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Interleukin (IL)-1 Beta and IL-6 Levels in Human Embryo Culture Supernatants and their Role in Implantation Following IVF: A Prospective, Non-randomized Study

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Abstract

Objective: Interleukin-1 beta (IL-1β) and Interleukin-6 (IL-6) are pro-inflammatory cytokines involved in the mother-embryo interaction during blastocyst adhesion and invasion into the endometrium. IL-1β is also considered as a first signal delivered from blastocyst to the endometrium to influence uterus receptivity. The aim of the present prospective, non-randomized study was to explore whether the measurement of IL-1β and IL-6 secretion by blastocysts could serve as a non-invasive method to predict blastocyst implantation competence following *in vitro* fertilization (IVF).

Methods: IL-1β and IL-6 were measured in the supernatant culture media of 683 blastocysts transferred into 245 women following IVF cycles, and their levels were correlated with implantation and pregnancy rates per transfer. Measurements were performed using a Luminex 200 and commercially available interleukin kits. Statistical analyses were performed using GraphPad Prism 5 software, and a probability of $p<0.05$ was used to indicate a significant difference.

Results: IL-1β was detected in 26.5% of the blastocyst supernatants (179/683), with a mean value 0.099 pg/ml, and IL-6 was detected in 20.35% of blastocysts (139/683), with a mean value of 0.046 pg/ml. Cytokine levels showed no correlation with blastocyst quality or developmental stage. The mean values of IL-1β and IL-6 in the implanted blastocysts were 0.073 and 0.036 pg/ml, respectively. In the non-implanted blastocysts, the corresponding values were 0.0141 and 0.060 pg/ml. The pregnancy and implantation rates in women with detectable IL-1β levels (pregnancy rate (PR): 56.3%; implantation rate (IR): 21.8%) and non-detectable IL-1β levels (PR: 60.23%; IR: 28.37%) and in women with detectable IL-6 levels (PR: 58%; IR: 25%) and non-detectable IL-6 levels (PR: 59.5%; IR: 26.83%) were not significantly different.

Conclusion: Blastocysts secrete IL-1β, and to a lesser extent, IL-6. No significant differences in implantation or pregnancy outcomes were identified between patients with detectable and undetectable IL-1β and IL-6 levels. Therefore, the non-invasive measurement of IL-1β and IL-6 secreted by blastocysts prior to transfer should not be considered a useful biomarker of blastocyst development and implantation competence.

Keywords: Interleukin-1β; Interleukin-6; Blastocyst; Embryo viability; Implantation markers

Introduction

A major goal of reproductive medicine is to identify and transfer embryos with the highest potential for implantation into a woman's uterus. To achieve this goal, researchers have focused on finding new techniques and methods for the selection of viable embryos for embryo transfer (ET) [1-6]. Since the culture of human embryos to the blastocyst stage (day 5 or 6) appears to yield the highest pregnancy rate (PR) and implantation rate (IR) following IVF [7,8], the development of the fertilized egg to the blastocyst stage has been used as a marker of embryo viability. Blastocyst transfer increases the clinical pregnancy rates following IVF to greater than 50%, thus offering the opportunity to include fewer embryos in the ET, without compromising the success rate [7,9]. To further improve the IR, there is an essential need to identify additional markers of implantation potential, apart from morphological criteria or embryo development to the blastocyst stage. Finding such markers could lead to single-embryo transfer without compromising pregnancy rates, thereby eliminating the possibility of multiple pregnancies, which constitutes the biggest default of IVF [7,8,10,11].

The recognition that the mechanism of implantation is similar to that of inflammation has led to new insights in the field of IVF. Immune factors, such as cytokines, chemokines, and several growth factors, have been shown to play vital roles in the mother-embryo "dialogue" that occurs during the blastocyst adhesion and invasion to the endometrium [12,13]. Studies have also suggested that non-invasive measurements of soluble forms of biomarkers, such as cytokines secreted by embryos into the culture media, could have prognostic value in pregnancy and implantation outcomes [14].

