Evaluation of in vitro fertilization outcomes using interleukin-8 in culture medium of human preimplantation embryos

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Objective: To investigate whether selected cytokines are detectable in the embryo culture medium (EM) of human preimplantation embryos (HPE) and what the relationship is of the cytokines with clinical outcomes.
Design: Cross-sectional study.
Setting: University-affiliated tertiary teaching hospital.
Patient(s): Three-hundred and thirty infertile women who underwent fresh cycle in vitro fertilization (IVF) between January and December 2014.
Intervention(s): Collection on the day of transfer of the EM of each embryo that was transferred in all patients for measurement of cytokine levels.
Main Outcome Measure(s): Measurement of 13 selected cytokines in the EM of day-3 HPE to analyze the relationship of the cytokine with embryo quality and clinical outcome.
Result(s): Of the cytokines measured, only interleukin-8 (IL-8) was statistically significantly associated with clinical outcome. The rate of detectable IL-8 in the EM was 32.42%, and the pregnancy rate, implantation rate, and number of live births per in vitro fertilization (IVF) or intracytoplasmic sperm injection patient (N LBPP) were higher, and 0 IR was lower in patients for whom the medium from transferred embryos was positive for IL-8 (IL-8 positive group) compared with the patients for whom the medium tested negative for IL-8 (IL-8 negative group). Compared with the IL-8 negative group, a higher pregnancy rate was observed in the IL-8 positive group when the patients received equal good–ordinary quality embryos.
Conclusion(s): In the EM from HPE, IL-8 is associated with higher pregnancy rates, higher IRs, and higher N LBPP, so IL-8 may be an independent predictor for pretransfer assessment of the embryo development potential in IVF patients. (Fertil Steril® 2017;107:649–56. ©2016 by American Society for Reproductive Medicine.)
Key Words: Culture medium, embryo quality, interleukin-8, IVF outcome

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As the number of assisted reproduction cycles increases worldwide, improvement in the ability to identify quickly and noninvasively the best embryos for transfer remains a critical goal for reproductive medicine. To improve the implantation rate (IR) for individual embryos and achieve high pregnancy rates, multiple embryos are transferred into the majority of patients undergoing in vitro fertilization with or without intracytoplasmic sperm injection (IVF-ICSI).

Consequently, IVF-ICSI has a high risk of multiple pregnancies. The high rate of multiple pregnancies or births resulting from IVF-ICSI is a major health issue and imposes a heavy burden on government health services (1). With increased awareness of the medical and socioeconomic problems related to multiple pregnancies, their prevention has become one of the central aims of successful assisted reproduction technology (ART) programs (2).

Elective single-embryo transfer after IVF-ICSI has been suggested as the only effective means to avoid multiple pregnancies (3). To enable the use of single-embryo transfer without diminishing pregnancy rates, great efforts have been made to improve the IR and to establish criteria for the identification of the embryo with the highest potential for implantation after transfer. Currently, embryo quality is usually assessed using morphologic criteria (4) that are based largely on cell number and morphologic appearance, which are relatively poor predictors of successful implantation. The limitations of morphologic embryo evaluation have led many researchers to seek additional technologies to assess the reproductive potential of a given embryo (5, 6). The technologies under investigation include the evaluation of glucose, lactate, pyruvate, or amino acid levels in the embryo culture medium (EM), measurement of oxygen consumption by the embryo, and genomic and proteomic profiling.

Noninvasive methods are currently thought to be the most promising candidates for the prediction of reproductive success after ART (2,5–10). Several metabolic parameters of developing embryos have been assessed by these techniques, and a relationship has been demonstrated between metabolites in the culture medium and the outcome of clinical pregnancy. Noninvasive observation of embryo development by capturing images with a time-lapse device has also been used for assessment of embryo viability, and the application of such technologies has already shown positive effects on clinical outcomes (11). However, because of the lack of larger, randomized clinical studies, it remains to be elucidated whether embryo selection using dynamic parameters improves clinical outcomes and which parameters are statistically significant (12, 13).

