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Intrauterine insemination of cultured peripheral blood mononuclear cells prior to embryo transfer improves clinical outcome for patients with repeated implantation failures

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Summary

Implantation failure is a major limiting factor in assisted reproduction improvement. Dysfunction of embryo–maternal immuno-tolerance pathways may be responsible for repeated implantation failures. This fact is supported by immunotropic theory stipulating that maternal immune cells, essentially uterine $CD56^{+}$ natural killer cells, are determinants of implantation success. In order to test this hypothesis, we applied endometrium immuno-modulation prior to fresh embryo transfer for patients with repeated implantation failures. Peripheral blood mononuclear cells were isolated from repeated implantation failure patients undergoing assisted reproductive technology cycles. On the day of ovulation induction, cells were isolated and then cultured for 3 days and transferred into the endometrium cavity prior to fresh embryo transfer. This immunotherapy was performed on 27 patients with repeated implantation failures and compared with another 27 patients who served as controls. Implantation and clinical pregnancy were increased significantly in the peripheral blood mononuclear cell test versus control (21.54, 44.44 vs. 8.62, 14.81%). This finding suggests a clear role for endometrium immuno-modulation and the inflammation process in implantation success. Our study showed the feasibility of intrauterine administration of autologous peripheral blood mononuclear cells as an effective therapy to improve clinical outcomes for patients with repeated implantation failures and who are undergoing *in vitro* fertilization cycles.

Keywords: Endometrium immuno-modulation, *In vitro* fertilization and fresh embryo transfer, Peripheral blood mononuclear cells, Repeated implantation failure

Introduction

In human-assisted reproduction, *in vitro* fertilization (IVF) success rates are strongly dependent on embryo implantation, which occurs in only 20–30% of cases after fresh embryo transfer. Moreover, about 10% of patients have unexplained repeated implantation failures (RIF) after several IVF treatments. Understanding and mastering the molecular mechanisms that govern this key phase of human reproduction would therefore improve significantly the IVF success rate and provide a therapeutic response to numerous RIF cases.

At present, many hypotheses have been put forward to explain RIF, one of these is the exploration of immuno-physiological aspects of implantation and

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pregnancy for these kinds of patients and also to elucidate maternal–fetal immuno-tolerance mechanisms, based on the fact that the embryo is a semi-allogenic conceptus to which the maternal immune system should become adapted (Hill *et al.*, 1995). Therefore, it appears likely that any dysfunction that affects the maternal immune response could explain the RIF for the same patient. Nevertheless, the maternal immune system should modulate implantation and pregnancy more by local mechanisms gathered at the maternal–fetal interface rather than by systemic mechanisms across the organism.

Indeed, it is accepted today that implantation much depends on endocrine mechanisms widely described as immune local mechanisms involving a broad spectrum of cytokines. A real cross-talk, particularly rich and complex and not yet elucidated, is established between endometrium and embryo even before and during implantation through a myriad of molecules such as cytokines, chemokines, growth factors, adhesion molecules etc. (Johnson *et al.*, 1999). This accurate and organized cross-talk involves maternal immune cells, particularly lymphocytes.

In fact, early before implantation, semen presence in the uterus causes an influx of T lymphocytes and macrophages that initiate uterine inflammatory conditions suitable for implantation. Later, natural killers (NK) cells begin to accumulate in the endometrium during the latter middle period of the menstrual cycle (Bulmer *et al.*, 1991; King, 2000). These large granular lymphocytes called uterine NKs (uNKs) are phenotypically different from peripheral NKs (pNKs) as they are characterized by the expression of CD56, but do not express CD16 and CD3 (Bulmer & Lash, 2005). At the time of implantation these cells are the most represented immune cells at the maternal–fetal interface and are 50–90% of all decidual leukocytes, while pNKs represent only 5–15% of circulating leukocytes (Parham, 2004). Infiltration of uNK cells is clearly influenced by sex hormones, particularly progesterone. Nevertheless, it has been reported that endometrium-derived IL15 and IL11 have also been implicated in proliferation and differentiation of these cells (Ashkar *et al.*, 2003; Becknell & Caligiuri, 2005). This increase in uNK cell levels in the uterus suggests that they play an important role in the implantation mechanism (Saito *et al.*, 2007). The endometrium uNK rate seems to be correlated with IVF success rate (Johnson et al., 1999) and therefore should be a good indicator of predictive IVF success.

Many studies have investigated the uNK contribution and action mechanism in implantation. It has been shown that uNKs control endometrium decidualization and vascularization through vascular endothelial growth factor (VEGF), placental growth factor (PLGF), leukaemia inhibitory factor (LIF) and interferon (IFN) secretion (Kimber, 2005; Croy *et al.*, 2003). They participate in embryo–maternal interactions during implantation and fetal development by decidual spiral artery remodelling to provide the embryo with the necessary nutrients (Ashkar *et al.* 2003; Hanna *et al.* 2006; Fettback *et al.* 2009; Lash & Bulmer, 2011).