Cytokines are small, multifunctional glycoproteins, that have biological actions mediated by specific cell-surface receptors and that act as signals, regulating functions during embryo-maternal interactions [15]. Imbalances in cytokine expression levels and cytokine signaling could lead to implantation failure or abnormal placentation [16]. Of the cytokines and growth factors studied to date, IL-1 [17], IL-2 [18], IL-4 [19], IL-6 [20], tumor-necrosis factor- alpha (TNF-a) [17], IL-10, interferon-gamma (IFN-γ) [21], leukemia inhibitory factor (LIF) [22], IL-11 [23], and transforming growth factor-b (TGF-b) [24], epidermal growth factor (EGF) [25], heparin-binding epidermal growth factor (HB-EGF) [26] and insulin growth factor (IGF) [27] have been suggested to be crucial for the embryo implantation process.

During early embryonic development, IL-1 and IL-6 are of particular interest: IL-1 has been shown to function at both maternal and embryonic sites during implantation, whereas mice deficient in IL-6 have exhibited reduced fertility and implantation rates [20]. Both IL-1 and IL-6 have been detected in embryo culture media, but no significant correlation has been observed between cytokine concentrations and embryo morphology [28]. In contrast, Baranao et al. [29] were unable to detect IL-6 in embryo culture media; however, they detected IL-1β, and its levels, ranging from 14 to 82 pg/ml, correlated positively with implantation outcome. Another study by De los Santos et al. [30], reported embryonic production of IL-1 (ranging from 4 to 12 pg/ml) only in co-culture with endometrial cells.

The aim of the present study was to measure the embryonic production of IL-1β and IL-6 in a large number of samples and to look whether their levels correlate with blastocyst morphology and/or can predict implantation competence.

Methods

Patient recruitment and stimulation protocols

A total of 245 patients were recruited for this non-randomized prospective study (Table 1) after they provided signed informed consent for embryo transfer on day 5 post-insemination (p.i.) (insemination day was designated as day 0). The analysis and measurement of cytokines were performed using a non-invasive test on "spent" culture medium. The study received ethical approval by the Institutional Board of Genesis Athens Clinic (Ref No: 64, 03/01/2011). The main goal of this study was to identify a clinically applicable marker for embryo selection; thus, patient recruitment was not limited according to age, infertility cause, number of previous IVF attempts or method of fertilization used (classical IVF or Intracytoplasmic Sperm Injection-ICSI). The only recruitment criterion was that the embryo transfer occurred on Day 5.

All patients received human menopausal gonadotrophin (HMG) stimulation (Menopur-HMG HP; Ferring GmbH, Kiel, Germany) or recombinant follicle stimulation hormone (FSH) stimulation (Gonal-F; Merck Serono Europe Ltd, London, U.K. or Puregon; N.V. Organon, Oss, The Netherlands) at doses ranging from 150 to 450 IU daily for up to 14 days. The gonadotrophin-releasing hormone (GnRH) agonists triptorelin (Arvekap; Ipsen, PharmaBiotech, France) or leuprorelin acetate (Daronda; Abbott Lab, France) were administered according to either long (down regulation) or short (flare) protocols. The ovarian response was monitored by means of serum estradiol assays and vaginal ultrasonographic scans of follicles. When more than three follicles measured ≥ 19 mm and the estradiol concentration was ≥ 545 pg/ml, a dose of 10,000 IU of HCG (Pregnyl; N.V. Organon, Oss, The Netherlands) or recombinant hCG (Ovitrelle 250 μg; Merck Serono Europe Ltd., London, U.K.) was administered. Oocyte retrieval was scheduled 36 h after hCG administration. Oocytes were retrieved by transvaginal needle aspiration under ultrasonographic guidance. Luteal-phase support commenced on the next day of oocyte retrieval by means of the vaginal administration of progesterone at a dose of 600 mg/day for 16 days.

Number of Patients	245
Ovarian stimulation protocol	GnRH (l)+ or GnRH(s)
Day of embryo transfer (ET)	Day 5
Women's mean age (years)	38.08 ± 2
Day 3 FSH (IU/l)	8.60 ± 2.71
AMH (ng/ml)	0.90 ± 0.23
Number of previous IVF/ICSI cycles	2.10 ± 1.00
Retrieved oocytes	19.93 ± 6.00
Blastocysts per ET	2.81
Number of blastocysts on ET (total)	683
Number of samples analyzed	683
Causes of infertility	
Male factors	86 (35.10%)
Female factors	122 (49.80%)
Both male and female factors	17 (6.90%)
Unexplained	20 (8.10%)
Oocyte fertilization technique	
IVF	33
ICSI	212

Table 1: General characteristics of the study population (values are means ± SDs).