Cytokines are a large group of proteins that are secreted by cells and act as humoral regulators that modulate the functional activities of individual cells and tissues at nanomolar to picomolar concentrations under normal or pathologic conditions. Their fundamental function is to change the properties of a wide range of cells and tissues. Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). Cytokines bind to their specific receptor on cell membranes and thereby induce a cascade of signal pathways that results in the actual effect of the cytokine on a cell or a tissue. In reproductive immunology, cytokines have been implicated in menstruation, ovulation, parturition, and embryo implantation as well as in pathologic processes such as preterm delivery and endometriosis (14, 15).

The possibility of assessing embryo quality and predicting IVF outcomes by measuring the level of cytokines in the EM of human preimplantation embryos (HPE) has been explored for many years. In the 1990s, researchers measured interleukin-1 (IL-1), IL-6, colony-stimulating factor-1 (CSF-1), tumor necrosis factor α (TNF-α), and transforming growth factor-β levels in the EM of human embryos, and raised the possibility that the concentrations of these cytokines in EM might be predictive for pregnancy in patients undergoing IVF-ICSI (16–19). Zollner et al. (20) demonstrated that TNF-α concentrations in HPE EM were statistically significantly lower in women who conceived than in those who did not conceive, and leukemia inhibitory factor (LIF) concentrations were statistically significantly higher in women who conceived. The results from this study suggested that LIF could be required for embryo implantation and that high TNF-α concentrations were predictive of implantation failure.

Luminex's xMAP (Multi-Analyte Profiling) technology is based on existing technologies—flow cytometry, microspheres, lasers, digital signal processing, and traditional chemistry—that have been combined in a unique way. The technique allows the simultaneous analysis of up to 500 bioassays from a small sample volume, typically a single drop of fluid, and can be configured to perform a wide variety of protein or nucleic acid assays quickly, cost effectively, and accurately, Using Luminex's xMAP technology, we were unable to detect elevated levels of granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1β, IL-6, or TNF-α (data not shown), but we found elevated levels of IL-8 in the EM of human embryos. In this study, we analyzed the level of IL-8 in the EM of HPE, the relationship of the IL-8 concentration with embryo quality, and the subsequent outcomes of clinical pregnancies.

MATERIALS AND METHODS

Patients

Infertile couples undergoing IVF-ICSI treatment at our center between January and December 2014 were included in this study. The indications for ART were tubal factor infertility, endometriosis, male factor infertility, or idiopathic sterility. In cases of male subfertility, immunologic factors, or unexplained fertilization failure in a previous IVF cycle, we performed ICSI. No patient selection or exclusion criteria were used. Our study was approved by the ethics committee of the Affiliated Hospital of Guizhou Medical University, and all patients gave informed consent.

Ovarian Stimulation Protocols, Oocyte Retrieval, and Sperm Preparation

Natural, mild, or conventional ovarian stimulation for IVF-ICSI was performed depending on whether the patient was a poor responder. In conventional IVF-ICSI cycles, hyperstimulation was achieved by short, long, or ultralong protocols using a gonadotropin-releasing hormone agonist (Diphereline; IPSEN), or short protocols using 0.25 mg of gonadotropin-releasing hormone antagonist (Cetrotide; Merck Serono). Ovarian stimulation was started with doses of follicle-stimulating hormone (recombinant FSH, Gonal-f; Merck Serono) or human menopausal gonadotropin (Livzon

Pharmaceutical Group) varying from 150–300 IU/day according to the patient's age and ovarian reserve. The dose of recombinant FSH and human menopausal gonadotropin was adjusted and individualized for each patient based on the follicular growth.