It has been reported that the presence of lymphocytes during implantation contributes, in concert with the endometrium and blastocyst, to cytokine production, giving a profile that is dominated by Th1 proinflammatory cytokines: tumour necrosis factor (TNF), IFNγ, LIF, IL1, IL2, IL6, IL12 and IL15. The presence of these factors as inflammatory response actors seems to be essential for early stages of implantation (Kachkache *et al.*, 1991; McMaster *et al.*, 1992; Sanford & Wood, 1992; Stewart *et al.*, 1992). However, the initial inflammatory response must be selectively attenuated thereafter for maintenance of pregnancy (Chaouat *et al.*, 2007), that is why too early an inflammatory deficit would lead rather to embryonic implantation failure, while inflammatory excess leads to acute (spontaneous miscarriage) or chronic (pre-eclampsia, delayed intrauterine growth) rejection.

Conversely, the maintenance of pregnancy would necessitate a specific cytokine profile, characterized by the predominance of Th2 cytokines: IL3, IL4, IL10, IL5, IL13 and granulocyte–macrophage colony-stimulating factor (GM-CSF) that cause an anti-inflammatory state that is adequate for uterine receptivity and, therefore, for pregnancy (Miyazaki *et al.*, 2003; Challis *et al.*, 2009; David Dong *et al.*, 2009; Nagamatsu & Schust, 2010; Mor *et al.*, 2011).

Thus, the maternal immune system seems to be involved in the establishment and maintenance of pregnancy through the Th1/Th2 profile balance. An imbalance between these two systems could explain implantation failures in some patients (Wegmann *et al.*, 1993; Darmochwal *et al.*, 1999; Raghupathy *et al.*, 1999). In the mouse model, it has been demonstrated that embryo rejection is controlled by the Th1 system while the Th2 system plays a protective role (Vassiliadis *et al.*, 1998), knowing that autoimmune factors are the cause of 30% of human miscarriages (Pandey *et al.*, 2005). Many studies have explored the ability of T lymphocytes and uNKs to control the Th1/Th2 balance and it seems increasingly clear that uNK is much more relevant than other cells in the establishment of the Th1 cytokine profile.

Implantation failure is a main limiting factor in assisted reproductive technologies (ART) and many authors have shown clearly the contribution of cytokines and other factors to endometrium immunomodulation to improve ongoing pregnancy (Makrigiannakis *et al.*, 2006, 2008, 2011; Toth *et al.*, 2011; Hutter *et al.*, 2013). It has been estimated that inadequate

Table 1 Comparison of patient characteristics included in the study with at least 2 RIFs

Characteristic	PBMC test ($n = 27$)	Control ($n = 27$)	P-value
Age of the partner	42.56 ± 6.90	41.67 ± 5.41	0.60 (ns)
Age of the patient	34.74 ± 4.17	34.44 ± 3.86	0.79 (ns)
Number of RIFs	3.19 ± 1.75	3.63 ± 1.76	0.36 (ns)
AMH (ng/ml)	2.25 ± 1.48	1.98 ± 1.10	0.45 (ns)
Estradiol (pg/ml)	33.67 ± 17.88	36.63 ± 16.20	0.53 (ns)
Progesterone (ng/ml)	0.53 ± 0.17	0.47 ± 0.24	0.25 (ns)
Endometrial thickness (mm) on day of oocyte retrieval	9.91 ± 1.51	10.22 ± 1.65	0.47 (ns)

endometrial receptivity is related in up to two-thirds of cases (Simon *et al.*, 1998). As immune cells are known to modulate endometrial receptivity through a large panel of cytokines, it seemed important in patients with RIF to investigate the benefit on embryo implantation rate of intrauterine insemination (IUI) of their own lymphocytes *in utero* at the preimplantation stage. *In vitro* experiments in mice have demonstrated an increased embryo implantation rate with human peripheral blood mononuclear cells (PBMC) (Nakayama et *al.*, 2002; Yu *et al.*, 2014). Many recent studies have investigated this innovative immune approach to the implantation mechanism based on the hypothesis that, in RIF patients, lymphocyte recruitment is not able to induce an uterine inflammatory condition suitable for implantation (Nakayama et *al.*, 2002; Yoshioka *et al.*, 2006; Zhylkova *et al.*, 2010; Okitsu *et al.*, 2011; Yu *et al.*, 2014).

The practice of immuno-modulation techniques to improve implantation and pregnancy could be an interesting prospective therapeutic method for ART as reported by several studies (Yoshioka *et al.*, 2006; Zhylkova *et al.*, 2010; Okitsu *et al.*, 2011) and should even revolutionize the conventional IVF protocols actually practiced.

The aim of this work is to understand how PBMC culture and intrauterine transfer on the day of ovulation induction can modulate implantation rate in patients with RIF in order to improve pregnancy. This preliminary study was clearly beneficial to our patients and needs to be continued and developed for more improvement.

Materials and methods

Patients' selection

This is a prospective randomized study design of parallel group trials with the same size using block randomization. It includes a sample of 54 patients selected within the Anfa Fertility Center and spread over 1 year. Selected women were assigned randomly to receive autologous PBMCs cultures by an intrauterine non-invasive insemination 2 days before embryo transfer representing the PBMC test or no treatment group without receiving any cell transfer prior to embryo transfer to serve as control. The effect of immunotherapy PBMCs was evaluated by the implantation rate and clinical pregnancy. Each patient contributed a single cycle to this study, so there was no crossover between groups. As anticipated, PBMC test and control groups were comparable (Table 1). After the study recieved ethical approval, patients provided written informed consent.

