Levels of follicular G-CSF and interleukin-15 appear as noninvasive biomarkers of subsequent successful birth in modified natural in vitro fertilization/ intracytoplasmic sperm injection cycles

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Objective: To explore oocyte competence for subsequent birth. The modified natural IVF/intracytoplasmic sperm injection (ICSI) cycle was used as an experimental model by measuring levels of cytokines, chemokines, and growth factors in individual follicular fluids (FF).
Design: A retrospective blinded study.
Setting: European network of research, Embryo Implantation Control (EMBIC).
Patient(s): Single FF from 83 women were analyzed during a modified natural IVF/ICSI cycle, and reproducibility of follicular composition was evaluated over two cycles for 15 patients.
Intervention(s): Each FF sample was blindly tested to assess levels of 26 factors by bead-based immunoassays.
Main Outcome Measure(s): Each mediator was evaluated as a potential biomarker of subsequent birth by multivariate regression analysis.
Result(s): A combination of both FF G-CSF and IL-15 was the optimal model to predict birth (AUC_{ROC}, 0.85). Birth rates per cycle were 48.9% (16/33) if two good-prognosis criteria were present (FF G-CSF >12 pg/mL and IL-15 <7 pg/mL) and 8% (3/36) and 0% (0/14) if, respectively, one or none were present. FF G-CSF was significantly correlated over two cycles ($r = .71$), suggesting a possible prognostic value of its documentation.
Conclusion(s): Combined follicular G-CSF and IL-15 quantification appears as an efficient and noninvasive method to define oocyte competence for subsequent successful conception in modified natural IVF/ICSI cycles. (Fertil Steril® 2011;95:94–8. ©2011 by American Society for Reproductive Medicine.)

Key Words: G-CSF, interleukin-15, follicular fluid, oocyte, pregnancy, monitored natural cycle, IVF/ICSI, biomarker

The present study used the modified natural IVF/intracytoplasmic sperm injection (ICSI) cycle as an experimental model in which traceability from the follicle to the baby who is born is straightforward because only one follicle/oocyte/embryo is collected, fertilized, and, if possible, transferred. Premature ovulation is controlled by a GnRH antagonist administration with hMG support in the late proliferative phase to ensure successful collection of the dominant follicular fluid (FF) (1). The oocyte environment is directly explored in regard to subsequent pregnancy and birth.

The disadvantage of natural or monitored natural IVF cycle (MNC-IVF), impairing its large diffusion in routine, is related to the low percentage of cleaving embryos suitable for transfer and hence the low efficiency of such a policy in initiated cycles. Consequently, conventional ovarian hyperstimulation currently remains the rule for collecting oocytes for IVF/ICSI. The renewed interest in natural IVF cycles (and more broadly modified strategies including mild stimulation) is related to various observations that suggest that [1] oocyte quality and uterine receptivity would potentially be better when compared with the conventional scheme (2), [2] multiples pregnancies and ovarian hyperstimulation syndrome would be avoided, and [3] perinatal outcome for singletons would be optimized (3). Natural IVF strategies were recently described as an effective treatment in an extended cohort of young poor responders in which conventional related IVF results are disappointing (4).

To evaluate each factor as a putative potential biomarker of oocyte competence for subsequent birth, we retrospectively measured in a blinded manner the expression of cytokines, chemokines, and growth factors in the FF of dominant individual follicles using multiplexed bead-based technology.

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MATERIALS AND METHODS

Patients

In Clamart Hospital, from January 2005 to December 2006, 83 patients were enrolled in modified natural IVF/ICSI protocols. They had all previously failed to become pregnant in conventional IVF/ICSI cycles before they had reached 40 years of age (Table 1). The 83 modified natural IVF/ICSI cycles that were considered only included first attempts. Subsequently, reproducibility of follicular patterns over a second IVF/ICSI natural modified cycle was documented for 15 patients. The study was reviewed and approved by the Institutional Review Board. All patients undergoing IVF and participating in the study gave their informed consent.