Embryo culture and collection of culture media

Embryo culture to the blastocyst stage was based on the recommendations of Jones et al. [31], with a few modifications made to match the study's specifications. More specifically, oocytes were inseminated 4 to 6 h after their retrieval and were cultured either in groups of three in 20 μl microdrops after ICSI, or in groups of five in 0.5 ml Nunc wells (NUNC, Roskilde, Denmark) after conventional IVF, in pre-equilibrated G-IVF culture medium (Vitrolife Innovative

Cell and Tissue Technology; Goteborg, Sweden) under light mineral oil (Irvine Scientific; Santa Ana, California, USA). On day 1 following insemination, zygotes were cultured in groups of two to three in 20 µl microdrops of pre-equilibrated G1™V5 culture medium (Vitrolife Innovative Cell and Tissue Technology; Goteborg, Sweden), supplemented with 5% human serum albumin (HSA) (Irvine Scientific; Santa Ana, California, USA) under light mineral oil. Embryo culture was performed in Minc humidified incubators (MINC-1000, COOK, Australia) at 37°C and an atmosphere of 6% CO_2, 5% O_2 and 89% N_2.

Sixty-five to 76 h post-insemination (p.i.), embryos were transferred and individually cultured until Day 5 p.i., in 80 µl microdrops of pre-equilibrated G2™V5 medium (Vitrolife Innovative Cell and Tissue Technology; Goteborg, Sweden) supplemented with 5% HSA under light mineral oil. On the morning of Day 5 p.i., the blastocyst morphology was assessed [31-33] using a Nikon inverted microscope. The blastocysts that were selected for transfer were placed in fresh G2 medium, and 50 µl of the "spent" culture media was collected and stored at -20°C for a maximum of 2 months before analysis. A total of 683 samples of culture medium were collected and analyzed for the presence of IL-1β and IL-6.

Embryo transfer

All embryos were transferred on Day 5, and preference was given to the most morphologically advanced blastocyst (hatched>hatching>expanded>expanding> early blastocyst) [31], with the most prominent inner-cell mass (ICM) (many cells, tightly packed>many cells, loosely grouped>very few cells) and trophectoderm epithelium (TE) (many cells, cohesive epithelium>few cells, loose epithelium>very few cells) [32,33]. The embryos to be transferred were placed in fresh, pre-equilibrated G2™V5 medium supplemented with 10% HSA. The number of blastocysts selected for transfer was determined by the availability of embryos and the patient's IVF medical history and age. One to three blastocysts were transferred to the uterus of each patient.

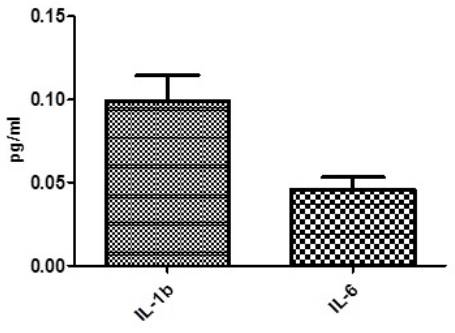

Figure 1: Mean values of IL-1β and IL-6 secretion levels. Mean values of IL-1β and IL-6 embryonic secretion in the 683 samples analyzed, corresponding to 245 IVF patients (P<0.05).

Measurement of IL-1 beta and IL-6 levels

IL-1β and IL-6 were measured at the Immunology-Histocompatibility Department of "Helena Venizelou" Maternity Hospital (Athens, Greece), using the Luminex IS 200 (Luminex Corporation, Texas, USA) and the commercially available IL-1β and IL-6 kits (Human IL-1β and Human IL-6 Singleplex Bead Kit; Invitrogen Carlsbad, California, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., California, USA). A comparison of the mean values of IL-1β and IL-6 between groups of patients was performed using the Mann-Whitney U test and their correlations with pregnancy and implantation rates were calculated using Fischer's exact test. One-way analysis of variance (ANOVA) was used to show the correlation between the mean values of IL-1β and IL-6 and the developmental stages of the blastocyst. A probability of P<0.05 was used to indicate a significant difference.

Results

IL-1β and IL-6 secretion by human blastocysts before embryo transfer

The investigation of IL-6 and IL-1β secretion was performed using the supernatant culture medium drops in which the embryos were cultured from Day 3 to Day 5 p.i. Because 1 to 3 blastocysts were included in each ET, the mean values of IL-1β and IL-6 per patient were calculated, and the detection levels are presented as pg/ml. Of the 683 samples (245 patients) collected and analyzed, IL-1β was detected in 26.5% (179/683) and IL-6 was detected in 20.35% (139/683) of samples, with mean values of 0.099 and 0.046 pg/ml, respectively. Data analysis showed higher secretion levels of IL-1β than of IL-6 (P<0.05) (Figure 1).

