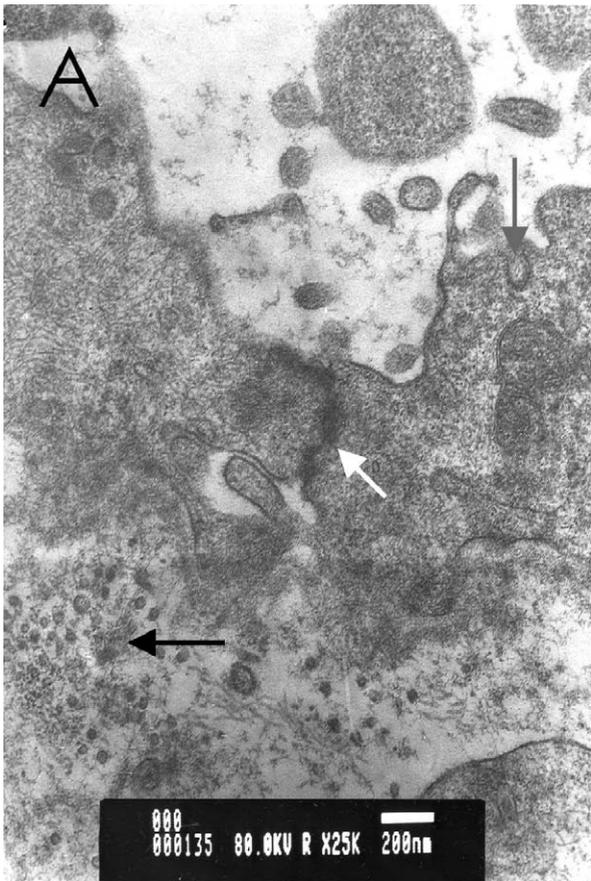
Huang and co-workers reported that the IL system is an important factor in embryo–maternal molecular communication during the implantation process (33). The IL system is composed of IL-1 $\alpha$ , IL-1 $\beta$ , and Interleukin 1 receptor antagonist (IL-Ira), and research suggests that of all the components of the IL system, IL-1 $\beta$  plays a key role in the proper orientation of the embryo to the uterine lining, a process known as apposition. The role of IL-1 $\beta$  is thought to be that of a “signal system” between the endometrium and the embryo (34). In addition to this, research has brought to light that IL-6 plays an important role at the time of implantation (35), and because cumulus cells are known to secrete IL-6, the technique of cumulus-aided embryo transfer might help in the communication system between the embryo and the endometrium.

In our study, cumulus cells were tested for the expression of IL-6, IGF-I, and VEGF, and these were present in the assay until day 10 of culture. Apoptosis occurred on day 10 of coculture. Around this time, the secretion of these factors was minimal.

Mammalian cumulus cells play a role during oocyte growth and maturation. Cumulus cells are known to nurture the oocyte while it is in the ovarian follicle and at the time of ovulation. It is speculated that it is through dynamic contacts via transzonal cumulus cell projections and via intercumulus projections that this close association between the cumulus cells and the oocyte is maintained (36). After oocyte maturation, however, the transzonal cumulus cell

## FIGURE 5

(A) Electron microscope photomicrograph of the expanded cumulus cells displaying enhanced cellular activity. The mitochondrion is indicated by the *gray arrow*. A gap junction is indicated by the *white arrow*. The *black arrow* indicates cytoplasmic vacuoles. (B) Electron microscope photomicrograph of the expanded cumulus cells displaying enhanced cellular activity. The *black arrows* indicate cytoplasmic projections.



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projections get disconnected (37) while the intercumulus projections still remain (36), and thus, the cumulus-cumulus cell communications are preserved.

The electron microscopy studies on the expanded cumulus cells demonstrated increased mitochondrial activity, cytoplasmic vacuoles, and gap junctions in these cells. These are all indicative of enhanced cellular activity. These studies gave us a better understanding of the intercumulus connections, which function in cell–cell adhesion, thereby also transferring essential nutrients to the embryo and appearing to provide an adhesive surface for uterine implantation.

The cytokine role of the cumulus cells was also demonstrated by observing increased colonization with cumulus cells in close proximity of the embryo and a paucity of these cumulus cells away from the embryo. In some situations, we

observed that the colonies embraced the embryo from all sides, and by day 3 to day 5 of coculture, the cumulus cells were actually observed growing above and below the embryo. The embryo was no longer free floating but appeared to be stuck.

The addition of cumulus cells into the uterus appears to be physiological because in many animal species, such as cattle, the cleaving embryo continues to be surrounded by cumulus cells.

Another indication of the efficacy of cumulus-aided embryo transfer vis-à-vis the implantation procedure is the observation of an increase in the higher-order multiple gestation rates (18.1% in the study group compared with 2.4% in the control group;  $P < .01$ ). In fact, we observed in vivo spontaneous embryo twinning occurring in 8 of the 23 embryo transfers categorized with higher-order pregnancy. In

**TABLE 3****Evaluation of VEGF, IL-6, and IGF-1 in cumulus cells by ELISA.**

Treatment	VEGF (pg/mL)	IL-6 (pg/mL)	IGF-I (ng/mL)
Day of oocyte retrieval			
0	10	0	0.08
1	13	0	— <sup>a</sup>
2	24	0	2.2
3	37	0	— <sup>a</sup>
4	90	0	— <sup>a</sup>
5	1,530	78	1
6 <sup>b</sup>	—	—	—
7 <sup>b</sup>	—	—	—
8	3,500	13	1.2
9	3,500	24	0.74
10	450	2.5	1.4
Distilled water	0	0	0
Normal saline	0	0	0
Quinn's Advantage medium with HEPES	0	0	0
Quinn's Advantage Fertilization medium	0	0	0
Quinn's Advantage Cleavage medium	0	0	Trace
Quinn's Advantage blastocyst medium	0	0	Trace

*Note:* VEGF = vascular endothelial growth factor; IL = interleukin; IGF = insulin-like growth factor; ELISA = enzyme-linked immunosorbent assay.

<sup>a</sup> Readings were not taken due to technical difficulty.

<sup>b</sup> Analysis not done on day 6 and day 7 due to nonavailability of samples.

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these eight transfers, the number of sacs observed was higher than the number of embryos transferred. We therefore speculate that the presence of growth factors in cumulus-aided embryo transfer is perhaps enhancing the efficiency of implantation. Spontaneous twinning was not observed in the control group.

The study has certain limitations. We have not been able to always deliver a standard number of cumulus cells along with the embryos into the uterus. Enzyme immunoassay was performed for only three growth factors, although several other growth factors may also contribute to the implantation process. We are also planning to use immunocytochemistry to test other growth factors. Further mRNA studies have been planned to demonstrate the presence of important growth factors.

Within the framework of these limitations, this study has demonstrated a significant increase in implantation rates ( $P < .001$ ) and a significant increase in the PRs ( $P < .01$ ), using the combination of coculture and cumulus-aided embryo transfer with autologous cumulus cells. These cells may not only aid in embryonic development, but also provide a natural mechanism to improve embryo–uterine adhesion. This study also suggests that sequential media could be modified to contain those embryotrophic factors that are present in cumulus cells to enhance PRs. We hope to use

this technique in the future to select a limited number of healthy embryos with a potential to implant, thereby increasing the PRs but limiting the rates of higher-order multiple pregnancy.

The study also advocates the need for randomized studies, preferably at multiple centers, to further examine some parts of the hypothesis that it has evoked.

Further studies are being performed to elucidate which of the cytokines and growth factors, or combinations thereof, lead to the increased adhesiveness of the embryos, thereby leading to an increase in the PR.

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