Modified Natural IVF/ICSI Protocol

From day 8 onward, selection of the dominant follicle was monitored by ultrasound. To prevent the risk of a premature LH peak, when the mean diameter of the dominant follicle exceeded 13 mm, 0.5 mg of a GnRH antagonist (Cetrotide 0.25 mg; Serono Pharmaceuticals, Boulogne, France) was administered SC each day until the hCG injection. At that time, 150 IU hMG (Menopur; Ferring Pharmaceuticals, Gentilly, France) was administered simultaneously to prevent a subsequent fall in the E_2 level (5). The criteria used for triggering ovulation with 5,000 IU of IM hCG (Gonadotrophine Chorionique Endo; Organon Pharmaceuticals, Saint-Denis, France) was a follicle size of $\geq$16 mm in mean diameter. The single oocyte was retrieved 34 hours afterward, and the embryo was transferred 2 days later. On day 2, the embryos were analyzed in relation to [1] their fragmentation (grade 1, 10% or less; grade 2, 10%–30%; grade 3, 30%–50%; grade 4, over 50%; an embryo was downgraded when blastomeres were unequal in size); and [2] their embryo score, defined as the number of blastomeres $\times$ (5 $-$ embryo grade) (6).

FF Collection and Samples

Under transvaginal ultrasound guidance, the FF from the dominant follicle was gently and thoroughly aspirated by a 10-mL syringe. If no oocyte was recovered, the follicle was flushed up to 3 times to improve oocyte recovery rate (7). FFs were centrifuged and frozen at -80°C for subsequent analysis. For multiplex analysis, each sample was masked to be blindly analyzed.

Multiplex Sandwich Assays

Multiplex bead-based immunoassays were performed to measure multiple analytes simultaneously in individual FF samples by flow cytometric resolution of spectrally distinct microspheres coupled with monoclonal antibodies specific for cytokines and reporter fluorochromes bound to detection antibodies as described elsewhere (8). The bead-based multiplex sandwich immunoassay (purchased from Bio-Rad Laboratories, Hercules, CA) was read with a Luminex system (Luminex Map Technology, Milan, Italy) and used to measure the concentrations of the following cytokines and chemokines: IL-1α, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL–9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, TNF-α, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, CCL5, eotaxin, MIP-1α, and MIP-1-β. Means and limits of detection are detailed in Table 1.

Statistical Analysis

To identify predictive factors of subsequent birth, multivariable logistic regression analysis was performed, and receiver operating characteristic (ROC) analysis was used to determine respective performances and sensibility/specificity. Analytes with >50% of values below the limit of sensitivity (IL-5, IL-1beta, IL-10, IL-13, TNF-α, FGF, and PDGF) were excluded, owing to a certain level of uncertainty. Each analyte was first evaluated on the entire cohort (n = 83) with known covariates factors able to influence the outcome "birth/per initiated cycle" as age, antral follicle count (AFC) on day 3 of the cycle, FSH levels on day 3, range of the attempt, endometrial thickness, and E_2 level the day of hCG injection. We subsequently performed a second run including in the analysis the embryo score for the 54 patients from whom an embryo was successfully obtained (Table 2). A value of $P>.1$ was used as a criterion for exclusion according to the literature on multivariate prognostic modeling (9). We then combined significant analytes to evaluate the performance of their combination with no correction for multiple testing as required for a hypothesis-based study. The following thresholds were used to interpret the area under the ROC curve (AUC_{ROC}): 0.9–1, perfect separation; 0.8–0.9, excellent discrimination; 0.7–0.8, acceptable discrimination; 0.6–0.7, poor discrimination; 0.5–0.6, no discrimination. Comparisons between distinct categories according to the outcome or the cytokine profile were performed using an analysis of variance (ANOVA) test. $P<.05$ was considered statistically significant.