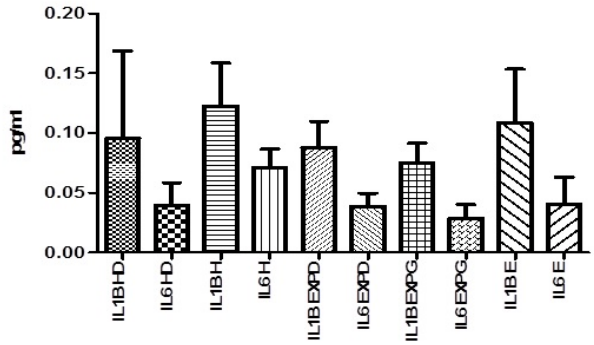

Figure 2: IL-1β and IL-6 levels at different blastocyst developmental stages. IL-1β and IL-6 levels (mean values) according to the blastocyst developmental stage (245 women; 683 samples) (HD: Hatched; H: Hatching; EXPD: Expanded; EXPG: Expanding; E: Early blastocysts).

IL-1β and IL-6 secretion by human blastocysts according to the morphology of embryos before embryo transfer

The mean values of IL-1β and IL-6 were also evaluated based on the developmental stage of the blastocysts being transferred (Figure 2). Overall, there were no significant differences in the levels of

interleukins secreted by blastocysts according to morphological stage. However, a comparison of the two cytokine secretion levels between the different blastocyst developmental stages showed higher IL-1β levels than IL-6 levels (P<0.05) in the less advanced blastocyst stages (expanding and early blastocysts).

IL-1 beta and IL-6 secretion by human blastocysts with respect to pregnancy and implantation outcome following embryo transfer

The mean values of IL-1β and IL-6 were 0.073 and 0.036 pg/ml, respectively, in the implanted blastocysts and 0.141 and 0.060 pg/ml, respectively, in the non-implanted blastocysts.

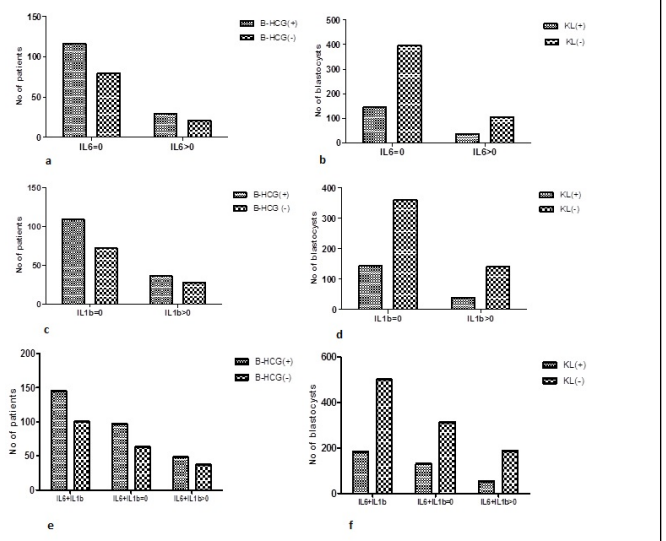

Figure 3: Pregnancy and implantation outcomes with respect to IL-1 beta and IL-6 levels. The total number of women with positive or negative β-hCG, as well as the total number of blastocysts implanted, is presented (KL(+): implanted, KL(-): non-implanted). Implantation was recorded after ultrasonography of β-hCG(+) patients.

Undetectable IL-1β levels in all supernatant medium samples occurred in 109 patient cycles of positive and 72 cycles of negative beta-human chorionic gonadotrophin (β-hCG) levels (P>0.05). Detectable IL-1β levels in at least one blastocyst supernatant culture medium sample per transfer were associated with 36 cycles of positive and 28 cycles of negative β-hCG (p>0.05) levels (Figure 3c). Regarding IL-6, undetectable levels in all samples were associated with 116 cycles of positive and 79 cycles of negative β-hCG (p>0.05) results. Detectable levels of IL-6 in at least one supernatant medium sample per transfer were found in 29 and 21 cycles corresponding to positive and negative pregnancy results, respectively (p>0.05) (Figure 3a). Furthermore, undetectable levels of both IL-6 and IL-1β levels were found in 97 and 63 cycles corresponding to positive and negative pregnancy outcomes, respectively (p>0.05), and the detection of both IL-6 and IL-1β was found in 48 and 37 ETs with positive and negative outcomes, respectively (p>0.05) (Figure 3e).

