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Intrauterine administration of paternal and maternal peripheral blood mononuclear cells mix as solution for repeated implantation failure

Hanen Elloumi[1], Mariem Ben Khelifa[1], Sonia Mnallah[1], Mohamed Khrouf[1], Sabrine Rekik[2], Fethi Zhioua[2], Moncef Ben khalifa[3], Marouen Braham[2], Mohamed Jemaà[4,5] and Khaled Mahmoud[1]

[1]FERTILLIA ART Center, Clinique La Rose, Tunis, Tunisia; [2]Gynecology Department of Aziza Othmana Hospital, Tunis, Tunisia; [3]Reproductive Medicine, Reproductive Biology & Genetics, CHU Amiens Picardie, Amiens, France; [4]Human Genetics Laboratory, Faculty of Medicine of Tunis, Tunis El Manar University, Tunis, Tunisia and [5]Department of Biology, Faculty of Science of Tunis, Tunis El Manar University, Tunis, Tunisia

Research Article

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Corresponding author:
Hanen Elloumi;
Email: elloumichaabanehanen@yahoo.fr

Summary

To date, implantation is the rate-limiting step for the success of *in vitro* fertilization (IVF) treatment. Accumulating evidence suggests that immune cells contribute to embryo implantation, and several therapeutic approaches have been proposed for the treatment of recurrent implantation failure (RIF). Endometrial immune modulation with autologous activated peripheral blood mononuclear cells (PBMCs) is one of the most widely used protocols. However, the effect of intrauterine insemination of mixed paternal and maternal-activated PBMCs has not yet been attempted and studied. The aim of our study is to test the effect of the addition of paternal lymphocytes on the implantation rate in RIF patients. Mononuclear cells were isolated from the peripheral blood of 98 RIF patients and cultured for 72 h before insemination into the endometrial cavity 48 h before embryo transfer. Our patients were divided into 4 groups according to the type and number of PBMCs inseminations. Our study shows that activated PBMCs promoted clinical pregnancy rates (CPR) in all groups. Moreover, we found that the groups injected with more than 2 million cells showed a better clinical outcome and, more interestingly, patients inseminated with both paternal and maternal activated PBMCs showed the highest CPR, reaching 47.2%, in addition to the highest implantation rate 31. 2% and the live birth rate 41.39%. Our work demonstrates the importance of administering a large number of activated PBMCs with the addition of paternal activated PBMCs to immunomodulate the endometrium for the success of *in vitro* fertilization in RIF patients.

Introduction

To date, implantation has been the rate-limiting step in the success of *in vitro* fertilization (IVF). The process of implantation is complicated and requires the orchestration of a series of events involving both the embryo and the endometrium (Kim and Kim, 2017). Successful implantation requires a series of complex morphological and functional events, including decidualisation of endometrial stromal cells, epithelial cell adhesion, vascular remodelling and immune regulation (Mor *et al.*, 2017; Ochoa-Bernal and Fazleabas, 2020).

Embryo implantation and pregnancy maintenance are associated with important changes in the levels of immune cells in the endometrium, including macrophages, natural killer (NK) cells and a distinct cytokine profile, particularly between T helper type 1 (Th1) and T helper type 2 (Th2) cells, and between Th17 and regulatory T (Treg) cells (Wang *et al.*, 2020).

The presence of lymphocytes during embryo implantation has been reported to increase cytokine production, with a predominant profile of pro-inflammatory cytokines, mainly tumour necrosis factor (TNF), leukaemia inhibitor factor (LIF) and the interleukins IL1, IL2, IL12 and IL15 (Silasi and Mor, 2012).

In addition, the decidual transformation of stromal cells, which begins before implantation in women, is facilitated by local dendritic cells and uterine NK cells (King, 2000; Plaks *et al.*, 2008). These leukocytes produce cytokines to participate in complex interactions with ovarian steroid hormones and growth factors that drive the decidual phenotype transition (Dimitriadis *et al.*, 2005; Salamonsen *et al.*, 2003). The presence of these factors at appropriate levels, as actors in the inflammatory response, appears to be critical for early implantation.