All patients without exception were selected on the following inclusion–exclusion criteria:

- Inclusion criteria: at least two previous failures of implantation after IVF/intra-cytoplasmic spermatozoa injection (ICSI) (mean = 3), primary infertility, endometrial thickness < 6 mm in ovulation induction, age < 40 years (mean 35 years) (Table 1), regular menstrual cycles, BMI < 30, absence of uterine pathology and infectious negative balance.
- Exclusion criteria: polycystic ovary syndrome and uterine pathology.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. All patients who participated in this study signed an informed consent after being informed about the terms and issues of study.

Stimulation protocol

In order to eliminate the effect of protocol and better align the sample and follicular cohort, it was preferable to use the antagonist protocol using r-FSH (Cetrotide 0.25 and Gonal-F) (Fig. 1). Further r-FSH administration (Gonal-F; Serono Laboratories, Saint Cloud, France) was started by daily subcutaneously injections (150–225 IU/day), which were maintained constantly for 5 days and were adjusted according

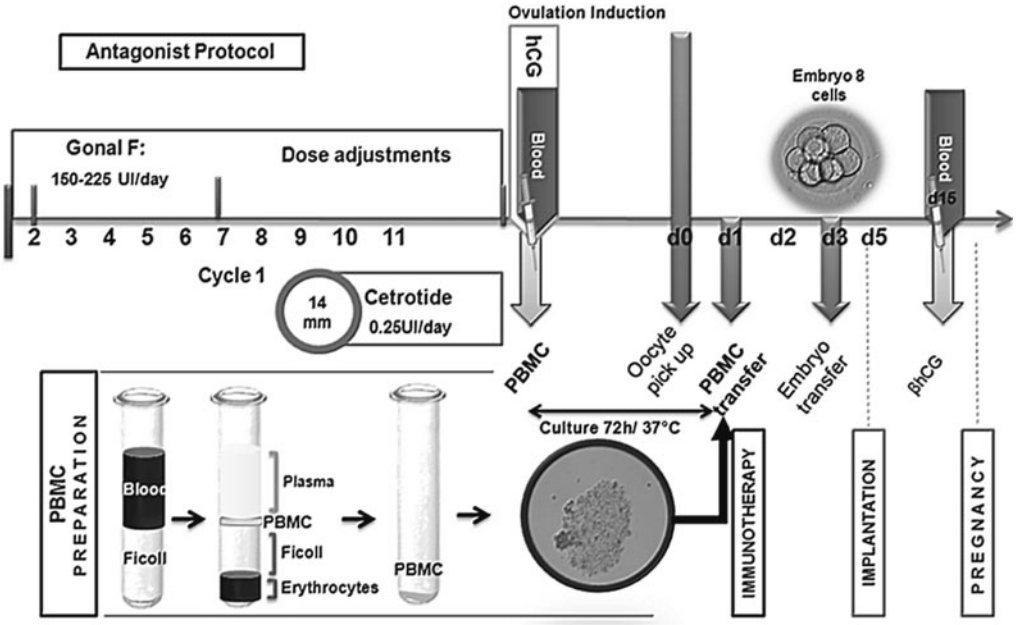

Figure 1 Immunotherapeutic strategy by transferring peripheral blood mononuclear cells (PBMCs) in patients with repeated implantation failures (RIF). After antagonist ovarian stimulation protocol, a blood sample is scheduled on the day of ovulation induction in order to isolate PBMCs using a separation protocol based on Ficoll (PREPARATION PBMC). PBMC are well prepared after a culture for 72 h and then transferred to the patient *in utero* at D1 (immunotherapy).

to usual parameters of follicle growth determined by serum estradiol concentrations and ultrasound monitoring. Ultrasound scans were performed to evaluate the number and sizes of early antral follicles and to calculate mean ovarian volume. When at least one follicle exceeded 14 mm in diameter, a potent, third-generation GnRH antagonist, cetrorelix acetate (Cetrotide, 3 mg; Serono Laboratories), was administered subcutaneously (0.25 IU/day). An intramuscular injection of 10,000 IU of human chorionic gonadotrophin (HCG; Gonadotrophines Chorioniques Endo®, Organon) was performed after obtaining follicles ≥ 17 mm. Luteal phase was supported by vaginal administration of micronized progesterone 400 mg/day (Utrogestan®, Besins International, Montrouge, France) from the day of oocyte pick-up to the day of the pregnancy test. If a pregnancy occurred, progesterone administration was extended until evidence of fetal heart activity at ultrasound was found.

Collection, culture and transfer of PBMCs

Each sample of blood was taken from RIF patients undergoing ART cycles on the day of ovulation induction and it was collected in citrated tubes to perform a separation based on Ficoll-Hypaque solution with a volume of 3 ml (Histopaque®-1077; Sigma). After centrifugation at 18–20°C for 30–40 min at 400 *g*, we obtained a distinct layer of PBMC under the median plasma (Fig. 1). The PBMC layer was transferred to another tube and three volumes of phosphate-buffered saline (PBS) was added and then centrifuged at 18–20°C and 60–100 *g* for 10 min to obtain a PBMC pellet. The lymphocyte pellet was washed twice with PBS and then resuspended at 37°C in complete culture medium ready for use (supplied by ATL R & D laboratory. 78 320 La Verrière France) with 75 IU of HMG-Menoupur®. After 72 h of incubation, 1×10^6 cells in 0.4 ml were transferred into the endometrium cavity 2 days prior to embryo transfer.