With respect to the implantation outcome, IL-1β was undetectable in a total of 504 supernatant medium samples, corresponding to 504 blastocysts that were transferred, 143 of which implanted. Detectable levels of IL-1β were found in 179 samples and resulted in the implantation of 39 embryos (p>0.05) (Figure 3d). Regarding IL-6, undetectable levels were found in a total of 544 samples corresponding to the implantation of 146 blastocysts, whereas detectable levels of IL-6 were observed in 139 samples, corresponding to the implantation of 35 blastocysts (P>0.05) (Figure 3b). Last, both IL-1β and IL-6 were undetectable in 443 samples, corresponding to the implantation of 129 embryos, whereas both cytokines were detected in 240 samples corresponding to the implantation of 53 blastocysts (p>0.05) (Figure 3f).

Overall, the pregnancy and implantation rates in women with detectable IL-1β levels (PR: 56.3%; IR: 21.8%) and women with non-detectable IL-1β levels (PR: 60.23%; IR: 28.37%) were not significantly different (P>0.05). Similarly, no significant differences (P>0.05) were observed between women with detectable IL-6 levels (PR: 58%; IR: 25%) and women with non-detectable IL-6 levels (PR: 59.5%; IR: 26.83%) (Figure 4).

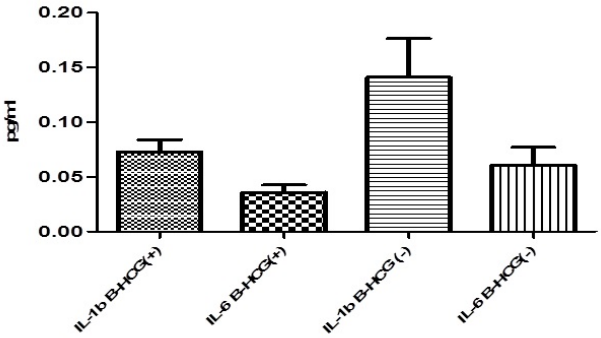

Figure 4: Correlation of IL-1 beta and IL-6 levels with the β-hCG results. IL-1 beta and IL-6 levels in correlation with the β-hCG result in 683 samples analyzed (β-hCG (+): positive pregnancy result; β-hCG (-): negative pregnancy result). IL-1beta is secreted at significantly higher levels than IL-6 under both β-hCG (+) and β-hCG (-) conditions (P<0.05). The IL-1 beta and IL-6 levels were not significantly different between the two categories (P>0.05), although both were elevated in the β-hCG (-) category.

Finally, a multiple regression analysis showed no correlation between IL-1β or IL-6 embryonic secretion and patient age, infertility causes, previous IVF attempts or the fertilization method used.

Discussion

The pro-inflammatory cytokines IL-1β and IL-6 are considered to play crucial roles in the implantation process [34,35]. Several studies have implicated IL-1β expression and secretion by blastocysts [29,30,36] and have suggested that this cytokine acts as a mediator with respect to the invasion of the blastocyst into the endometrium. IL-6 expression by blastocyst-stage embryos has been implied to be very limited and most likely related to the non-expression status reflected in earlier stages of pre-implantation development [29,37]. However, recent studies have reported IL-6 expression and secretion by cleavage-stage embryos [28,38] and have proposed the use of IL-6 as a marker of embryo developmental competence [38]. It has also been

indicated that the supplementation of culture media with IL-1β or IL-6 can improve the implantation rate following IVF, by enhancing embryo development to the blastocyst stage and by increasing the number of blastocyst cells [13,39-41]. In contrast, it has been shown that the reduction of culture medium IL-6 levels compromises pregnancy and implantation rates [42,43]; consequently, it is postulated that IL-6 is related to embryo viability.

The purpose of the present study was to investigate whether there is a relationship between the secretion of IL-1β and IL-6 by embryos at the blastocyst stage and implantation or pregnancy outcomes. In our study, IL-1β was detected in the supernatant culture media of 26.5% of the blastocysts. In contrast, IL-6 was detected in 20.35% of the blastocyst culture medium samples at levels significantly lower than those of IL-1β. The analysis of the IL-1β and IL-6 blastocyst secretion levels revealed no significant differences between patients with positive and negative pregnancy results. Similarly, there were no significant differences in implantation and pregnancy outcomes between patients with detectable and non-detectable levels of IL-1β and IL-6.