Several therapeutic strategies have been proposed to improve conception in patients with recurrent implantation failure (RIF). Indeed, although RIF remains based on clinician judgement, the consensus is that it is defined as failure to implant after at least three IVF cycles in which 4 high-grade quality embryos have been transferred in a woman under 40 years of age (Cimadomo *et al.*, 2021; Coughlan *et al.*, 2014). Among these strategies, Immunotherapeutic

approaches have been described, including granulocyte colony-stimulating factor (Madkour *et al.*, 2016), anti-tumour necrosis factor (TNF) agents (etanercept and adalimumab) and intravenous immunoglobulin (Parhizkar *et al.*, 2021). Other experimental approaches are also described, in particular, local endometrial injury (Gui *et al.*, 2019) or administration of platelet-rich plasma (Maleki-Hajiagha *et al.*, 2020).

Cell-based therapies are also among the proposed strategies to help RIF patients. These include stem cell and peripheral mononuclear cell intrauterine perfusion (Esmaeilzadeh *et al.*, 2020). Several studies have reported maternal lymphocyte intrauterine insemination at the pre-implantation stage as a therapy for patients with recurrent implantation failure (Nobijari *et al.*, 2019; Pourmoghadam *et al.*, 2020). Indeed, this process provokes a local inflammatory response and de facto increases the production of pro-inflammatory cytokines.

Insemination with PBMCs aims to regulate the dialogue between the endometrium and the embryo (Billington, 2003). PBMCs are mainly composed of T lymphocytes, B lymphocytes and monocytes and are involved in several mechanisms. Indeed, PBMCs injection promotes implantation rate (IR) and clinical pregnancy rate (CPR) and could optimize *in vitro* fertilization (IVF) results in patients suffering from repeated IVF/intracytoplasmic sperm injection (ICSI) failures (Nobijari *et al.*, 2019; Wu *et al.*, 2019; Yoshioka *et al.*, 2006).

The local immune cells at the endometrial site contribute to embryo implantation. Indeed, recent data suggest that embryo-specific tolerance can be induced prior to this process (Mayoral Andrade *et al.*, 2020). However, many studies have suggested that an unbalanced maternal immune response against the embryo may lead to its rejection (Bashiri *et al.*, 2018; Cakiroglu and Tiras, 2020; Chaouat *et al.*, 2005; Garrido-Gimenez and Alijotas-Reig, 2015). In fact, the female immune system first comes into contact with paternal antigens before the implantation process and during coitus. In fact, the presence of semen in the uterus causes the migration of a large number of leukocytes, accompanied by an intense inflammation suitable for implantation (Song *et al.*, 2016).

Despite the importance of male antigens in the recruitment of the immune system to the implantation site and the embryo implantation process (Robertson *et al.*, 2013), no studies have been reported to evaluate the consequences of co-administration of both maternal and paternal PBMCs to improve the success of IVF treatment.

The main objective of our study is to evaluate the effect of co-administration and protocol modulation of enriched paternal PMBCs on the success of IR, CPR) and live birth rate (LBR) in infertile patients with a history of RIF.

Materials and methods

Patient selection and study design

Our prospective study was conducted between February 2018 and January 2023 at the Assisted Reproductive Technology (ART) Clinic, Fertilia, Tunis Tunisia. The study included 98 couples with RIF who underwent another round of thawed embryo transfer.

Inclusion criteria's were defined as:

(a) The impossibility to achieve clinical pregnancy after at least five high-grade embryos (embryos on day 5 with integrated morphology cleavage (IMC) (A), TE (A) and expansion >3) (Gardner and Schoolcraft, 1999) transfer in a minimum of three fresh or frozen cycles.

Table 1. Characteristics of our cohort of patients

Characteristics	Mean ± SD
Age of the patient	36.19 ± 3.22
Age of the partner	43.51 ± 7.3
Duration of infertility	6 ± 2.7
Type of infertility: female, male and mixed	
Number of RIFs	5.23 ± 1.86
Anti-mullerian hormone	2.70 ± 0.99
Endometrial thickness (mm) on day of embryos transferred on previous cycles	8.5 ± 0.9
Number of embryos transferred per patient	6.76 ± 1.71
Number of PBMCs inseminated	2.53 ± 1.23

Results are expressed as mean ± standard deviation (SD).