RESULTS

IVF/ICSI Results and Pregnancy/Delivery Rates in the Cohort

The outcome after the first IVF/ICSI procedure was pregnancy for 25 patients (19 deliveries and six spontaneous abortions) and no pregnancy for 29. For 19 patients, an oocyte was collected but not successfully fertilized (12 ICSI, 11 matures oocytes, and seven IVF), and in 10 cases no oocyte was retrieved. Infertility was due to sperm abnormalities (39%), tubal abnormalities (30%), or endometriosis (12%) or was unexplained (19%). Table 1 details the characteristics of the patients in these four groups. Standard IVF was used for 44 oocytes, and ICSI for 39. Embryo score was significantly higher in the pregnant group ($P = .002$). The AUC_{ROC} of the embryo

TABLE 1

Characteristics of patients.

Characteristic	Pregnant group		No pregnancy	No oocyte collected	No ET	*P* value
	Birth	Abortion				
No. of patients	19	6	29	10	19	
Mean age	33	34	33.8	33.7	34.3	.87
Mean no. of previous conventional attempts	2.5	2	3	2.4	2.5	.67
FSH at day 3	7.4	8.2	7.9	7.3	7.4	.34
AFC at day 3	13.4	11.2	12.3	13	13	.74
E_2 level day of triggering, pg/mL	249	233	255	344	201	.23
Endometrial thickness day of triggering	8.3	9.1	8.48	8	8.5	.31
Optimal embryo transferred, %	78	66	58	—		.22
Embryo score	17.3	14.5	11			.002

Note: These covariables were included in multivariate regression models.

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TABLE 2

Mean concentration, detection limit, and predictability for subsequent birth in univariate and multivariate regression models.

Mediator	Mean ± SE (pg/mL)	Limit of detection, mean ± SD (pg/mL)	AUC_{ROC} to predict birth (univariate analysis)	AUC_{ROC} to predict birth/per cycle (n = 83; multivariate analysis including known covariates)	AUC_{ROC} to predict birth/per transfer (n = 54; multivariate analysis including embryo score and known covariates)
Embryo score			0.71 ($P = .006$)		0.76 ($P = .0002$)
G-CSF	13 ± 0.76	3.26 ± 4.01	0.81 ($P < .0001$)	0.81 ($P < .0001$)	0.85 ($P < .0001$)
GM-CSF	46 ± 5.8	1.79 ± 1.32	0.59 ($P = .29$)	—	
IL-1Ra	267 ± 36	1.54 ± 0.99	0.60 ($P = .14$)	—	
IL-2	17.7 ± 1.9	2.69 ± 2.04	0.50 ($P = .95$)	—	
IL-4	15.2 ± 2.2	1.01 ± 0.88	0.58 ($P = .07$)	—	
IL-6	8.52 ± 0.6	1.91 ± 0.04	0.52 ($P = .81$)		
IL-7	23.8 ± 1.7	1.01 ± 0.95	0.76 ($P = .0001$)	0.74 ($P = .002$)	0.74 ($P = .005$)
IL-8	195 ± 15	1.69 ± 0.40	0.49 ($P = .88$)	—	
IL–9	31 ± 2.5	1.62 ± 0.12	0.61 ($P = .13$)	—	
IL-12	2.8 ± 0.3	1.56 ± 0.64	0.48 ($P = .88$)		
IL-15	6.58 ± 0.62	1.37 ± 0.48	0.60 ($P = .07$)	0.65 ($P = .03$)	0.87 ($P = .0001$)
IL-17	9.53 ± 1.6	0.8 ± 0.84	0.76 ($P = .001$)	0.75 ($P = .0002$)	0.85 ($P < .0001$)
Eotaxin	204 ± 22	4.80 ± 3.84	0.68 ($P = .004$)	0.68 ($P = .08$)	Excluded from model
MIP-α	3.8 ± 0.3	1.53 ± 0.53	0.51 ($P = .61$)	—	
MIP-β	75 ± 4.5	1.97 ± 0.21	0.56 ($P = .38$)		
RANTES	64 ± 16	1.68 ± 0.22	0.61 ($P = .13$)		
VEGF	2107 ± 147	1.90 ± 0.25	0.65 ($P = .03$)	0.67 ($P = .01$)	Excluded from model
MCP-1	3.8 ± 0.3	1.48 ± 0.22	0.53 ($P = .63$)		
IP-10	192 ± 18.5	9.09 ± 5.11	0.68 ($P = .01$)	0.73 ($P = .004$)	0.82 ($P = .002$)
IFN-γ	51.6 ± 6.3	1.83 ± 0.32	0.53 ($P = .68$)		
G-CSF +IL-15				0.85 ($P < .0001$)	0.85 ($P < .0001$)