During the 3 days of PBMC incubation, special attention was paid to monitoring the culture including the convergence of lymphocyte cells PBMC forming clusters of cytoplasmic processes (Fig. 2), this criteria is crucial for transfer PBMC efficiency. Therefore, two patients were excluded from the study due to unsatisfactory PBMC culture.

IVF/intra-cytoplasmic spermatozoa injection (ICSI) protocol

Follicles were collected 35–36 h post HCG injection. The oocytes obtained were denuded by hyaluronidase (hyaluronidase, LifeGlobal). After washing in buffered medium (Ferticult, JCD, France), oocytes were placed in a Petri dish that contained culture medium (Global®, LifeGlobal) and subsequently transferred to a box containing sperm microinjection immobilized in polyvinylpyrrolidone (PVP, LifeGlobal). Under a

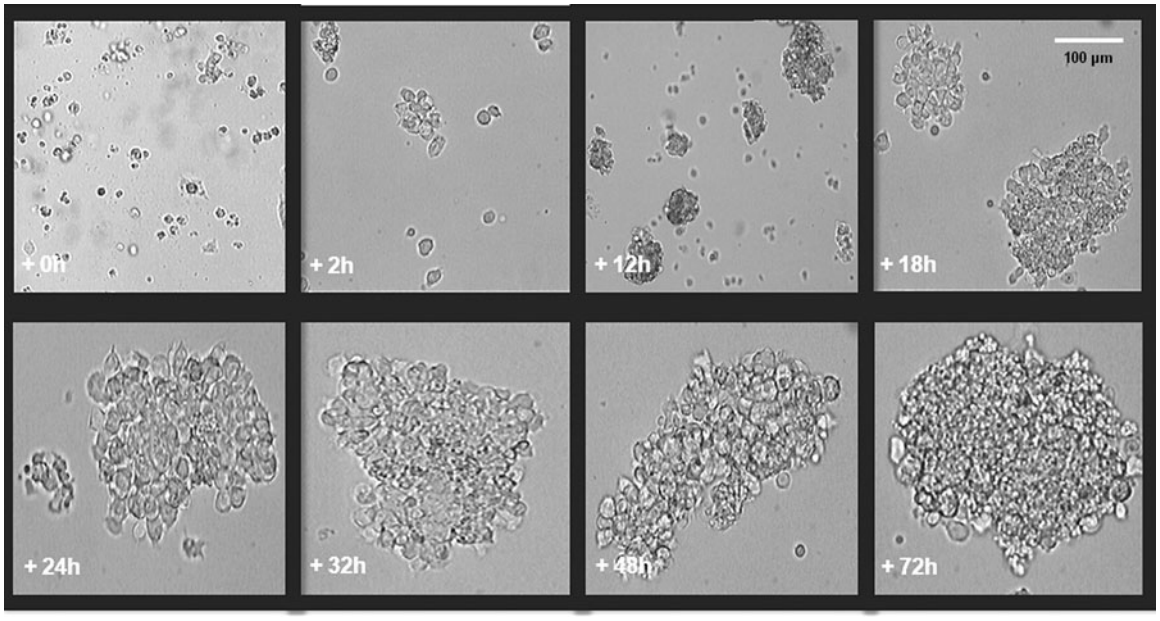

Figure 2 Photomicrograph (original magnification ×40; scale bar, 100 µm) of peripheral blood mononuclear cells (PBMCs) cultures isolated from blood samples of patients. After a separation phase using protocol based on Ficoll, PBMCs are in isolated mononuclear cells and inactive form (0 h). Throughout incubation (72 h, 0 h), cells are activated and thus have a more granular appearance to bind them and increase convergence more condensed form clusters.

Table 2 Comparison of patient embryological outcomes included in the study with at least 2 RIFs

Embryological outcomes	PBMC test ($n = 27$)	Control ($n = 27$)	*P*-value
Total number of oocytes	247	295	0.26 (ns)
Number of oocytes per patient	9.15 ± 3.72	10.93 ± 7.16	0.26 (ns)
Maturation rate	(167/247) 67.61%	(183/295) 62.03%	0.42 (ns)
Fertilization rate	(109/167) 65.27%	(124/183) 67.76%	0.97 (ns)
Cleavage rate	(109/109) 100%	(123/124) 99.19%	0.32 (ns)
Number of embryos per patient	4.04 ± 1.76	4.56 ± 3.50	0.49 (ns)
Number of good quality embryos (A+B) per patient	2.26 ± 1.02	2.59 ± 2.10	0.46 (ns)
Total number of embryos transferred	65	58	0.49 (ns)
Number of embryos transferred per patient	2.41 ± 0.64	2.15 ± 0.60	0.13 (ns)