In the natural or mild IVF-ICSI cycles, patients were given 50–150 mg of clomiphene citrate (GKH Pharmaceutical), or 5 mg of letrozole (letrozole tablets; Jiangsu Hengrui Medicine) daily for 5 days, or no medication, followed by administration of 75–150 IU of either recombinant FSH or human menopausal gonadotropin daily. Ovarian stimulation was monitored by transvaginal ultrasound and measurement of estradiol plasma levels. Ovulation was induced with 250 μg of recombinant human chorionic gonadotropin (hCG, Ovidrel; Merck Serono) when there were three or more follicles and the largest follicle had reached 18 mm in diameter. Oocytes were retrieved 36 hours after hCG administration by transvaginal aspiration of follicles under ultrasound guidance.

All semen samples collected for IVF-ICSI were analyzed according to the World Health Organization (WHO) criteria, and were prepared using a double-density gradient technique (80% and 40% gradient; SAGE In-Vitro Fertilization) and swim-up procedure. After several centrifugation and washing steps, the semen pellet was incubated in Quinn's Advantage fertilization medium (ART-1020; SAGE In-Vitro Fertilization) supplemented with 5% human serum albumin (SAGE In-Vitro Fertilization) for 20 to 60 minutes at 37°C for the swim-up of motile sperm.

Fertilization, Pronuclear Scoring, and Embryo Culture

The insemination of the oocytes was performed using standard procedures for IVF or ICSI as previously described elsewhere (21–23) and was performed 39 to 42 hours after the hCG injection. Fertilization was confirmed 16 to 20 hours after oocyte retrieval by the presence of two distinct pronuclei and polar bodies under the inverted microscope. The zygotes were then placed individually into 30-μL droplets of fresh Quinn's Advantage Cleavage medium (ART-1026, SAGE In-Vitro Fertilization) supplemented with 10% human serum albumin (SAGE In-Vitro Fertilization), covered with mineral oil (SAGE In-Vitro Fertilization, Inc.) and maintained in an incubator at 37°C under 5% CO_2 and air until embryo transfer.

Embryo Assessment and Embryo Transfer

Embryos were assessed between days 2 and 3 of culture according to the standard morphologic assessment methods based on cleavage rate and morphology (24), and the cleavage stage was noted on days 2 and 3. Every embryo was graded on day 3 on the basis of cell number, regularity of blastomeres, and degree of fragmentation. Embryos with a cell count ≥ 6 and class I or II were considered morphologically good quality embryos, while embryos with cell count <6 but ≥ 4 and class I or II were considered ordinary-quality embryos, and those with a cell count ≥ 6 but grade III or a cell count ≤ 3 were considered morphologically inferior embryos. Only good quality embryos and ordinary quality embryos were selected for transfer. Embryo selection for day-3 transfer was based solely on morphologic parameters, and only the embryos with the highest number of blastomeres and the least fragmentation were transferred.

The number of embryos transferred was based primarily on the age and number of previous IVF-ICSI cycles of the patient. Generally, patients under 35 years of age received two embryos, and those aged 35 years or older received three embryos. However, all patients who had undergone two or more IVF-ICSI cycles received three embryos regardless of their age. The embryos not selected for transfer were cryopreserved on day 3 or placed into fresh culture medium followed by further cultivation to the blastocyst stage.

Sample Collection and Assay

On the day of transfer, the EM of each embryo that was transferred was collected for all patients. Samples of medium incubated under the same conditions without an embryo were also collected and served as negative controls. The collected specimens and negative controls were immediately frozen and stored at −20°C until analyzed.

Luminex's xMAP assay was performed to simultaneously determine the concentrations of 13 selected cytokines (GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, interferon-γ, and TNF-α) in the EM of embryos being transferred to each patient. The samples were analyzed in a 96-well plate using High Sensitivity Human Cytokine Kits (EMA Millipore Corporation) according to the manufacturer's instructions. The plates were read on a MEGPIX System (EMA Millipore Corporation).The median fluorescence intensity from each well was recorded, evaluated, and converted into the respective concentration (pg/mL) using Milliplex Analyst software (EMA Millipore Corporation). All samples were tested by Shanghai Tellgen Life Co. Ltd. of the People's Republic of China.