(b) Age ≤40 years.
(c) Primary infertility (Table 1), the cycles with testicular biopsy, cryptoazoospermia, extreme Oligo-Astheno-Teratospermia and ovarian insufficiency were excluded.
(d) Normal karyotype.
(e) Regular menstrual cycles
(f) Not having any systemic, immunologic, endocrine disease and thrombophilia.

Patients were subdivided into 4 groups according to the type and the number of PBMCs transplantation.

Group A ($n = 24$): Patients with autologous PBMCs transplantation

- Group A1 ($n = 13$): Number of PBMCs <2 millions
- Group A2 ($n = 11$): Number of PBMCs ≥2 millions

Group B ($n = 74$): Patients with co-cultured maternal and paternal PBMC transplantation

- Group B1 (29): Number of PBMCs <2 millions
- Group B2 (45): Number of PBMCs ≥2 millions

5 patients were excluded from the study due to an unsatisfactory PBMCs culture (< 1Million).

Ethical standard

The authors declare that all procedures contributing to this work met the ethical standards of the relevant national and institutional human subjects committees. All patients who participated in this study signed an informed consent form after being informed of the conditions and issues of the study.

IVF protocol

Ovarian stimulation was performed using either gonadotropin-releasing hormone (GnRH) analogue or GnRH antagonist with human menopausal gonadotropin (HMG) or recombinant-follicle-stimulating hormone (rFSH). Human chorionic gonadotropin was administrated when optimal follicle development was reached, as evaluated by serial transvaginal ultrasound and oestrogen determinations. Oocyte retrieval was performed via a transvaginal approach with sonographic guidance 36 h after the administration of rhCG (Ovitrelle, Merck Serono). Maturity of the oocytes was evaluated using the inverted microscope at x400. After

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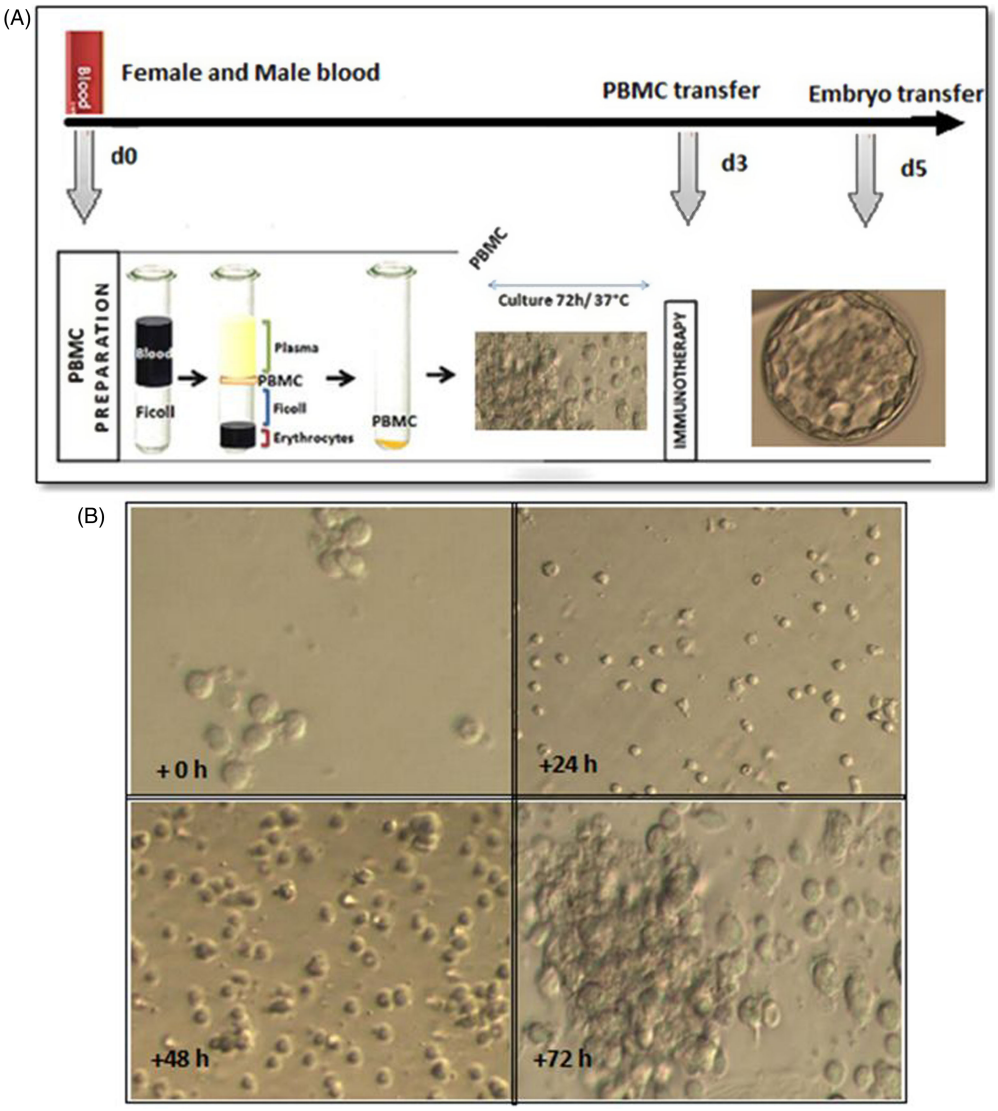