microscope, ICSI sperm is injected by pipette into the oocyte injection held in place with a drop of culture medium (Global®, LifeGlobal) through a contention pipette. The microinjected oocytes are cultured in microdroplets of 50 µl IVF30 medium (VitroLife) in each box in a 60 mm Nunc dish, covered with NidOil (Nidacon), then incubated in an oven at 37°C and in 5% CO_2 in air. Thereafter, fertilization was confirmed by observation of the second polar body expelled and two pronuclei (2PN) in the oocyte cytoplasm. Embryos were kept in culture until the day 3 and their quality was evaluated in order to transfer the two best day 3 embryos into the uterus of the patient-treated PBMC or not. Adequate embryo quality was defined as embryos that had uniformly sized and shaped blastomeres, embryos with ooplasm that had no granularity and a maximum fragmentation of 10% were considered good quality embryos (A + B). Depending on the patient, one, two or three embryos were transferred *in utero* using a Frydman catheter (CCD Laboratories, Paris, France) (Table 2).

Management of clinical pregnancy

The success of implantation is estimated by ultrasound via the observation of the embryo sac. The occurrence of clinical pregnancy is controlled by observing by ultrasound the development of embryo after 6–8 weeks.

Statistical analysis

Data were presented as mean ± standard deviation (SD) or standard number representing the total. Thus, these data were analyzed by Student's

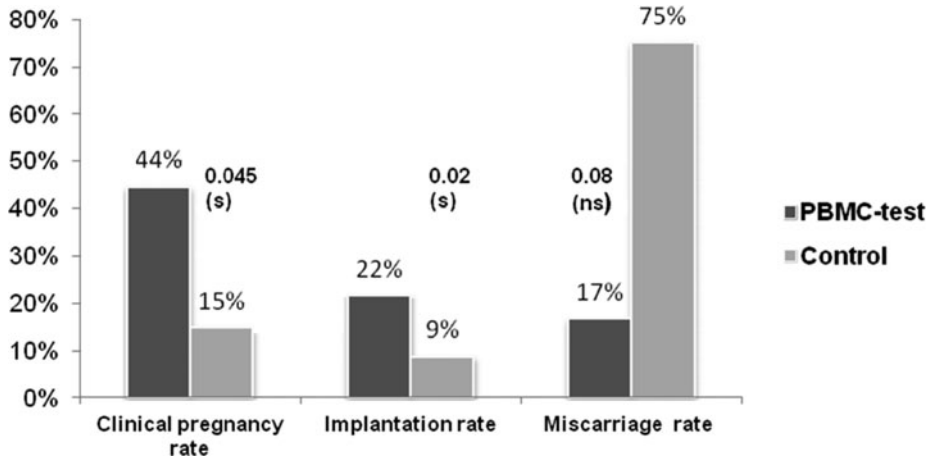

Figure 3 PBMC immunotherapy effect on clinical outcomes of patient with at least two RIFs. Note: Results are expressed as *n*, *n*(%). A statistically significant difference is considered when $P < 0.05$ (s). $P > 0.05$ is not significant (ns). Implantation rate is expressed by calculating the total number of embryonic sacs by total number of transferred embryos.

t-test for comparison of mean values or chi-squared test for comparison of percentages using the Statistical Package, Statistica (version 6.0) to compare significantly different populations: a *P*-value < 0.05 indicates significant difference. Then, mean values of clinical outcomes were evaluated to calculate study power by post-hoc test using G*Power software (version 3.0.10).

Results and Discussion

This study shows that *in utero* administration of autologous PBMC in patients with at least two RIF at improves implantation rate significantly (21.54 vs. 8.62%) compared with untreated patients and also the clinical pregnancy rate (44.44 vs. 14.81%) (Fig. 3). Such immunotherapy had no significative effect on the miscarriage rate of these patients when implantation has occurred, which could be a consequence of chromosomal disorders.

Our results are in agreement with the immunotropic theory, which stipulates that maternal immune cells positively influence implantation, placental and fetal growth. However, the precise mechanism of PBMC action is still unclear.

One of the hypotheses we can postulate for these patients with RIF is a poor recruitment of their lymphocytes at the endometrial level, which could be due to inadequate or insufficient endometrial signals. Therefore, in those patients, the inflammatory endometrial state usually required for implantation success should be affected. Then, uterine PBMCs supplementation 2 days before embryo transfer should compensate the endometrial initial inflammatory deficit.

Many studies complement our hypothesis, showing that uNK and T lymphocyte rates are decreased in patients with unknown infertility reporting a dysregulation of cytokines production (Klentzeris *et al.*, 1992; Fukui *et al.*, 2006; Karami *et al.*, 2012; Chernyshov *et al.*, 2010; Sacks *et al.*, 2012; Zhou *et al.*, 2012; Dons'koi *et al.*, 2013a,b). Furthermore, an interesting and recent study demonstrated that PBMCs intrauterine administration had no effect on implantation rate in patients with only one IVF failure (34 vs. 33%) but significantly increased in patients with at least three IVF failures (42 vs. 25%) (Okitsu *et al.*, 2011).