Note: The multivariate logistic regression models included age, range of attempt, basal FSH on day 3, AFC on day 3, endometrial thickness, E_2 level the day of triggering, and embryo score if specified (last column). Embryo score = [no. of blastomeres × (5 − grade)] on day 2.

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score to predict subsequent birth was set at 0.71 ($P<.0006$) in univariate analysis and reached 0.76 ($P=.0002$) in multivariate analysis including known variables and will be used as a reference for subsequent analysis (Table 2). Distribution of age, FSH, logarithm of range of the attempt, and AFC were normal in the cohort.

Levels of Cytokines, Chemokines, and Growth Factors and Prediction of Cycle Outcome

In regression models including known covariates, FF G-CSF, IL-7, IL-15, IL-17, eotaxin, VEGF, and IP-10 were significantly predictive (Table 2). Inclusion of embryo score in the model increased the AUC_{ROC} for FF G-CSF from 0.81 to 0.86 ($P<.0001$) to predict birth. Introduction of embryo score also increased the power of predictability of IL-15, IL-17, and IP-10, while VEGF and eotaxin were excluded (Table 2).

The optimal threshold according to the ROC curve for FF G-CSF was 12.08 pg/mL, with a sensitivity of 89.5% for a specificity of 54.7%. FF G-CSF concentrations were below that point in 45% (low group, 37 FF and 21 transferred) and at or above it in 55% (high group, 46 FF and 33 transferred). The rates of birth per cycle and per transfer were 5.3% and 9.5% in the low G-CSF group versus 37.8% and 51.5% in the high G-CSF group ($P=.001$), respectively . Pregnancy rates per cycle and per transfer were, respectively, 18.9% and 33% in the low G-CSF group versus 39% and 56% in the high G-CSF group ($P=.02$). G-CSF, IL-7, and IL-17 were significantly correlated with one another. The correlation coefficient between G-CSF and IL-17 was 0.68 ($P<.0001$); between G-CSF and IL-7, 0.62 ($P<.0001$); and between IL-17 and IL-7, 0.77 ($P>.0001$).