Figure 1. Peripheral blood mononuclear cells (PBMCs) preparation. (A) Intrauterine insemination of cultured PBMCs prior to embryo transfer. On the day of the introduction of exogenous progesterone (J0), each sample of blood (10 ml) was taken from recurrent implantation failure patients and their partners, in order to isolate PBMCs using Ficoll separation, and the PBMCs were cultured for 72 h. Finally, 0.3 ml of cultured PBMCs were transferred into the uterine cavity using an embryo transfer catheter. (B) Isolated PBMCs before culture 0 h, throughout incubation (0 h, 24 h, 48 h 72 h).

2 h of incubation, in human tubal fluid at 37° and 5% CO2, cumulus corona complex was removed with hyaluronidase. ICSI was performed in metaphase II oocytes using fresh or frozen-thawed spermatozoa prepared.

Endometrium and embryo preparations

Hormone replacement therapy for endometrial preparation was started on the first day of the cycle with oestradiol valerate (2 mg/day). Endometrial echography was performed. When endometrial thickness reached 8 mm with progesterone valerate <1 mg/ml, progesterone (400 mg) was administered daily. All embryos were cryopreserved at the blastocyst stage using a vitrification kit (Kitazato, BioPharma, Shizuoka, Japan) and thawed using a thawing kit (Kitazato, BioPharma, Shizuoka, Japan) according to the manufacturer's protocol. Embryo vitrification remains the best option and has been shown to significantly improve clinical outcomes, both in terms of post-thaw survival, clinical pregnancy rates and pre-implantation genetic diagnosis (Rienzi *et al.*, 2017, Simopoulou *et al.*, 2014).

PBMCs preparation

Blood samples were taken 5 days before the planned embryo transfer. A volume of 10 ml was collected in citrated tubes from RIF patients and their partners. Mononuclear cells were isolated by density gradient centrifugation using a commercially available lymphocyte preparation and then cultured in complete ready-to-use culture medium (supplied by ATL R et D laboratory, La Verrière, France) at 37°C and 5% CO2. Lymphocytes from each partner were cultured in separate tubes for 72 h and then cells were selected for insemination under both conditions, mixed population of lymphocytes (autologous and paternal) or autologous only. A minimum of 1×10^6 cells in 0.3 ml was transferred into the endometrial cavity 48 h before embryo transfer (Figure 1).

