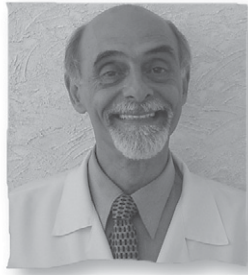


## Article

# Endometrial expression of IL-1RtI in patients undergoing miscarriage or unsuccessful IVF cycles



Jorge Haddad-Filho obtained his medical degree in 1976 and worked as a general clinician until 1985. He then took up a post in the Federal University of São Paulo, taking part in the reorganisation of the Human Reproduction Division of the medical school there. In 1989-1990 he worked at the Withington Hospital (South Manchester University Hospitals NHS Trust, UK) and Heidelberg University, Germany. He participated in the introduction of in-vitro techniques in the Federal University of São Paulo, and is currently Director of the assisted reproduction programme there. His major area of interest is the role of the endometrium in embryo implantation.

Dr Jorge Haddad-Filho

Jorge Haddad-Filho<sup>1,2</sup>, Agnaldo Pereira Cedenho<sup>2</sup>, Sima Godosevicius Katz<sup>1,3</sup>

<sup>1</sup>Department of Histology, School of Medicine, Federal University of São Paulo, Rua Botucatu, 740 (Edif Lemos Torres 20 a), São Paulo, SP, Brazil, CEP 04023-900; <sup>2</sup>Human Reproduction Division, Federal University of São Paulo, Rua Botucatu, 725 São Paulo, SP, Brazil. CEP 04023-062

<sup>3</sup>Correspondence: Tel/Fax: +55 11 55764268; e-mail: simagkatz.morf@epm.br

## Abstract

This study sought to compare the endometrial expression of interleukin-1 receptor type I in patients with a history of spontaneous recurrent miscarriage and patients with unsuccessful IVF cycles. Eight patients who had undergone two or more consecutive unsuccessful IVF cycles (group I), in which at least one good quality embryo was transferred, and 18 patients with at least three spontaneous late miscarriages (group II) were included in the study. Endometrial and follicular development were evaluated by ultrasonography. Plasma concentrations were evaluated for FSH and prolactin on cycle day 3 and for progesterone on day 7 after ovulation, when an endometrial biopsy was performed; samples were cryopreserved for interleukin-1 receptor type I immunohistochemistry and embedded in paraffin for endometrial dating. Patients with no ovulation or any anomalies in hormone concentrations, uterine cavity or endometrial histology were excluded. Interleukin-1 receptor type I was expressed in the luminal epithelium of both groups. There was a significant difference ( $P = 0.0357$ , two-tailed Fisher's exact test) in the glandular epithelium expression of interleukin-1 receptor type I between groups I (87.5% of patients) and II (38.9% of patients). Endometrial expression of interleukin-1 receptor type I does not seem to be involved in embryo implantation in IVF patients, but may play a role in spontaneous recurrent miscarriage.

**Keywords:** embryonic implantation, human endometrium, interleukin-1 receptor type I, IVF, recurrent miscarriage

## Introduction

Embryo implantation rate, one limiting factor for the success of in-vitro procedures, ranges between 25 and 30% (De Croo *et al.*, 2000). It decreases when poor quality (Giorgetti *et al.*, 1995; Hsu *et al.*, 1999) or cryopreserved embryos are transferred (Stafford-Bell and Copeland, 2001). Nevertheless, even transferring excellent quality embryos does not ensure pregnancy (excellent embryos defined as those with  $\leq 20\%$  fragmentation and no multinucleation; Van Royen *et al.*, 2001), suggesting that endometrial factors or the embryo-endometrial interaction may not be adequate.

Interleukin-1 $\beta$  (IL-1 $\beta$ ) and its receptor type I (IL-1RtI) seem to play a role in this interaction (Tabibzadeh, 1992; Simón *et al.*, 1993a, 1994a,b; Sims *et al.*, 1993). The human embryo produces and secretes IL-1 $\beta$  when co-cultured with endometrial epithelium (Simón *et al.*, 1998), and the trophoblast expresses IL-1RtI (Simón *et al.*, 1995), suggesting that these molecules may mediate, in some way, the interaction between embryo and endometrium. In fact, receptor blocking by a specific antagonist (IL-1Ra) reduces the probability of pregnancy in mice (Simón *et al.*, 1994a; Abbondanzo *et al.*, 1996). Furthermore, the binding of IL-1 $\beta$  to its receptor stimulates production of leukocyte inhibition factor (Arici *et al.*, 1995) and increases the endometrial

expression of integrin- $\beta 3$  (Simón *et al.*, 1998), a marker for uterine receptivity (Lessey *et al.*, 1995).