Optimal Model

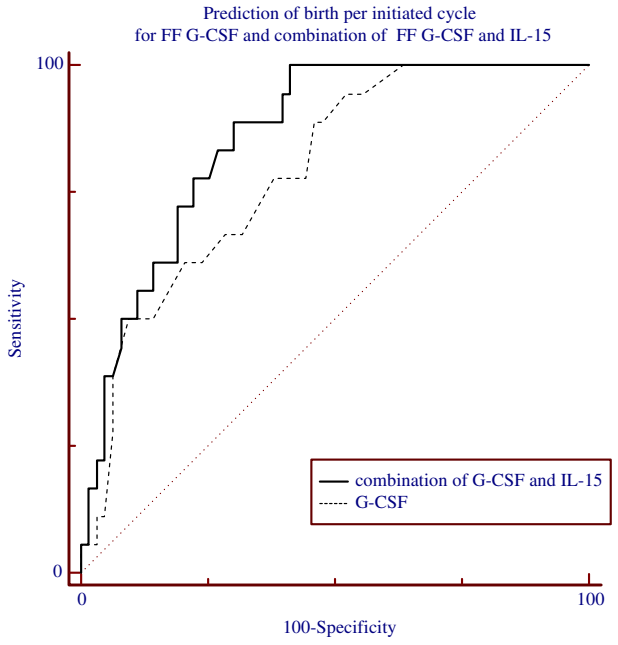
To optimize the model of prediction of birth, all the analytes found to be significant in the multivariate regression model (G-CSF, IL-7, IL-15, IL-17, eotaxin, VEGF, IP-10) were integrated into the analysis. Only FF G-CSF and IL-15 were retained in the model to predict birth with an AUC_{ROC} at 0.85 ($P<.0001$; Fig. 1). Birth rates per cycle were 48.88% (16/33) if two good-prognosis criteria were present (FF G-CSF>12 pg/mL and IL-15 <7 pg/mL) and 8% (3/36) and 0% (0/14) if, respectively, only one or none were present (Table 3).

FF Levels of Cytokines, Chemokines, and Growth Factors and Prediction of Oocyte Retrieval and Fertilization

Ten patients had no oocyte retrieved. We evaluated whether any quantification was associated with failure to collect the oocyte after the same logistic regression models as previously described. High concentrations of both GM-CSF and IL-15 were predictive of the failure to collect the oocyte with AUC_{ROC}, respectively, at 0.85 ($P=.0068$) and 0.88 ($P=.0001$) if evaluated with covariates. GM-CSF and IL-15 were correlated. Integrated with all the other analytes in a prediction model, only IL-15 and E_2 on the day of triggering were retained in the model with an AUC_{ROC} at 0.89 ($P=.0001$) to

FIGURE 1

Comparison of ROC curves evaluating FF G-CSF and combination of FF G-CSF and FF IL-15 as predictors of birth/cycle (n = 83). AUC_{ROC} were 0.81 [0.70–0.89], $P<.0001$, for FF G-CSF and 0.85 [0.74–0.92], $P<.0001$, for a combination of FF G-CSF and FF IL-15.



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predict failure of oocyte retrieval. An IL-15 threshold above 7 pg/mL had a sensitivity of 90% and a specificity of 77.8% to predict oocyte failure retrieval. Thirty-one percent (26/83) of patients had an FF IL-15 value above 7 pg/mL. Using the same methodology, we failed to construct a model that was able to predict failure of fertilization.

Exploration of FFs over Two Natural Modified IVF/ICSI Cycles

Sixteen patients who failed to be pregnant at the first cycle [no transfer (n = 6), no oocyte collected (n = 1), no pregnancy despite ET (n = 7), and abortion (n = 2)] were explored through a subsequent modified natural IVF/ICSI cycles. The second cycles resulted in six births, one abortion, five no ET, and four no pregnancy. FF G-CSF was the only cytokine significantly correlated over two cycles ($r = 0.71$, $P = .008$), while FF IL-15 showed variations from a cycle to another. All six women who delivered had the optimal combination (FF G-CSF over 12 pg/mL with IL-15 below 7 pg/mL) over the second cycle and five out of six over the first observational cycle (one case of elevated FF IL-15).

DISCUSSION

In the present cohort, the combined noninvasive analysis of FF G-CSF and IL-15 allows us to distinguish, among patients with apparently equal clinical presentations, a subgroup of patients—40%, (33/83)—with high chances of conception but also a subset of patients (17%, 14/83) with no chance of conception in MNC-IVF. Independent of FF G-CSF, and so adding value to the model, high levels of IL-15 were found to be predictive of a failure in oocyte retrieval with potential variations from one cycle to another. Poor prognostic values of high FF IL-15 concentrations on subsequent pregnancy rates have been reported in conventional IVF (10, 11) and seem predictive of a failure, especially if combined with poor embryo morphology.