Clinical best practice guidelines stipulate to look into possible underlying causes that may necessitate therapeutic investigation only after three implantation failures have taken place, and only if four good quality embryos of fresh or frozen cycles have been transferred minimally in women under the age of 40 (Coughlan et *al.*, 2014). However, RIF as a clinical entity is yet to have a universally accepted definition but it refers to repeated failure in achieving clinical pregnancy. We, therefore, carried out a comparative analysis of the PBMC-treatment effectiveness as a function of the number of RIFs (e.g., two RIFs vs. three RIFs), as depicted in Table 3.

Clinical pregnancy rates strongly increase in patients with at least three RIFs as compared with control patients (70 vs. 29%; Fig. 4), in contrast with a lack of a significant difference between implantation and miscarriage rates in the same patients.

Table 3 Comparison of patients' characteristics in the two RIFs subgroup and the three RIFs at least subgroup

Characteristics	Two RIFs ($n = 17$)	Three RIFs at least ($n = 10$)	P-value
Age of the patient	33.82 ± 4.03	37.20 ± 2.57	0.03 (s)
Number of RIFs	2	4.50 ± 1.96	—
Total number of embryos transferred	40	23	0.84 (ns)
Number of embryos transferred per patient	2.35 ± 0.49	2.30 ± 0.82	0.84 (ns)
Number of good quality embryos (A + B) per patient	2.41 ± 1.12	1.80 ± 0.79	0.14 (ns)

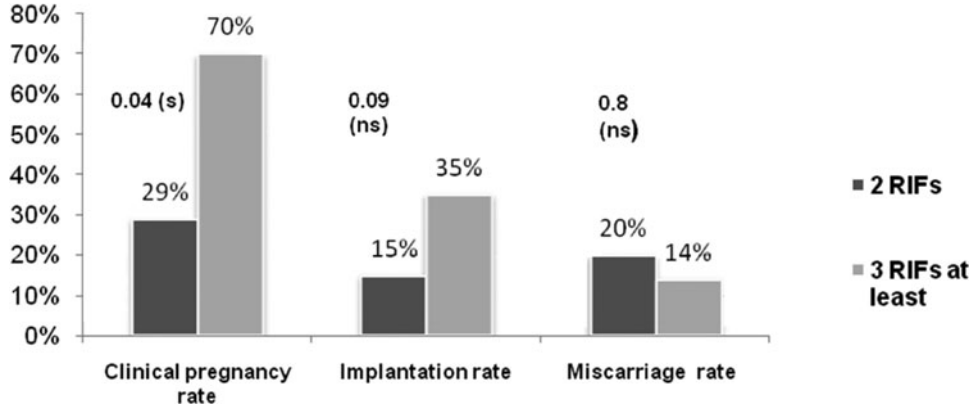

Figure 4 Comparison of PBMC immunotherapy effect on clinical outcomes between two RIFs patients subgroup and at least three RIFs patients subgroup. Note: Results are expressed as n, n(%). A statistic significant difference is considered when $P < 0.05$ (s). $P > 0.05$ is not significant (ns). Implantation rate is expressed by calculating the total number of embryonic sacs by total number of transferred embryos.

Table 4 Characteristics of three RIFs at least patients subgroup

Characteristics	PBMC test ($n = 10$)	Control ($n = 17$)	P-value
Age of the patient	37.20 ± 2.57	34.65 ± 3.76	0.07 (ns)
Number of RIFs	4.50 ± 1.96	4.59 ± 1.54	0.90 (ns)
Total number of embryos transferred	23	37	0.64 (ns)
Number of embryos transferred per patient	2.30 ± 0.82	2.18 ± 0.53	0.64 (ns)
Number of good quality embryos (A + B) per patient	1.80 ± 0.79	2.65 ± 2.26	0.27 (ns)

In addition, our study showed that when analyses were restricted to patients with at least three RIF ($n = 27$), which included 10 patients from the PBMC test versus 17 patient controls (Table 4), clinical pregnancy and implantation rates were approximately tripled in these treated patients compared with the controls (70, 35 vs. 24, 14% respectively) (Fig. 5), agreeing with Okitsu *et al.*'s (2011) findings. In addition, a significant five-fold decrease in miscarriage rate could be noted (14.29% in PBMC test vs. 75% in control ($P = 0.048$) and power $1 - \beta = 84\%$) compared with patients with at least two RIFs . Generally, other studies also support our observations (Nakayama et *al.*, 2002; Zhylkova et *al.*, 2010), confirming the effectiveness of our immunotherapy treatment in the patients who suffered from multiple location failures probably caused by insufficient presence of Th1 cytokines or from high miscarriages rate induced by Th2/Th1 deficit.

Those observations were supported by the statistical evaluation of our study power, which was about 66% for all patients and 74% in the three RIFs subgroup. Thus, our implantation and clinical pregnancy rates showed great improvements over previously published studies and were 59% for two RIFs and 81% for three RIFs (67% for Yoshioka *et al.*, 2006 and 40% for Okitsu *et al.*, 2011) (Table 5).