The IL-1 system has been shown to play an important role in achieving and maintaining a successful pregnancy, and some cases of infertility may be related to altered expression of IL-1 system components in the endometrial tissue. The expression of IL-1RtI in women with habitual abortion or undergoing IVF remains unclear.

The present study examined endometrial dating and the expression of IL-1RtI in two groups of women, IVF patients with a history of at least two unsuccessful stimulated cycles, and patients with three or more consecutive spontaneous abortions, with at least 8 weeks gestation. It was investigated whether habitual abortion and unsuccessful implantation in IVF patients might be associated with abnormal endometrial expression of IL-1RtI. The fact that embryonic implantation is naturally achieved in habitual abortion patients suggests that IL-1RtI expression in these patients is adequate for embryo implantation, thus making habitual abortion patients a suitable control regarding embryo implantation in IVF patients.

## Materials and methods

### Patients

Tissue samples were obtained from patients ( $n = 8$ ) who did not achieve pregnancy after undergoing two or more in-vitro fertilization cycles within a year (group I), and from women ( $n = 18$ ) who had had three or more consecutive pregnancies which ended in spontaneous abortion after week 8 of pregnancy (group II).

The study design was approved by the Institutional Ethics Committee (UNIFESP/EPM-CEP no. 799/01) and written consent was obtained from all patients.

### Group I

In the patients of group I, two or more IVF cycles were performed. The long protocol was used, suppression being achieved with napherelin 200  $\mu\text{g}$  nasally twice daily. After ultrasonographic determination of suppression, stimulation started with a daily intramuscular administration of human menopausal gonadotrophin (HMG) (a combination of FSH/225 IU and LH/75 IU). Patients were monitored by ultrasonography, and HMG doses were adapted to their ovarian response after day 6 of stimulation. A microscopically assessed morphological criterion was used to score embryo quality (Giorgetti *et al.*, 1995; Hsu *et al.*, 1999; grade 1 = maximum; grade 5 = minimum), transferring at least one embryo with grade 1 into each patient. Luteal phase was supplemented with intravaginal progesterone, 600 mg/daily. Blood concentrations of  $\beta$ -human chorionic gonadotrophin ( $\beta$ -HCG) were zero on day 15 after transfer, in all cycles, menstruation occurring up to 20 days after transfer.

### Group II

These patients had had three or more pregnancies, diagnosed by plasma concentrations of  $\beta$ -HCG  $>30$  IU/l (measured using the IMx System, Abbott, USA) and ultrasonograph showing a topical gestational sac enclosing an embryo with a heartbeat, in at least one pregnancy. All patients had spontaneous abortions after gestational week 8, characterized by uterine bleeding, with concomitant reduction of plasma concentrations of HCG, and ultrasonographic demonstration of disappearance of the gestational sac. None of the patients underwent curettage.

### Cycle for tissue collection

Blood samples for measurement of plasma concentrations of FSH and prolactin (IMx System, Abbott; normal range: 3.0–20.0 IU/l for FSH and 0.33–27.33 ng/ml for prolactin) were obtained between menstrual cycle days 1 and 5. From cycle day 9 onward, daily transvaginal ultrasonography was performed (ALOKA-500, with a 5 MHz probe), observing endometrial thickness and aspect (Forrest *et al.*, 1988) and follicular diameter (mean of two measurements). The day on which signs of ovulation were observed by ultrasonography (i.e. sudden decrease in follicular size, increase in follicular echogenicity and crenation of the follicular wall; Ritchie, 1986) was considered the first luteal day (L+1). An endometrial biopsy was obtained with a Pipelle de Cornier (Prodimed, Neuilly-en-Telle, France), on day L+7; on the same day, a blood sample was collected for progesterone measurement. Between days 9 and 12 of the next cycle, a hysterosalpingogram was performed in all patients.