Interestingly, predictability of birth based on FF G-CSF/IL-15 concentrations was independent of, higher than, and complementary to the one based on the embryo score alone (our referential in routine), whereas the high correlation of FF G-CSF over two cycles suggests that documentation over a single cycle may inform on the cumulative chances of conception over natural IVF/ICSI cycles.

Because the reported results are based on the observation of a limited retrospective series, our findings must clearly be confirmed. A randomized multicenter prospective study should evaluate whether the documentation of FF G-CSF/IL-15 over one MNC is effective to identify patients with a high chance of conception in MNCs when compared with a cohort of patients using a conventional scheme.

Ovarian hyperstimulation is widely applied to compensate for the inefficiency of the IVF procedure by increasing the number of collected oocytes to provide a large cohort of embryos, undergoing a morphological selection to maximize the likelihood of implantation (12). The basis for such a policy is the lack of criteria to define individual oocyte quality. If at the oocyte collection step we can accurately evaluate the potentiality and competence of the oocyte, the basis of systematic ovarian hyperstimulation could be restricted to patients who would benefit from it.

TABLE 3

Predictive value of combinations of follicular G-CSF and IL-15 on births rates.

G-CSF and IL-15 combination, pg/mL		Pregnancy/initiated cycles, %	Pregnancy/transfer, %	Birth/initiated cycles, %	Birth/transfer, %
G-CSF >12	IL-15 <7	51.5 (17/33)**	63 (17/27)*	48.48 (16/33)***	59.3 (16/27)***
	IL-15 >7	8.3 (1/12)	16.6 (1/6)	8.3 (1/12)	16.67 (1/6)
G-CSF <12	IL-15 <7	20.8 (5/24)	23 (3/13)	8.3 (2/24)	15.4 (2/13)
	IL-15 >7	14.3 (2/14)	25 (2/8)	0 (0/14)	0 (0/8)

Note: Cutoff values were defined from the area under ROC curves. The *P*-values between FF with FF G-CSF >12 pg/mL and FF IL-15 <7 pg/mL if compared with the three others combinations were .001 (***) for birth/cycle and birth /transfer, .005 (**) for pregnancy/cycle, and .02 (*) for pregnancy/transfer using ANOVA test.

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Indeed, a previous study performed in conventional ovarian hyperstimulation with traceability of individual FFs until birth also identified FF G-CSF as a biomarker correlated with the implantation potential of the corresponding embryo (6). Although FF cytokine profiles cannot be precisely compared between conventional and MNC-IVF/ICSI cycles (measurements assessed in the same laboratory with the same assay system but with different kit batches), the mean FF G-CSF concentration in conventional hyperstimulation was higher than in natural modified cycles (21 vs. 13 pg/mL). Furthermore, some cytokines (IL-7, IL-17), never detected in conventional hyperstimulation, were successfully detected in MNCs, suggesting some crucial differences of expression related to ovarian hyperstimulation itself. Influence of antagonist administration with hMG support should also be investigated to be compared with "pure" natural cycles.

G-CSF has been previously shown to be secreted by granulosa cells at ovulation (13, 14), then within the endometrium during the luteal phase, and finally during gestation in the placenta, and early serum increases occurred in case of pregnancy (15). More recently, a randomized controlled trial reported that G-CSF administration significantly increased live-birth rates in patients with recurrent miscarriages (16). In the present study, almost all miscarriages (five of six) occurred in the low G-CSF group. Therefore, our main hypothesis is that FF G-CSF reveals the competence of the oocyte to promote a local immune dialog between mother and conceptus. Such a dialog seems crucial for an effective placentation, but the underlying mechanism remains unknown and needs to be elucidated. Basic and clinical studies need to be conducted to elucidate such promising issues.

We applied for a patent of application in July 2009.