Post-IVF-ICSI Treatment and Follow-Up Evaluation

Luteal phase supplementation (dydrogesterone, 40 mg/d, orally) was given on the evening of the day of oocyte pickup until 14 days later. Follow-up procedures were set up in the reproductive center, and the pregnancy rate, chemical pregnancy rate, abortion rate, clinical pregnancy rate, IR, and live-birth rate were monitored between the time of embryo transfer and the end of the pregnancy. The pregnancy rate was defined as the proportion of patients who received an embryo who had a positive serum β-human chorionic gonadotropin (β-hCG) concentration 14 days after embryo transfer. Chemical pregnancy was defined as a positive β-hCG pregnancy test, followed by a subsequent failure of the pregnancy to progress to an ultrasound-confirmed pregnancy, and the chemical pregnancy rate was defined as the number of chemical pregnancies divided by the total number of pregnancies. The abortion rate was defined as the proportion of pregnancies with total loss of gestational sacs before 28 weeks of gestation. The IR was defined as the number of gestational sacs divided by the total number of embryos transferred,

0 IR was defined as the total number of embryos transferred from the patients in which no embryos implanted divided by the total number of embryos transferred from the patients who received embryos, 100% IR was defined as the total number of embryos transferred from patients in which all transferred embryos implanted divided by the total number of embryos transferred from the patients who received embryos, and the clinical pregnancy rate was defined as the number of patients with at least one gestational sac divided by the total number of patients who received embryos. The loss at follow-up evaluation ratio was defined as the number of pregnant women who had lost the pregnancy at follow-up evaluation divided by the total number of pregnant women. The live-birth rate was defined as the percentage of women with a living baby relative to the number of women who achieved a pregnancy, and the number of live births per patient (N LBPP) was defined as the total number of live births divided by the total number of patients who received embryos.

Statistical Analysis

Data for cytokine concentrations are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. All analyses were performed using SPSS software version 19.0 (SPSS Inc.). The mean differences between groups were compared by one-way analysis of variance. The chi-square test was used to analyze the associations between categorical variables. All *P*-values were two-tailed, and $P<.05$ was considered statistically significant.

RESULTS

IL-8 in the EM from HPE

To investigate whether the 13 selected cytokines (GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, interferon-γ, and TNF-α) were present in the EM from human reimplantation embryos, nine negative control samples and 330 patient samples (study group) were tested by Luminex's xMAP assay using the High Sensitivity Human Cytokine Kits. The results showed that of the 13 cytokines measured, only IL-8 was statistically significantly associated with the clinical outcome. The minimum detectable concentration +2 standard deviations (SD) for IL-8 was 0.16 pg/mL, and samples with IL-8 concentrations above 0.19 pg/mL were defined as positive.

When the study group was divided into IL-8 positive and IL-8 negative groups, the analysis showed that the IL-8 concentration in the IL-8 positive group was higher than that of the negative control group, the overall study group, and the IL-8 negative group ($P=.003$, .000, and .000, respectively), and that the concentrations of IL-8 in the whole study group were higher than those in the IL-8 negative group ($P=.000$). However, no statistically significant difference in the concentrations of IL-8 was observed between the negative control group and the IL-8 negative group ($P=.94$) (Table 1).

Evaluation of Association of IVF-ICSI Outcome with Presence of IL-8 in EM from HPE

Characteristics of patients for whom the EM was tested for IL-8. Overall, 330 samples of EM from embryos transferred to patients were tested for IL-8. Because each sample represented all the embryos transferred to one patient in one cycle, there were 330 patients involved in the study. Of the 330 patients whose samples were tested by Luminex's xMAP assay, seven missed the first follow-up evaluation when they underwent IVF-ICSI and were confirmed to be pregnant by the presence of gestational sacs on ultrasonic examination at 6 to 8 weeks. Of the 330 women whose samples were tested by Luminex's xMAP assay, 107 had samples positive for IL-8 (IL-8 positive group), and 223 had samples that tested negative for IL-8 (IL-8 negative group). The age range for these patients was from 21 to 46 years. There were no statistically significant differences between the two groups in age ($P=.38$), years of infertility ($P=.84$), endometrial thickness on the day of hCG administration ($P=.87$), number of embryo transfer cycles ($P=.98$), ratio of loss at follow-up evaluation or presence of gestational sacs ($P=.56$), or the cell number (mean ± SEM) of embryos being transferred ($P=.27$).