### Exclusion criteria

The exclusion criteria used in the present study aimed at selecting only participants who showed morphologically suitable uterine conditions and who did not show any abnormal hormonal conditions. The following results excluded patients from the study: (i) FSH or prolactin plasma concentrations out of the normal range of basal hormone concentrations (normal range: FSH 3.0–20.0 IU/l and prolactin 0.33–27.33 ng/ml); (ii) abnormal ultrasonographic aspect of endometrium; (iii) endometrial thickness  $<8$  mm on the day of ovulation; (iv) absence of ultrasonographic signs of ovulation: sudden decrease in the follicular size; increase in follicular echogenicity; crenation of the follicular wall; (v) progesterone plasma concentrations lower than 7 ng/ml; (vi) menstrual cycle length lower than 25 or higher than 35 days; and (vii) luteal phase lasting less than 12 days.

### Tissue processing

Endometrial samples were washed in cold phosphate-buffered saline solution (PBS; Sigma, St Louis, MO, USA), to remove contaminating blood. Half of the biopsy was immediately embedded in liquid nitrogen-cooled isopentane (Sigma), and stored at  $-70^{\circ}\text{C}$  until assayed for immunohistochemistry. The other half of the biopsy was immediately placed into formalin fixative and processed for routine paraffin embedding. Sections were cut at 5  $\mu\text{m}$ , hydrated and stained with haematoxylin and

eosin for endometrial dating, according to standard procedure (Li *et al.*, 1988).

## Immunohistochemistry

For the immunostaining procedure, samples were embedded in tissue freezing medium (Jung–Leica Instruments, Germany). Cryosections (7 µm) were mounted on slides (coated with 3-aminopropyl-triethoxy-silane; Sigma), air dried, fixed in acetone at –20°C and washed with PBS 0.05 mol/l, pH 7.4. Endogenous peroxidases were blocked with 0.6% hydrogen peroxide in methanol for 5 min, at room temperature, and non-specific binding was blocked with normal goat serum (1:20 in PBS). Thereafter, sections were incubated with polyclonal rabbit anti-human IL-1 receptor type I (primary antibody; Santa Cruz Biotechnology, CA, USA), at 1.6 µg/ml for 60 min, at 37°C. After testing various concentrations of the primary antibody, 1.6 µg/ml was the selected concentration because it allowed an optimal specificity/sensitivity ratio. Slides with different endometrial samples, one half of each study group, were processed at the same time, to maintain the same conditions for both groups.

Control incubations were performed by omitting the primary antibody and using normal goat serum instead.

After rinsing with PBS, pH 7.4, sections were incubated with biotin-conjugated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA, USA), at 18.7 µg/ml, for 30 min, at 37°C, rinsed with PBS, and incubated with ABC reagent (peroxidase conjugated streptavidin) (Dako A/S, Denmark) for 30 min, at 37°C. The chromogen (diaminobenzidine tetrahydrochloride/Tris–HCl with 2% hydrogen peroxide) was used for 5 min. Sections were counterstained with Carazzi's haematoxylin and then dehydrated in graded concentrations of ethanol, cleared in xylene and mounted in Entellan (Merck, Germany).

Immunostaining was assessed by two observers, in a double-blinded manner, by using an arbitrary scale: absent (–), weak (+), and intense (++). In the event of a difference between scores, the section was reanalysed by both observers together with a third reviewer.

## Statistical analysis

Fisher's two-tailed exact test was used for categorical variables and for non-categorical variables Student's two-tailed *t*-test was used. In both cases, significance level was 5%.

**Table 1.** Group I: patients (*n* = 8) with at least two consecutive unsuccessful IVF cycles, five with a diagnosis of bilateral tubal obstruction and three with male partner oligoasthenozoospermia.

Age (years)	Duration of infertility (years)	Number of patients	Number of cycles per patient
32–39	3–6	4	2
		3	3
		1	5

## Photography

Photographs of sections were taken using an Olympus PM30 automatic photomicrographic system mounted on an Olympus BX60 reflected light fluorescence microscope.

## Results

**Tables 1 and 2** show patients in groups I and II who met the inclusion criteria.

Both study groups were comparable for the following criteria: age of patients; normal concentrations of plasma hormones FSH, prolactin and progesterone; similar luteal and follicular phase length; follicle diameter on the day immediately before ovulation; and endometrial ultrasonographic aspect and thickness (**Table 3**).

None of the patients showed endometrial dating delay (**Table 4**); there was no significant difference in endometrial dating between groups I and II.