REFERENCES

1. Nargund G, Frydman R. Towards a more physiological approach to IVF. Reprod Biomed Online 2007;14:550–2.
2. Verberg MF, Macklon NS, Nargund G, Frydman R, Devroey P, Broekmans FJ, et al. Mild ovarian stimulation for IVF. Hum Reprod Update 2009;15:13–29.
3. Pelinck MJ, Keizer MH, Hoek A, Simons AH, Schelling K, Middelburg K, et al. Perinatal outcome in singletons after modified natural cycle IVF and standard IVF with ovarian stimulation. Eur J Obstet Gynecol Reprod Biol 2010;148:56–61.
4. Schimberni M, Morgia F, Colabianchi J, Giallonardo A, Piscitelli C, Giannini P, et al. Natural-cycle in vitro fertilization in poor responder patients: a survey of 500 consecutive cycles. Fertil Steril 2009;92:1297–301.
5. Rongières-Bertrand C, Olivennes F, Righini C, Fanchin R, Taïeb J, Hamamah S, et al. Revival of the natural cycles in in-vitro fertilization with the use of a new gonadotrophin-releasing hormone antagonist (Cetrorelix): a pilot study with minimal stimulation. Hum Reprod 1999;14:683–8.
6. Steer CV, Mills CL, Tan SL, Campbell S, Edwards RG. The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. Hum Reprod 1992;7:117–9.
7. Mendez Lozano DH, Brum Scheffer J, Frydman N, Fay S, Fanchin R, Frydman R. Optimal reproductive competence of oocytes retrieved through follicular flushing in minimal stimulation IVF. Reprod Biomed Online 2008;16:119–23.
8. Ledee N, Lombroso R, Lombardelli L, Selva J, Dubanchet S, Chaouat G, et al. Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: the role of granulocyte colony-stimulating factor. Hum Reprod 2008;23:2001–9.
9. Steyerberg EW, Eijkemans MJ, Harrell FE Jr, Habbema JD. Prognostic modelling with logistic regression analysis: a comparison of selection and estimation methods in small data sets. Stat Med 2000;19:1059–79.
10. Falconer H, Sundqvist J, Gemzell-Danielsson K, von Schoultz B, D'Hooghe TM, Fried G. IVF outcome in women with endometriosis in relation to tumour necrosis factor and anti-Mullerian hormone. Reprod Biomed Online 2009;18:582–8.
11. Vujisic S, Lepej SZ, Emedi I, Bauman R, Remenar A, Tiljak MK. Ovarian follicular concentration of IL-12, IL-15, IL-18 and p40 subunit of IL-12 and IL-23. Hum Reprod 2006;21:2650–5.
12. Fauser BC, Devroey P, Macklon NS. Multiple birth resulting from ovarian stimulation for subfertility treatment. Lancet 2005;365:1807–16.
13. Salmassi A, Schmutzler AG, Huang L, Hedderich J, Jonat W, Mettler L. Detection of granulocyte colony-stimulating factor and its receptor in human follicular luteinized granulosa cells. Fertil Steril 2004;81(Suppl 1):786–91.
14. Salmassi A, Zhang Z, Schmutzler AG, Koch K, Buck S, Jonat W, et al. Expression of mRNA and protein of macrophage colony-stimulating factor and its receptor in human follicular luteinized granulosa cells. Fertil Steril 2005;83:419–25.
15. Yanagi K, Makinoda S, Fujii R, Miyazaki S, Fujita S, Tomizawa H, et al. Cyclic changes of granulocyte colony-stimulating factor (G-CSF) mRNA in the human follicle during the normal menstrual cycle and immunolocalization of G-CSF protein. Hum Reprod 2002;17:3046–52.
16. Scarpellini F, Sbracia M. Use of granulocyte colony-stimulating factor for the treatment of unexplained recurrent miscarriage: a randomised controlled trial. Hum Reprod 2009;24:2703–8.