In the IL-8 positive and negative groups, the primary infertility incidence was 38.32% (41 of 107) and 45.74% (102 of 223), and the secondary infertility incidence was 61.68% (66 of 107) and 54.26% (121 of 223), respectively; obviously no statistically significant difference in primary infertility incidence and secondary infertility incidence was observed between the two groups ($P=.20$). In the IL-8 positive and negative groups, the incidence of tubal factor infertility was 84.11% (90 of 107) and 83.41% (186 of 223), respectively; no statistically significant difference of the incidence was observed between the two groups ($P=.87$). The number of retrieved oocytes in the IL-8 positive group was greater than that in the IL-8 negative group (10.63 ± 0.45 vs. 9.61 ± 0.30), but this difference was not statistically significant ($P=.055$) (Table 2).

Relationship of IVF-ICSI outcome with the presence of IL-8 in EM from HPE. In the IL-8 positive group, the pregnancy rate, IR, 0 IR, and 100% IR were 54.21% (58 of 107), 27.62% (66 of 239), 52.30% (125 of 239), and 13.39% (32 of 239), respectively. In the IL-8 negative group, the pregnancy rate, IR, 0 IR, and 100% IR were 40.81% (91 of 223), 19.92% (97 of 487), 63.86% (311 of 487), and 9.24% (45 of 487), respectively; therefore, obvious differences in the pregnancy rate, IR, and 0 IR were observed ($P=.02$, .02, and .003,

TABLE 1

Concentrations of interleukin-8 (IL-8) in the culture medium from human preimplantation embryos tested with the Luminex xMAP assay.

		IL-8
Group	**n**	**Concentration (pg/mL)**
Negative control group	9	0.019 ± 0.006
Study group	330	0.60 ± 0.10[a]
IL-8 negative group	223	0.06 ± 0.003
IL-8 positive group	107	1.74 ± 0.27[b]

[a] $P=.000$ compared with IL-8 negative group.
[b] $P=.003$, .000, and .000, compared with negative control group, study group, and IL-8 negative group, respectively.

Huang. Assessment of embryo quality with IL-8. Fertil Steril 2016.

TABLE 2

Characteristics of patients for whom medium from transferred embryos tested positive or negative for interleukin-8 with the Luminex xMAP assay (n = 330).

Characteristic	IL-8 Positive	IL-8 Negative	*P* value
Age (y) (mean ± SEM)	32.69 ± 0.46	32.20 ± 0.31	.38
Years of infertility (mean ± SEM)	5.50 ± 0.39	5.40 ± 0.28	.84
Etiology			
Primary infertility (%)	38.32 (41/107)	45.74 (102/223)	.20
Secondary infertility (%)	61.68 (66/107)	54.26 (121/223)	
Tubal factor (%)	84.11 (90/107)	83.41 (186/223)	.87
Embryo transfer cycles (mean ± SEM)	1.18 ± 0.05	1.18 ± 0.04	.98
Endometrial thickness (mm) on the day of hCG administration (mean ± SEM)	11.39 ± 0.24	11.44 ± 0.18	.87
No. of retrieved oocytes (mean ± SEM)	10.63 ± 0.45	9.61 ± 0.30	.055
No. of embryos transferred (mean ± SEM)	2.23 ± 0.04	2.18 ± 0.03	.36
Ratio of loss at follow-up of gestational sacs in pregnancy	3.45% (2/58)	5.49% (5/91)	.56
Cell no. of embryos being transferred (mean ± SEM)	6.86 ± 0.09%	6.73 ± 0.07%	.27

Note: hCG = human chorionic gonadotropin; IL-8 = interleukin 8; SEM = standard error of the mean.