IL-1RtI was expressed in the endometrial luminal epithelial cells of all patients (**Figure 1a–d**) and in the glandular epithelial cells (**Figure 1b, e**) of seven patients (87%) in group I and seven patients (38.9%) in group II (*P* = 0.0357, two-tailed Fisher's exact test) (**Table 5**). In the cases where the glandular epithelial cells did not express IL-1RtI at all, a clear transition between luminal and glandular cells could be observed (**Figure 1a**). Stromal cells showed positive reaction in two patients in group I and in four patients in group II (**Table 5**).

The immunostaining of IL-1RtI was more intense in the luminal epithelial cells than in the glandular epithelial cells (**Figure 1b**). When observed in detail, luminal epithelial cells showed intense immunostaining in their apical side and fainter staining distributed throughout the cytoplasm (**Figure 1d**).

Immunoreactive IL-1RtI was localized only in the apical side of the glandular epithelial cells (**Figure 1b, e**). In the stroma, only a few isolated cells showed very weak staining (**Figure 1c**).

The immunoprecipitation reaction was negative when antibody was omitted from the medium (**Figure 1f**).

**Table 2.** Group II: patients (*n* = 18) with a history of spontaneous recurrent miscarriage.

Age (years)	Number of patients	Number of miscarriages per patient
25–40	16	3
	2	5

**Table 3.** Comparison of the parameters of cycle evaluation between patients in group I (at least two consecutive unsuccessful IVF cycles,  $n = 8$ ) and group II (spontaneous recurrent miscarriage,  $n = 18$ ).

Parameter	Group I	Group II
Age (years)	35.4 ± 2.50	33.2 ± 3.70
Follicle diameter (mm) <sup>a</sup>	21.3 ± 3.56	19.5 ± 1.33
Endometrial thickness (mm) <sup>b</sup>	10.8 ± 0.91	10.3 ± 0.86
FSH <sup>c</sup> (IU/l)	5.6 ± 1.04	5.6 ± 1.28
Prolactin <sup>c</sup> (ng/ml)	16.2 ± 5.07	16.3 ± 5.31
Progesterone (ng/ml)	16.4 ± 2.42	15.5 ± 3.43
Follicular phase length (day)	15.9 ± 2.73	15.1 ± 2.67
Luteal phase length (day)	13.8 ± 1.13	14.4 ± 1.13

Values are means ± standard deviation. There were no statistically significant differences between the two groups.

<sup>a</sup>On the day immediately before ovulation.

<sup>b</sup>On the day of ovulation.

<sup>c</sup>On day 3 of the cycle.

**Table 4.** Endometrial dating for patients in group I (at least two consecutive unsuccessful IVF cycles,  $n = 8$ ) and group II (spontaneous recurrent miscarriage,  $n = 18$ ).