Although the observed effects are in all likelihood due to the transferred PBMC, we could not totally

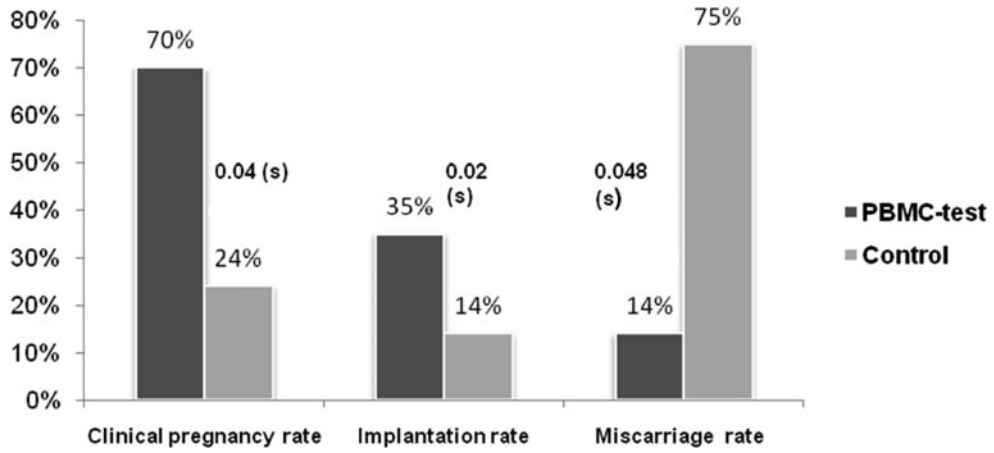

Figure 5 PBMC immunotherapy effect on clinical outcomes in the at least three RIFs patients subgroup. Note: Results are expressed as *n*, *n*(%). A statistically significant difference is considered when $P < 0.05$ (s). $P > 0.05$ is not significant (ns). Implantation rate is expressed by calculating the total number of embryonic sacs by total number of transferred embryos.

Table 5 Statistic power evaluation of different PBMC immunotherapy studies on clinical outcomes

	Our study	Our study's subgroup three RIFs at least	Yoshioka *et al.* (2006)	Okitsu *et al.* (2011)
Study characteristics				
Sample size (PBMC-treated vs. control)	27 vs. 27	10 vs. 17	17 vs. 18	19 vs. 32
Number of RIFs at least	2	3	4	3
Embryo transfer of IVF-cycle type	Fresh embryo D3	Fresh embryo D3	Fresh blastocyst D5	Frozen embryo D3
Clinical outcomes (PBMC-treated vs. control)				
Clinical pregnancy rate	44% vs. 14% ($1-\beta = 79\%$) s	70% vs. 24% ($1-\beta = 83\%$) s	41% vs. 11% ($1-\beta = 75\%$) s	42% vs. 16% ($1-\beta = 53\%$) s
Implantation rate	21% vs. 8% ($1-\beta = 39\%$) s	35% vs. 14% ($1-\beta = 78\%$) s	23% vs. 4% ($1-\beta = 59\%$) s	25% vs. 9% ($1-\beta = 28\%$) s
Miscarriage rate	17% vs. 75% ($1-\beta = 79\%$) ns	14% vs. 75% ($1-\beta = 62\%$) s	—	—

exclude the possibility that the increased implantation rates in treated patients could be due to possible endometrial inflammation caused by the intrauterine devices used to deliver the PBMCs. While this can be controlled in women that undergo the same procedure minus the PBMCs, such a practice is ethically controversial and was not proposed in similar studies (Yoshioka *et al.*, 2006; Okitsu *et al.*, 2011). Furthermore, others have shown that uterine mechanical stimulation prevents implantation in women (Rivera *et al.*, 1999), and intrauterine devices in rats evoked a minor inflammatory reaction that did not induce implantation (Schulten *et al.*, 1975). Together, it seems improbable that the increased implantation rates observed in our study would be attributable to uterine mechanical stimulation.

In the context of our study, it was interesting to investigate how administrated PBMCs could be activated to produce cytokines. It is well known that leukocyte activation begins during diapedesis when leukocytes move through blood vessels to colonize tissues. Furthermore, leukocytes are exposed *in uteri* to many signals that emanate from the endometrium and blastocyst. In our IVF protocol, it seems evident that many of these activating signals are short-circuited as *in vivo* embryo migration in maternal genital tracts does not occur. However, it is well established that PBMC, especially NK and monocytes, have the property to be activated by contact with their *in vitro* culture support. Therefore, we can easily suppose that, in our study, administered PBMCs were activated during the 3 days of *in vitro* culture knowing that

after 24 h of PBMCs culture, IL1α, IL1β and IL8 transcripts are abundant (Ideta et *al.*, 2010). In addition, PBMCs in our protocol stay *in uteri* for 2 days before embryos transfer and then can be submitted to various activating endometrial signals to produce cytokines.