Huang. Assessment of embryo quality with IL-8. Fertil Steril 2016.

respectively) in comparing the IL-8 positive and negative groups. The clinical pregnancy rate was higher and abortion rate was lower in the IL-8 positive group than those in the IL-8 negative group, but the differences were not statistically significant (P=.12 and .09, respectively); the chemical pregnancy and live-birth rates were also not statistically significantly different between the two groups, but the N LBPP in the IL-8 positive group was higher than in the IL-8 negative group (P=.05) (Table 3). The results suggest that the presence of IL-8 in the EM from HPE is associated with higher pregnancy rates and higher implantation potential of the transferred embryo, and may be related to a lower abortion rate after IVF-ICSI. Although the live-birth rate was unchanged, the N LBPP increased because the pregnancy rate and IR increased in patients for whom the medium from the transferred embryos tested positive for IL-8.

Relationship of IL-8 in EM from HPE with embryo quality. The patients for whom the medium from transferred embryos was tested for IL-8 were classified into two groups according to the qualities of the embryos they received: good–ordinary quality embryos (GOQE) and ordinary quality embryos (OQE). In the GOQE group, each patient received only good quality embryos, or each patient received both good and ordinary quality embryos. In the OQE group, each patient received only ordinary quality embryos. There was no statistically significant difference in the quality of embryos being transferred between the IL-8 positive and IL-8 negative groups (P=.76; Table 4). No statistically significant difference in pregnancy rate was observed between the IL-8 positive and negative groups when the patients received OQE (P=.82; see Table 4). However, a statistically significant difference in pregnancy rate was observed between the IL-8 positive and negative groups when the patients received GOQE (P=.02; see Table 4). These results suggest that, compared with the IL-8 negative group, the presence of IL-8 in the EM from HPE was related to a higher pregnancy rate when patients received equal quality embryos, and that the pregnancy rate was 58.16% when patients were transferred with GOQE for which the culture medium tested positive for IL-8 (see Table 4).

TABLE 3

Comparison of IVF-ICSI outcome in patients for whom medium from transferred embryos tested positive or negative for interleukin 8 (IL-8).

Rate	IL-8 positive	IL-8 negative	*P* value
Pregnancy	54.21% (58/107)	40.81% (91/223)	.02
Clinical pregnancy	44.86% (48/107)	35.87% (80/223)	.12
Implantation	27.62% (66/239)	19.92% (97/487)	.02
No (0) implantation	52.30% (125/239)	63.86% (311/487)	.003
100% implantation	13.39% (32/239)	9.24% (45/487)	.09
Chemical pregnancy	10.34% (6/58)	9.89% (9/91)	.93
Abortion	6.90% (4/58)	16.48% (15/91)	.09
Live birth	68.97% (40/58)	65.93% (60/91)	.70
No. of LBPP	37.38% (40/107)	26.90% (60/223)	.05

Note: No. of LBPP denotes the number of live births per in vitro fertilization/intracytoplasmic sperm injection (IVF-ICSI) patient.

Huang. Assessment of embryo quality with IL-8. Fertil Steril 2016.

DISCUSSION

Implantation of the embryo is essential for successful pregnancy, and approximately half of all human embryo implantations result in a failed pregnancy. Failure of the embryo to implant is clinically relevant to recurrent pregnancy loss and the low success rates of IVF-ICSI, and implantation remains the rate-limiting step for the success of ART. At the time of implantation, proinflammatory chemokines dominate in the implantation microenvironment (25, 26).

Chemokines are a family of small cytokines or signaling proteins secreted by cells. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells. Chemokines are best known for their functions in leukocyte trafficking. In reproductive medicine, chemokines play important roles in ovulation, menstruation, embryo

TABLE 4

Comparison of the quality of embryos with pregnancy rate in the interleukin-8 positive and negative groups.