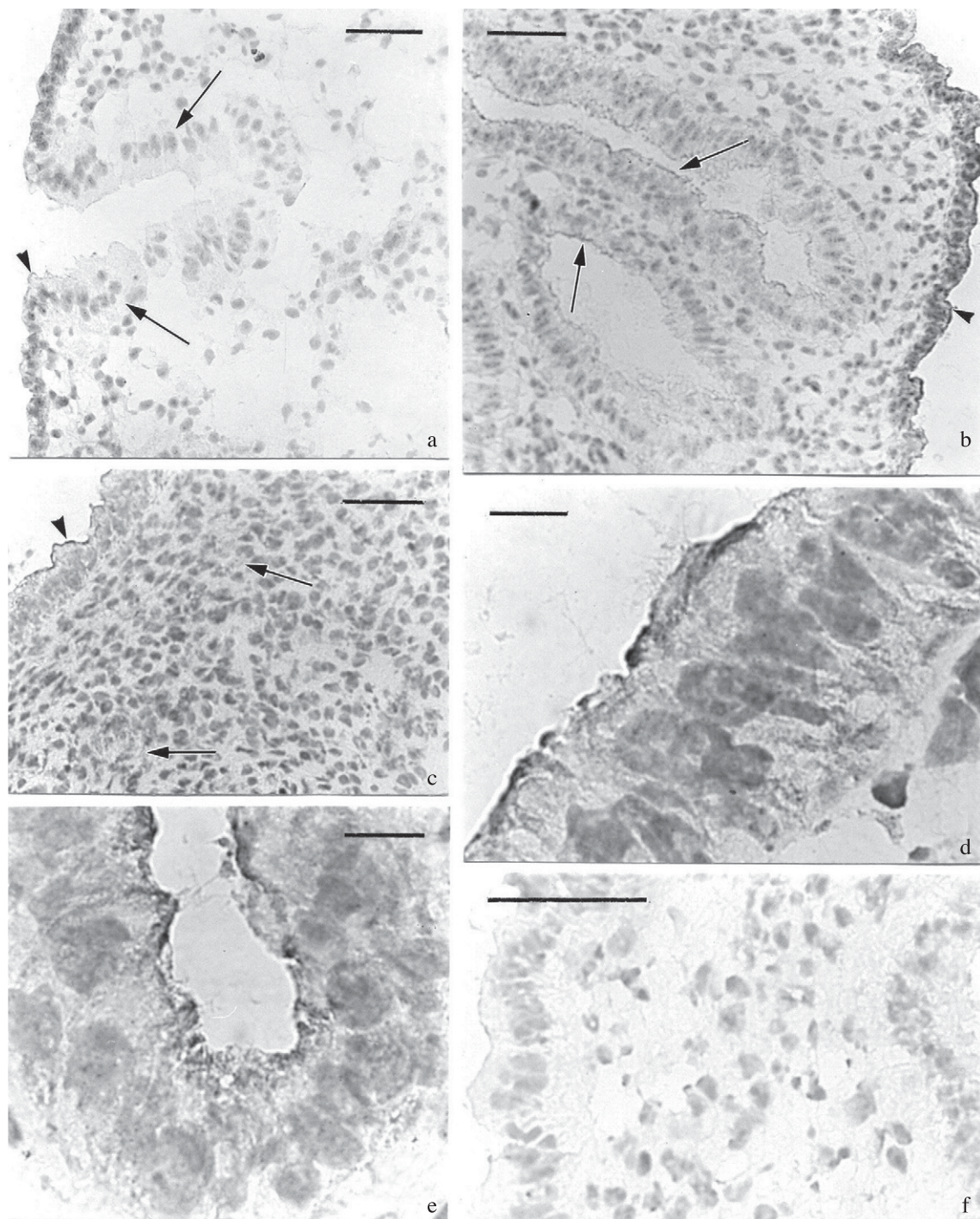
Histological dating (day)	Group I	Group II
6 or 8	2	8
7	6	10

**Table 5.** Interleukin-1 receptor type-1 expression in the endometrial, epithelial and stromal cells of patients in group I (at least two consecutive unsuccessful IVF cycles,  $n = 8$ ) and group II (spontaneous recurrent miscarriage,  $n = 18$ ), as measured by the intensity of immunostaining.

Intensity of staining	Group I	Group II
Luminal epithelial cells		
–	0	0
+	0	0
++	8	18
Glandular epithelial cells		
–	1	11
+	7	7
++	0	0
Stromal cells		
–	6	14
+	2	4
++	0	0

Intensity of staining: – = absent; + = weak; ++ = intense.





**Figure 1.** Immunohistochemical staining for interleukin-1 receptor type-1 (IL-1RtI) in human endometrium using the avidin-biotin-peroxidase (ABC) method. Sections were counterstained with haematoxylin. (a) Luminal epithelial cells show strong positive immunohistochemical staining (dark grey). No staining is detected in the glandular epithelial cells (arrows). Arrowhead = transition between luminal and glandular epithelium. Group II patient,  $\times 285$ . Bar =  $50\ \mu\text{m}$ . (b) Strong positive immunoreaction (dark grey) is detected in the luminal epithelial cells (arrowhead). Glandular epithelial cells (arrows) are weakly immunoreactive. Group I patient,  $\times 285$ . Bar =  $50\ \mu\text{m}$ . (c) Stromal cells exhibit very weak staining (arrows). Luminal epithelial cells show positive immunostaining (arrowhead). Group II patient,  $\times 310$ . Bar =  $50\ \mu\text{m}$ . (d) Higher magnification view of luminal epithelial cells seen in (b). Cells show strong immunostaining in the apical surface and fainter staining throughout the cytoplasm.  $\times 1450$ . Bar =  $10\ \mu\text{m}$ . (e) Higher magnification view of the glandular epithelial cells seen in (b). Cells show positive immunoprecipitation reaction in the apical side.  $\times 1450$ . Bar =  $10\ \mu\text{m}$ . (f) No immunostaining is observed in negative immunohistochemical control. Group II patient,  $\times 580$ . Bar =  $50\ \mu\text{m}$ .