When transferred *in utero*, PBMCs can modulate implantation quality using direct or indirect pathways by cytokine and growth factor secretion or by acting on adhesion proteins. It has been shown that uNKs but not pNKs produce at implantation specific cytokines such as GM-CSF, CSF1, IL2, IFNγ and LIF (Jokhi *et al.*, 1994; King *et al.*, 1995; Ideta *et al.*, 2010; Yu *et al.*, 2014). This observation suggests an important local action for these maternal immune cells to promote implantation. It is now well established that uNKs induce endometrial decidualization phenomena in order to prepare implantation through LIF secretion (Kimber, 2005; Yu *et al.*, 2014) and stimulate angiogenesis in the decidua (Croy *et al.*, 2002; Hanna *et al.*, 2006; Oh & Croy, 2008) remodeling endometrial spiral arteries by VEGF, PLGF and angiogenic substances (Segerer *et al.*, 2009; Lash & Bulmer, 2011; Yu *et al.*, 2014). Furthermore, VEGF concentration peaked significantly in first-trimester decidua and seemed indispensable for implantation success and the maintenance of pregnancy (Salamonsen, 2003; Segerer *et al.*, 2009).

Aberrant maternal immune responses and an unbalanced cytokine profiles specially the ones secretes by uNKs are influencing feto-maternal immune tolerance during implantation and could induce RIF or even recurrent pregnancy losses (Makrigiannakis *et al.*, 2006, 2011; Toth *et al.*, 2011). Patients with miscarriages present over-expression of IL15 which be related to vascularization with consecutive placental and fetal rejection (Toth *et al.*, 2010).

uNKs also promote an increase in decidual monocyte-attracting chemokines secretion, especially GM-CSF, which decreased level has been clearly correlated with implantation failure rate in RIF patients (Perricone *et al.*, 2003). In such patients, embryos cultures supplementation with GM-CSF demonstrated an increase in implantation rate or even included in the basic treatment for RIF patients for immune modulation (Ziebe *et al.*, 2013; Toth *et al.*, 2011).

The function of uNK cells includes more than cytokine production; they contribute to regulate trophoblastic invasion through cytotoxicity, which is controlled by activating signals and inhibitory receptors. Excessive cytotoxicity is known to provoke implantation failures. However, in this study, PBMC supplementation has a positive effect on implantation rate so it is not very probable that their action includes, at least in this protocol, an increase in cytotoxicity. An interesting study has shown that most uNK cells are considered to be cytokine-producing cells rather than cytotoxic cells (Tabiasco *et al.*, 2006). It is interesting to note that once implantation has occurred in patients with two RIFs, we found no significant effect of immunotherapy on miscarriage rate. This result suggests that PBMC transfer has an effect only when the implantation process fails. Thus immunotherapy appears to be ineffective in patients with RIF caused by an early dysregulation in the maintenance of pregnancy. Patient with unexplained recurrent spontaneous abortions (URSA) probably present with over-activation of cytotoxic uNKs characterized by up-regulation of CD69stim and CD158a as reported elsewhere (Chernyshov *et al.*, 2010; Sacks *et al.*, 2012; Dons'koi *et al.*, 2013a,b; Wu *et al.*, 2014). In these patients, another therapeutic strategy needs to be developed.

In this study, we have not considered the PBMCs-T lymphocytes fraction role, as it is known that there is no adhesion of these cells in *in vitro* culture support in contrast with NKs and monocytes. This reason is why T lymphocytes are probably eliminated during culture cell washing.

Another action of PBMCs on implantation and clinical pregnancy rates in our patients may be mediated by macrophages. Maternal macrophages represented 30–35% of all decidual leukocytes in first-trimester deciduas (Bulmer *et al.*, 1988) and should be implicated through their cytokine secretion in the control of trophoblastic invasion (Abrahams *et al.*, 2004). Furthermore, uNK activation requires IL2 produced by macrophages and macrophage interactions through the NKG2D signalling pathway (Basu *et al.*, 2009). Indeed, decidual macrophages are considered as local immuno-modulators during implantation and eventually essential for normal pregnancy with abundance of M2 phenotype. Conversely, hypothetically, the over-expression of Fas ligand (FasL) as proapoptotic cytokine by M1 phenotype induced trophoblast apoptosis in interaction with decidual corticotropin-releasing hormone (CRH) (Guenther *et al.*, 2012; Kalantaridou *et al.*, 2007). Fas/FasL and petformin expressed by trophoblasts play a crucial role in feto-maternal immune tolerance during implantation (Makrigiannakis *et al.*, 2008). For implantation, CRH is initially expressed in the endometrium to finely control stromal cell decidualization; for maintenance of pregnancy, CRH is produced more by the placenta following urocortin action (Zoumakis *et al.*, 2009). However, the increase in the decidual macrophages population is present in patient with spontaneous miscarriages and other gynaecological disorders as pre-eclampsia and endometriosis with over-expression of G-protein-coupled oestrogen receptor (GPER) (Guenther *et al.*, 2012; Heublein *et al.*, 2012; Hutter *et al.*, 2013).

Our results support the hypothesis that in RIF patients lymphocytes recruitment is not able to

induce uterine inflammatory condition suitable for implantation. As this uterine infiltration of uNK cells is clearly influenced by endometrial cytokines secretion we can suppose that RIF patient endometrium has inadequate cytokine secretion, especially IL15 and IL11, which are known to be implicated in proliferation and differentiation of uNKs (Ashkar *et al.*, 2003; Becknell & Caligiuri, 2005).

Thus, *in utero* administration of autologous PBMCs increases implantation rate in patients with RIF should be an effective therapeutic response to implantation repeated failures in order to improve classical IVF protocols success rate knowing that implantation is the most determinant mechanism in ART.

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