Outcomes

14 days after frozen embryo transfer, a positive pregnancy test was assessed by β-human chorionic gonadotropin (hCG) dose and confirmed by the detection of a gestational sac. The primary outcome measured was live birth rate; LBR (number of live births per transfer cycle) and secondary outcomes included clinical pregnancy rate CPR (number of gestational sac confirmation per transfer cycle) and implantation rate; IR (number of gestational sacs seen at 6.5 weeks per number of embryos transferred).

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Table 2. Characteristics of our cohort of patients depending on subgroups

Characteristics	Group A1	Group A2	Group B1	Group B2
Number of patients (*n*)	13	11	29	45
Woman's age (year)	31.27 ± 0.56	37 ± 0.77	35.93 ± 0.14	35 ± 3.9
Age of the partner	38 ± 3.98	41.54 ± 5.2	40.13 ± 7.8	41 ± 5.37
Duration of infertility	6.61 ± 3.44	5.9 ± 2.3	5.13 ± 3.96	6.25 ± 2.9
Number of embryos transferred per patient on previous cycles	4.61 ± 1.5	5.09 ± 1.66	5.86 ± 1.8	5.9 ± 1.84
Mean number of embryos transferred per patient on PBMC insemination cycle	1.84	1.63	2	1.78
Number of PBMCs inseminated	1.49 ± 0.31	3.13 ± 0.8	1.54 ± 0.76	3.36 ± 1.3
Clinical pregnancy rate (%; per transfer cycle)	23%	36.3%*	12.5%	**47.2%*#**
Implantation rate (%; per transferred embryo)	12.5%	27.7% *	6.2%	**31.2%*#**
Live birth rate (%; per transfer cycle)	15.3%	18.1%*	12.5%	**41.6%*#**

Results are expressed as *n*, *n* (%) or mean ± standard deviation (SD).
*($P < 0.05$) indicates significant difference between groups 2 and 1
#($P < 0.05$) indicates significant difference between group B2 compared to all other groups (SPSS).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). As indicated in the table legend, statistical analysis was performed using SPSS software (version 23). Power analysis for comparing mean for each group, given the studied sample size, was performed using SPSS. The alpha level was set at .05.

Differences between PBMCs-treated groups with regard to Clinical pregnancy rate, Implantation rate, and Live birth rate were analyzed using the two-tailed t test. Moreover, to ensure the robustness of our findings and account for potential violations of assumptions associated with small sample sizes, we also implemented bootstrapping techniques. Additionally, receiver-operating characteristic (ROC) curve analysis to estimate the discriminatory power of administering a large number of activated PBMCs with the addition of paternal activated PBMCs, and Youden's method selected suitable threshold. This analysis was performed using SPSS Software. ROC curves and the corresponding area under the curve (AUC) were utilized as diagnostic tools to assess the specificity and sensitivity of the indicators (Figure S1).

Results

Our study included 98 women with a mean age of 36.19 ± 3.22 years and a mean partner age of 43.51 ± 7.3 years. The duration of infertility in our cohort was 6 ± 2.7 years. Patients had 5.23 ± 1.86 IVF attempts with an average of 6.76 ± 1.71 embryos transferred (Table 1). Following the European Society of Human Reproduction and Embryology standard protocol, the transfer was performed 5 days after the progesterone supplementation.

To investigate the validity of PBMC insemination (both autologous and paternal mix or autologous only) to improve clinical pregnancy rates, we divided our cohort into 4 groups (Table 2). Groups A1 and A2 for patients inseminated with autologous PBMCs and groups B1 and B2 for patients inseminated with parental PBMCs mix. It is important to note that there were no significant differences in clinical history or characteristics between the groups (Table 1).

Our first observation was that administration of activated PBMCs promoted CPR, regardless of the concentration and type of inseminated PBMCs (Table 2). This result is consistent with previous studies highlighting the importance of endometrial immunomodulation in preventing RIF (Benkhalifa *et al.*, 2022).

Furthermore, and importantly, we have shown here that with a high number of activated and injected PBMCs, we significantly increase the CPR, IR and LBR. In fact, groups A2 and B2 with a number of injected PBMCs of 3.13 ± 0.8 and 3.85 ± 1.81 million cells, respectively, showed a significant clinical outcome compared to groups A1 and B1 with 1.49 ± 0.31 and 1.50 ± 0.31 million cells, respectively ($P < 0.05$) (Table 2).