## Discussion

The aetiology of recurrent implantation failure in assisted reproduction is complex and not completely understood. Embryonic aneuploidy, abnormalities of the uterine cavity and altered endometrial receptivity have all been reported as potential causes (Urman *et al.*, 2005).

Urman *et al.* (2005), for example, point out that despite transferring a good quality embryo, pathological lesions of the uterine cavity, the presence of hydrosalpinges, fibroids, endometriosis, or even a less than optimal embryo transfer technique, may be overlooked as causes of implantation failure.

Recurrent miscarriage has been attributed to a host of anatomical, endocrine and immunological abnormalities, although 50% of miscarriages are unexplained (Carrington *et al.*, 2005). Except for genetic factors, neither sporadic nor recurrent pregnancy losses can usually be explained with certainty (Munné *et al.*, 2005).

Abnormal embryo–endometrium dialogue may account for some cases of recurrent implantation failure, over- or under-expressed genes being a particular abnormality (Hoozemans *et al.*, 2004; Urman *et al.*, 2005).

Focusing on the cytokine embryo–endometrium dialogue as a potential cause of unsuccessful IVF treatment of a number of patients referred to the authors' medical school clinic, the expression of IL-1RtI was investigated in the uterine tissue of these patients.

The number of reports on the expression of the cytokines IL-1 themselves regarding both successful and unsuccessful IVF fertilization seems to be very small, and reports on the expression of their receptor, IL-1RtI, are virtually non-existent (Laird, 2006). It was therefore decided to examine the complimentary aspect of IL-1 signalling, its receptor. At the time the present research work was performed, the investigation on IL-1RtI was a first approach, as genetic screening of preimplantation embryos was not sponsored by the institution, which provides all referred patients with free treatment.

Women with spontaneous recurrent miscarriage were chosen for controls, as, in these individuals, pregnancy had gone beyond the embryo implantation phase.

The exclusion criteria aimed at selecting only participants who showed morphologically suitable uterine conditions.

Transvaginal ultrasound examination, which can show uterine intracavitary abnormalities and lesions, was performed in all patients. Patients with uterine morphological problems, hysterosalpingography suggesting abnormalities, poor ovarian reserve (Seifer *et al.*, 1999), hyperprolactinaemia (Alila *et al.*, 1987) and luteal insufficiency (Lessey, 2000) were excluded.

Both study groups were comparable regarding some variables that directly or indirectly play a role in fertilized egg implantation. These groups had normal concentrations of plasma hormones FSH, prolactin and progesterone. Transvaginal

ultrasonographic observations, which also provide convenient monitoring of endometrial and follicular development, showed that both unsuccessful IVF patients and spontaneous recurrent miscarriage controls had similar luteal and follicular phase length, follicle diameter on the day immediately before ovulation, endometrial aspect and thickness.

None of the IVF patients included in this study had a history of recurrent miscarriage but were referred for IVF treatment either because of bilateral tubal obstruction (5 patients) or partner's oligoasthenozoospermia (3 patients).

The selection of embryos for transfer was performed according to the institutional standard procedures based on morphological criteria described by Giorgetti *et al.* (1995) and Hsu *et al.* (1999) (grade 1 representing the best morphological condition: embryo with blastomeres of equal size, without multinucleation and showing no cytoplasmic fragments).

Previous studies have shown that morphological selection of embryos alone is not enough to select against all chromosome abnormalities. Morphologically excellent embryos may show aneuploidy, which may lead to an unsuccessful pregnancy (if any implantation had occurred at all). Prenatal genetic screening (PGS) of preimplantation embryos allows for the selection of euploid embryos, improving the chances of successful implantation and pregnancy (Hoozemans *et al.*, 2004; Munné *et al.*, 2005). PGS is indicated for patients who have recurrent miscarriages (Munné *et al.*, 2005) or a history of failed implantation (Caglar *et al.*, 2005), as well as patients aged 35 or older (Munné, 2005; Munné *et al.*, 2005).