Based on previous findings reporting that IL-6 expression is under the control of several factors including IL-1β [44], the current study also investigated a possible role of the combination of IL-6 and IL-1β secretion by the human blastocyst with respect to implantation and pregnancy outcomes. However, the results again showed no difference in pregnancy and implantation outcomes between patients with detectable and non-detectable levels of both IL-1β and IL-6.

The morphology and quality of the blastocysts prior to transfer were recorded, and the data were evaluated with respect to the detection levels of IL-1β and IL-6. Statistical analyses revealed no significant difference in the level of either cytokine and blastocyst morphology. Finally, comparisons between the levels of the two cytokines secreted at the different developmental stages of a blastocyst were performed, and although no difference was found in the three advanced blastocyst stages (hatched, hatching, expanded blastocysts), there was a significant difference in their secretion at the earlier stages (expanding, early blastocysts), with IL-1β levels being higher than IL-6 levels ($P<0.05$). The blastocysts in the earlier stages of development were also found to secrete the highest levels of both IL-1β and IL-6 among the samples analyzed.

To our knowledge there are no similar studies using the supernatant culture medium of the blastocyst-stage embryos to investigate a possible role of IL-1β and IL-6 secretion in the implantation outcome. Our results suggest that blastocysts secrete IL-1β before ET, whereas previous findings suggest that an embryo, prior to ET expresses and secretes IL-1β only in co-culture with endometrial tissue [13,30]. An explanation for this difference may be because the embryos analyzed in those studies were in earlier developmental stages, the different culture conditions and the different methods used (ELISA instead of Luminex) compared with our study. IL-6 secretion by the blastocyst-stage embryos was also detected, although at significantly lower levels than IL-1β, which was most likely related to differential IL-6 and IL-1β gene expression by the blastocysts. Previous studies of cleavage-stage embryos support our results regarding the relatively low secretion levels of IL-6. Recently, it has been suggested that IL-6 secretion levels are higher in the first 24 h of embryo development than in the second 24 h [28,38], indicating a gradual decrease in IL-6 secretion as the embryo develops.

Our results revealed no correlation of blastocyst quality and morphology with the detection levels of IL-1β and IL-6 that have been previously mentioned by other researchers studying the expression of those cytokines by the cleavage stage embryos [13,30,38,45]. However, a recent study identified a correlation of IL-1β with the developmental stage of embryos, but not their quality, instead showing IL-1β secretion levels to be proportional to the number of embryo cells [46]. The findings of our study do not seem to support this theory when applied to the blastocyst-stage embryos for either IL-6 or IL-1β. Conversely, it was observed that higher levels of both cytokines were detected in the earlier stages of blastocyst development (early and expanding blastocysts), characterized by fewer cells than the later blastocyst stages. This observation may lead to the assumption that the development of embryos beyond the 8-cell stage results in cytokine expression/secretion that is not proportional to the cell number of the developing embryo.

In the current study, the analysis of IL-1 beta and IL-6 secretion levels by blastocysts revealed no significant differences between patients with positive and negative pregnancy result. This result, however, may be affected by the transfer of multiple embryos per patient (2.81 blastocysts per patient); therefore, no direct correlation of cytokine secretion with implantation could be extrapolated from the results. The policy of our center, in accordance with the local IVF law with regard to the number of embryos transferred, represents an obstacle to obtaining a relationship between implantation and pregnancy outcomes using our results. However, the assessment of a large number of samples (683 samples) using x-MAP technology (Luminex), which represents a very sensitive method, allows us to draw some preliminary conclusions regarding the cytokine secretion exhibited by blastocysts. Although no correlation of IL-1β and IL-6 with implantation was demonstrated, a relationship between cytokine secretion and blastocyst development was found; however, further research using single-embryo transfer is needed for this relationship to be verified.

Conclusion

In conclusion, non-invasive measurement of IL-1β and IL-6, which are secreted by blastocysts in the supernatant culture medium, does not currently seem to serve as a biomarker for selection of the best human blastocyst for transfer. Furthermore, these factors are not useful even in conjunction with the existing selection criteria, such as morphological parameters, for improving the prediction of embryo viability.

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