More interestingly, our data showed for the first time the increase in clinical features when the women were inseminated with a high number of parental PBMCs mix (maternal and paternal). In fact, group B2 showed significantly the best CPR (47.2%), IR (31.2%) and LBR (41.6%) compared to all conditions groups ($P < 0.05$), highlighting the essential role of paternal adjunction in immunomodulation in RIF (Table 2).

It should be noted that we obtained relatively high statistical power (0.82), which means that there is an 82% chance of detecting a statistically significant effect if the alternative hypothesis is true (differences between PBMCs-treated groups). The ROC curve analysis demonstrated that administering a large number of activated PBMCs with the addition of paternal activated PBMCs to immune-modulate the endometrium for the success of *in vitro* fertilization in RIF patients. The ROC curve analysis yielded an AUC of 0.767 [0.651–0.883] [95%CI], with a threshold value of 2.84 that maximizes the model's performance (sensitivity: 0.63, specifcity: 0.23) (Figure S1).

Discussion

The combine effect of the number and the origin PBMCs uterine supplementation

Despite progress in assisted reproduction technologies, the lack of control of implantation remains a major obstacle to obtain successful pregnancies. It is of prime importance to determine the characteristic features of a receptive endometrium. Indeed, it has been suggested that endometrial immune cells, cytokines and chemokines promote endometrial receptivity and embryonic development (Leung *et al.*, 2000; Oliveira and Hansen, 2008; Robertson *et al.*, 2013). In this context, PBMC treatment induces

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Table 3. Methodological differences in studies and clinical outcomes of Peripheral blood mononuclear cells (PBMCs)-treated groups with three or more implantation failures

Study	No of patients	Volume of blood sample	IU PBMC administration	Summary of results concerning CPR
Yoshioka *et al.* 2006	35	NA	4×10^6 cells	PBMC group 41.2% vs. controls 11.1%
Okitsu *et al.* 2011	19	30 ml	1.5×10^7 cells	PBMC group 42.1% vs. controls 25% (The women with ≥3 RIF)
Yu *et al.* 2016	93	16–20 ml	$2–4 \times 10^6$ cells	PBMC group 46.2% vs. controls 20.9%
Li *et al.* 2017	48	NA	$2–4 \times 10^6$ cells	PBMC group 39.58% vs. controls 14.29%
Madkour *et al.* 2016	27	NA	1×10^6 cells	PBMC group 44.44% vs. controls 14.81%
Nobijari *et al.* 2019	122	NA	20×10^6 cells	PBMC group 38.6% vs. controls 19.7% (The women with ≥3 RIF)
Makrigiannakis *et al.* 2019	26	NA	20×10^6 cells	PBMC group 57.69% vs. controls 0%
Pourmoghadam *et al.* 2020	50	NA	$15–20 \times 10^6$ cells	PBMC group 42% vs. controls 22%

NA, Non available; CPR, clinical pregnancy rates; vs, versus.

the production of several cytokines, such as IL-1α, IL-1β, TNF-α and leukaemia inhibitory factor (LIF), which may have a positive impact on endometrial receptivity and actively contribute to blastocyst attachment and invasion.

Implantation of the embryo into the maternal endometrium is a crucial step in the reproductive process in several species, and both partners, the mother as well as the embryo, play an equal role in the embryo-maternal dialogue, they seem to communicate through signalling molecules. We can hypothesise here that our cohort of patients had problems with endometrial signalling, resulting in poor recruitment of their lymphocytes at the endometrial level. In fact, a normally functioning immune system is essential for successful embryo implantation and immune cells, including NK cells, macrophages and various cytokines, appear to play a central role (Garcia-Velasco, 2017; Wang *et al.*, 2020). During embryo implantation, the endometrium is found with a predominant profile of pro-inflammatory cytokines due to the presence of lymphocytes (Silasi and Mor, 2012). Therefore, implantation failure could be related to a deficit of inflammatory elements in the endometrium. Then, the addition of PBMCs would enhance the mobilization of specific inflammatory cells (uterine NK cells, macrophagic cells and regulatory T cells) and the maturation of immune players essential for the embryo implantation process.