Women between the ages of 35 and 39, the age range of patients and controls in the current study, show a frequency of 30% of chromosome abnormalities in embryos with apparently normal morphology (women aged over 40 years old show 60% frequency) (Munné, 2005).

Certainly PGS would have determined if aneuploidy was the cause of IVF failure in this patient group; however, such genetic tests could not be performed (as mentioned above), leading the researchers to focus the primary aim of the current study on the comparison of the natural endometrial expression of IL-1RtI in IVF patients and controls.

The present immunohistochemical study showed IL-1RtI expressed in the endometrial luminal epithelial cells of all patients, with the immunostaining of IL-1RtI showing more intense in luminal than in glandular cells, the latter even showing no staining in some patients in both groups, more frequently ( $P = 0.0357$ ) in group II. Stromal isolated aggregates also showed some immunostaining for IL-1RtI. These findings concerning epithelial cells and stromal cells in women on stimulated cycles and with recurrent miscarriage, as investigated in this study, were consistent with those reported in fertile women (Simón *et al.*, 1993a) and women with unexplained infertility (Bigonnesse *et al.*, 2001) in their natural cycles.

In the preimplantation phase, IL-1RtI present in the human endometrial epithelium would interact with IL-1 $\alpha$  and IL-1 $\beta$  synthesized and secreted by the embryo (De los Santos *et al.*, 1996; Simón *et al.*, 1998; Kauma, 2000), resulting in the secretion of leukaemia inhibitory factor (LIF) and in increased



expression of  $\alpha_v\beta_3$  integrin (Tabibzadeh *et al.*, 1990; Sawai *et al.*, 1997; Simón *et al.*, 1997) which are favourable to cellular adhesion. Maximal expression of IL-1RtI (Simón *et al.*, 1993a,b), LIF (Lindhard *et al.*, 2002) and  $\alpha_v\beta_3$  integrin (von Wolff *et al.*, 2001) coincides with the implantation window, and these factors might trigger the uterine attachment and implantation of the embryo.

The women in group I exhibited IL-1RtI expression in luminal cells similar to that shown by women with recurrent abortion, which can be taken as individuals with sufficient requirements for embryo adhesion and implantation. Therefore, a similar rate of embryo implantation would be expected in the IVF women. However, not even one case of successful implantation occurred in IVF women, suggesting that the expression of IL-1RtI in the endometrium of infertile patients undergoing IVF treatment is not associated with the patients' unsuccessful embryo implantation.

After embryo implantation, a number of factors are important for embryo development. In mammals endometrial gland secretion plays an important role in fetal growth and viability (Barker and Clark, 1997). The suppression of this glandular secretion in ewes by knocking out their uterine glands proved to compromise the survival and development of the conceptus (Gray *et al.*, 2002).

In humans, placental growth and development is regulated by, among other factors, the interaction of IL-1 produced mainly by the maternal decidua (Kauma, 2000) with the endometrial glandular epithelium IL-1RtI in a paracrine-autocrine way (Simón *et al.*, 1994b, 1996; Martín *et al.*, 2002).

IL-1 and IL-1RtI interaction induces the secretion of LIF and, via  $\beta_3$  integrin, of osteopontin (von Wolff *et al.*, 2001). Besides playing a decisive role in the embryo implantation, LIF has also been shown to support embryo development; for reproduction, the most important site of LIF production is in the endometrial glands (Lindhard *et al.*, 2002). Osteopontin is a component of histotroph that is synthesized and secreted by the endometrial glandular epithelium, which supports conceptus development for at least the first one-third of pregnancy in humans (Johnson *et al.*, 2003).

Fertile women show the expression of IL-1RtI in endometrial glandular epithelial cells during the embryo implantation phase (Simón *et al.*, 1993a).

Interestingly, women with a history of recurrent miscarriage investigated in the present study showed statistically significant ( $P = 0.0357$ ) absence of endometrial glandular epithelial cell expression of IL-1RtI, suggesting that this deserves further investigation in cases of habitual abortion.

In conclusion, the present study could not find a link between unsuccessful embryo implantation and IL-1RtI expression in endometrial luminal epithelial cells in the unsuccessful IVF sample studied. However, an unexpected aspect uncovered in the women with recurrent miscarriage included in this study is that deficient endometrial glandular expression of IL-1RtI seems to play a role in their recurrent miscarriage problem.

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