	IL-8 group			Pregnancy rate		
Embryo quality	Positive	Negative	*P* value	IL-8 positive	IL-8 negative	*P* value
GOQE	91.59% (98/107)	90.58% (202/223)	.76	58.16% (57/98)	43.56% (88/202)	.02
OQE	8.41% (9/107)	9.42% (21/223)		11.11% (1/9)	14.23% (3/21)	.82

Note: IL-8 = interleukin-8; GOQE = good–ordinary quality embryos; OQE = ordinary quality embryos.

Huang. Assessment of embryo quality with IL-8. Fertil Steril 2016.

implantation, and parturition (27–29). Chemokines at the maternal–fetal interface are well known to be essential for embryonic implantation.

It has been reported that nine chemokines are highly abundant in human endometrium: monocyte chemotactic protein-3, eotaxin, fractalkine, macrophage inflammatory protein-1β, 6Ckine, IL-8, hemofiltrate CC chemokines-1 and -4, and macrophage-derived chemokine, and that chemokine mRNA is generally up-regulated during endometrial receptivity and early pregnancy (28). Researchers have shown that these chemokines at the maternal–fetal interface possess three main functions in the early stages of pregnancy. First, they control the orientation of the embryo (30). Second, they regulate leukocyte trafficking (28, 30, 31) at the implantation site, where abundant expression of chemokines is accompanied by extensive leukocyte trafficking, and the large number of leukocytes is believed to maintain the delicate balance between protecting the developing embryo/fetus and tolerating its hemiallogeneic tissues (30, 31). Third, chemokines participate in the processes of placental development and promotion of human trophoblast migration (31, 32).

High levels of the proinflammatory T helper 1 (T_H1) cytokines (IL-6, IL-8, TNF-α) are present during early implantation; they can be secreted by the endometrial cells and by cells of the immune system that are recruited to the site of implantation. In fact, implantation, placentation, and the first and early second trimester of pregnancy resemble an open wound, which requires a strong inflammatory response. During this first stage, the blastocyst has to break through the epithelial lining of the uterus and damage the endometrial tissue to invade and implant. Then, the trophoblast replaces the endothelium and vascular smooth muscle of the maternal blood vessels to provide an adequate placental–fetal blood supply. To secure the adequate repair of the uterine epithelium and the removal of cellular debris, an inflammatory environment is required. Thus, the first trimester of pregnancy is a proinflammatory phase (25, 26). It has been suggested that the success of implantation may be secondary to the development of an injury-induced inflammatory reaction, that the levels of cytokines and chemokines at the implantation site correlate positively with the pregnancy outcome of IVF-ICSI patients, and that these cytokines are critical for the success of implantation (26).

Interleukin-8, a member of the chemokine family, is produced by monocytes and other cells such as fibroblasts, epithelial cells, and endothelial cells. There are two primary functions of IL-8: the induction of chemotaxis in its target cells, including neutrophils and granulocytes, and participation in the innate immune response that is often associated with inflammation; therefore, IL-8 is defined as a proinflammatory mediator (33). Throughout the menstrual cycle, IL-8 is localized at the luminal and glandular epithelium and endothelial cells, with its expression being significantly higher during the prereceptive and receptive periods (30, 34, 35). During the first and early second trimester of pregnancy, IL-8 at the maternal–fetal interface and implantation site may have an important role in maintaining pregnancy because of the dual functions of this interleukin as chemokine and proinflammatory cytokine.