We believed that the positive effect of intrauterine PBMC administration could be due to the concentration of inseminated cells. A previous literature reports indicated a high number of PBMCs to be injected in patients, not less than 2 million cells and, in some, reaching 10 million cells approximately (Li *et al.*, 2017; Madkour *et al.*, 2016; Okitsu *et al.*, 2011; Yoshioka *et al.*, 2006; Yu *et al.*, 2016) (Table 3). Our data further confirm this approach, with an improved clinical outcome in the group injected with more than 2 million cells (groups A2 and B2) compared to the group with 2 million cells (groups A1 and B1) (Table 2).

However, in certain cases of over-activated uterine immune profile, PBMC insemination may worsen the condition and cause deleterious effect. Over-expression of uNK cells results in an unfavourable implantation environment, so inadequate activation of uNK cells might be the cause of RIF (Lédée *et al.*, 2017).

The effect of the adjunction of paternal Lymphocyte

In our study, the positive effect of intrauterine administration of PBMCs could also be attributed to the number and origin of the cells inseminated. *In utero* administration of mixed autologous and paternal PBMCs in patients with at least three RIF significantly improves the pregnancy rate (47.2%) compared to patients treated with a lower concentration of PBMCs or with autologous PBMCs only.

CPR and LBR were significantly higher after intrauterine administration of mixed paternal and autologous PBMCs prior to thawed embryo transfer. These results confirm the efficiency of uterine supplementation with PBMCs of an appropriate type and concentration.

Vaccination of patients with husband's lymphocytes has been prescribed to stimulate the production of blocking antibodies (Hasegawa *et al.*, 1992; Hwang *et al.*, 1992; Takakuwa *et al.*, 1986). Indeed, previous studies have shown that injection of antipaternal lymphocytotoxic antibodies could prevent maternal rejection of the foetus by the endometrium (Hwang *et al.*, 1992). To ensure the same effect, our study follows the same idea in patients with unexplained recurrent spontaneous abortion since paternal lymphocyte cells could secrete blocking molecules.

Information about the presence of the developing embryo at the pre-implantation stage is transmitted to the endometrium not only by the endocrine system but also by the immune system. In fact, intrauterine insemination of paternal culture-activated PBMCs 48 h prior to embryo transfer provides biological signals, specifically paternal antigens and cytokines that have a significant impact on the female reproductive tract. The pioneering work of Robertson and colleagues suggests that exposure of the uterus to male seminal fluid promotes the maternal immune response. Indeed, seminal fluid contains cytokines and chemokines that prepare the local microenvironment for growth and attract Treg cells that react with paternal alloantigens. The addition of male PBMCs brings the same benefits as female PBMCs and overcomes the lack of paternal antigens (Robertson *et al.*, 2013). In line with this report, several publications have demonstrated that Treg cells are essential for productive implantation, as the absence of these cells has been associated with implantation failure. It is now well documented that both peripheral blood and uterine Treg cells increase in response to productive implantation. Accumulating evidence suggests that recognition of foreign paternal/foetal antigens by Tregs is critical for their development and function (Robertson *et al.*, 2013; Sasaki *et al.*, 2004; Schumacher and Zenclussen, 2014).

Many studies have provided considerable information on the intrauterine insemination of autologous PBMCs (Nobijari *et al.*, 2019;

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Pourmoghadam *et al.*, 2020). However, our study is the first to describe the use of paternal PBMCs for this immunomodulatory protocol. The exact mechanism of action of PBMCs is still unclear and both *in vitro* and *in vivo* experiments are needed to clarify the mechanism. In addition, immune profiling and personalized treatment approaches remain necessary to avoid worsening the condition and causing deleterious uterine immune overactivation.

Conclusion

In conclusion, our work demonstrated for the first time the importance of administering high numbers of activated PBMCs with the addition of paternal activated PBMCs to immunomodulate the endometrium for the success of *in vitro* fertilization in RIF patients.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199424000133

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