One study (34) that cocultured single human embryos to the blastocyst stage with human endometrial epithelial cells (EECs) demonstrated that the human blastocyst does not produce measurable amounts of IL-8; it showed, however, that EECs up-regulate IL-8 mRNA expression and protein production. In contrast, in our study, we found when using Luminex's xMAP assay or cytometric bead array (data not published) that IL-8 is present in the EM from human embryos. The reasons for these differences may be that in the study by Caballero-Campo et al. (34) the IL-8 enzyme-linked immunosorbent assays used to detect cytokines in culture medium from human embryos had low sensitivity (10 pg/mL) and the number of samples was small. In our study, it was possible to detect concentrations of IL-8 at $\geq$0.16 pg/mL, and we included 330 samples. Our results show that the presence or absence of IL-8 in EM from HPE is associated with the clinical outcome of IVF-ICSI. The presence of IL-8 in the EM from HPE is associated with a higher pregnancy rate, higher IR, and higher N LBPP, and may be related to a higher clinical pregnancy rate and lower abortion rate.

Because the presence of IL-8 in EM from HPE is related to a higher pregnancy rate, higher IR, and higher N LBPP when patients receive morphologically equal quality embryos, it is reasonable to believe that the presence of IL-8 in EM from HPE may predict the development potential of embryos and lead to a greater possibility of better quality embryos being transferred, and thus may predict a good clinical outcome of IVF-ICSI. Therefore, the presence of IL-8 in EM from HPE may be an independent predictive indicator of embryo development potential. Part of the reason why the presence of IL-8 is related to good clinical outcomes may be that IL-8 from HPE could function at the implantation site as a chemokine and proinflammatory cytokine.

In our study, each patient received two or three embryos transferred in one cycle; therefore, each sample consisted of

EM pooled from all of the two or three embryos transferred in the cycle, and the concentration of IL-8 in each sample was the mean concentration in EM from all these embryos. To evaluate accurately the range of IL-8 concentrations that predict the chance of pregnancy, we should determine the IL-8 concentration in the EM from each embryo. However, we were not able to do this because less than 25 μL of EM was collected from each embryo, which is insufficient for testing. As the culture media from two to three embryos were pooled, and in many cases only one of those embryos may have implanted, the results may become less dramatic; a more useful analysis might compare the IR, 0 IR, and 100% IR. The results suggested that 0 IR was more useful than IR for the implantation ability of transferred embryos that were tested for IL-8 in patients who received two or three embryos transferred in one cycle ($P=.003$ vs. $P=.02$). However, the sample size was not large enough to detect a statistically significant difference in 100% IR between the patients for whom the medium from the transferred embryos tested positive or negative for IL-8 ($P=.09$).

Studies of IL-8 and pregnancy show that the relationship of the level of IL-8 in blood and amniotic fluid with pregnancy is complicated. An elevation of IL-8 may be related to infection in pregnancy (36), and high levels of IL-8 have been observed in pregnant women with serologic evidence of *Toxoplasma gondii* infection (37). In patients with second-trimester abortions, the maternal plasma concentrations of IL-8 were elevated, so this chemokine might be a crucial factor in the defensive reaction of the mother during the second trimester of pregnancy (36). However, another study showed that the midtrimester amniotic fluid level of IL-8 was not a predictor of adverse pregnancy outcomes in terms of spontaneous abortion (38).

Our results demonstrate that some early embryos may produce IL-8. We suggest that, besides that derived from cells or tissues of the uterus, the abundant IL-8 at the implantation site could originate from the transferred embryo; this IL-8 produced at the implantation site by early embryos has at least two functions. First, by analogy with leukocyte trafficking caused by chemokines produced by the uterus at the implantation site, IL-8 from early embryos may cause further leukocyte accumulation around the embryo. Second, IL-8 from early embryos could have a compensatory effect in the absence or insufficiency of IL-8 produced by the uterus at the implantation site.

Overall, our results suggest that IL-8 can be measured in the EM from HPE, and that IL-8 from embryos may function at the implantation site as a chemokine and proinflammatory cytokine. The presence of IL-8 in culture medium from HPE is associated with a high pregnancy rate, high IR, and high N LBPP. The presence of IL-8 in the EM from HPE is also associated with better clinical outcomes when patients receive morphologically equal quality embryos. Thus, IL-8 may be a pretransfer independent predictor for assessment of the embryo development potential in IVF patients.

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