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Luteal progesterone level correlated with immunotherapy success of patients with repeated implantation failures

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Abstract

Problem*: Immunotherapy using PBMC administration demonstrated relatively its effectiveness to treat RIF patients but it is still unclear to explain some miscarriages. However, we could hypothesize a presence of dual interactive action between Luteal progesterone level (LPL) synthesized by corpus luteum after embryo implantation stage and the stimulated immune maternal system by immunotherapy. This issue could be informative basis data to personalize immunotherapy for RIF patients based on LPL predicting clinical outcomes.*

Method of Study: *This randomized controlled study included 70 patients undergoing ICSI program presenting at least 3 RIF: 39 for Control of untreated patients and 31 for PBMC-test concerning treated patients with immunotherapy. For the PBMC-test group, Peripheral Blood Mononuclear Cells (PBMCs) were isolated from patients on ovulation induction day and cultured three days to be administered to the intrauterine cavity of patients two days before fresh embryo transfer. LPL was analyzed at day 15 after embryo transfer and clinical outcomes were calculated including implantation, clinical pregnancy and miscarriage rates.*

Results: *Clinical outcomes were doubly improved after immunotherapy including implantation and clinical pregnancy rates comparing Control versus PBMC-test (10% and 21% vs 24% and 45%). On the other hand, this strategy showed an increase over double in LPL (4ng/ml for Control vs 9ng/ml for PBMC-test) while the latter was correlated to clinical pregnancy.*

Conclusions: *Bypassing the effectiveness of this immunotherapy approach for RIF patients, is directly correlated to LPL proving the interactive reaction between the immune profile of the treated patients and progesterone synthesis by corpus luteum.*

Keywords: *Luteal progesterone level, repeated implantation failure, immunotherapy, Peripheral blood mononuclear cells.*

Introduction

Though in vitro fertilization (IVF) success is generally limited to 30% depending on embryo implantation, the major part of implantation establishment is bypassing embryo quality and its genetic integrity highlighting the communication between the embryo and the mother. This cross-talk is essentially orchestrated by hormonal and immune dialogues to assure embryo invasion in the maternal endometrium without rejecting the fetal allograft. Furthermore, it is already known that progesterone (P4) is one of the most important implantations/pregnancy success keys for its effects on the endometrium and early pregnancy survival while its removal results in miscarriage.[1-3]

P4 is a hormonal key to modulate the maternal immune system by reducing natural killer (NK)-cell activity[4], inhibiting cytotoxic T-cell activity[5], increasing HLA-G production in trophoblast cells[6], increasing suppressor-cell levels[7] and as a special mechanism, it can induce lymphocyte-blocking proteins production such as progesterone-induced blocking factor (PIBF)[8-11]. Generally, its anti-inflammatory effect reported by several studies showing that it is essential to modify the cytokine response from pro-inflammatory profile presented by Th1 to anti-inflammatory profile presented by Th2.[12-19]

On the other side, at the pre-implantation stage, corpus luteum (CL) degeneration or luteolysis induced by intra-ovarian pro-inflammation under the action of PGF2α (Prostaglandin Factor 2 alpha), IL6, TNFα (Tumor Necrosis Factor-alpha) and caspase apoptotic cascade must be escaped.[20-26] This stage is determinant leading to the luteotropic status of developed CL for P4 production and requiring retardation at least luteoplacental shift between 7-9 weeks of pregnancy under hCG action effect from villous trophoblasts. [25-28]

Indeed, to shift from implantation to pregnancy stage, P4 is not the only controlling factor while a maternal immune system with Th1/Th2 balance is required.[29,30] In concert with endometrium and blastocyst, cytokines production profile is dominated by Th1 pro-inflammatory cytokines mainly IL1, IL2, IL12, IL15, IL18, and involving apoptotic factors such as TNFα and INFγ (Interferon-gamma), with the special intervention of some growth factors families; VEGF (Vascular Endothelial Growth Factor), TGFβ (Transforming Growth Factor Beta), IGF (Insulin Growth Factor) and LIF (Leukemia Inhibitory Factor) for angiogenesis and endometrial proliferation. This rich panel of various immune factors is essential for the early stages of implantation.[31-35]

Conversely, pregnancy maintenance would necessitate a cytokine profile; characterized by Th2 cytokines predominance (IL3, IL4, IL5, IL6, IL8, IL10, IL13, GM-CSF…) causing an anti-inflammatory state adequate to uterine receptivity.34-38 Thus, Th1/Th2 unbalance could explain implantation failures in some patients with RIF, RPL, or recurrent miscarriages (RM).[35,39,40]

Indeed, with a special interest in the immunology of reproduction, Yoshioka et al.41 was the first team that could to realize immunotherapy for patients with repeated IVF failures based on intrauterine administration of peripheral blood mononuclear cells (PBMC). Then, several studies developed this novel approach with some modifications on PBMC preparation protocol, including fresh or frozen cycles, embryo day 3 or blastocyst transfer, patients with at least 2, 3, or 4 RIF to improve significantly clinical outcomes and decrease the miscarriage rates.[40,42-45] Furthermore, the main goal of this immunotherapy approach was trying to activate the maternal immune system toward Th1 in endometrium before embryo transfer for implantation establishment and embryo invasion suggesting that RIF patients were suffering from a deficient Th1 system while RPL patients had a persistent Th1 after implantation despite Th2 cytokines secretion that is required for pregnancy maintain.35,39,40 Nevertheless, PBMC immunotherapy could be efficient for some RPL cases and avoid miscarriages40, 44 despite lack of clear definition toward RIF and RPL[46]. This issue led us to wonder about the mechanism of PBMC immunotherapy by what it could modulate maternal immune response homing Th1/Th2 balance required for implantation and pregnancy stage to manage RIF and RPL clinical cases. However, some anti-inflammatory therapies including P4 supplementation which is prescribed in 15-40% of women with RM could not be as much efficient as PBMC immunotherapy if we compare clinical outcomes throughout different studies.[39,47-49] Nevertheless, P4 is an indispensable factor for endometrium decidualization and for the early stage of clinical pregnancy to prepare an adequate immune environment for the fetus and low LPL results in an abnormal ongoing pregnancy.[18,19,49-52] Thus, the sensitive question lies on P4 responses involving the functional expression of progesterone receptors (PRs) on the endometrium and on some immune cells during the implantation and pregnancy stage. P4 supplementation treatment is probably insufficient for patients with miscarriages while some puzzle pieces are missed requiring the immune system involvement to occur the embryo-maternal cross-talk with synchronized between three elements embryo, endometrium and CL. Effectively, it was suggested that there is complex communication between the maternal immune system and embryo giving information of its presence to prepare an adequate immune environment for implantation requiring a differentiated CL for P4 production essential for endometrial immune modulation controlling the bias towards a pregnancy protective immune milieu.[35,39,53] Indeed, Fujiwara et al. hypothesized that there is an enigmatic dual positive regulation of CL function and endometrium differentiation by the endocrine-immune system.[35]

Forward, it is increasingly clear that embryo implantation is dependent on one side on immune local mechanisms calling a broad spectrum of cytokines and growth factors and on another side on endocrine mechanisms related to hCG action and luteal P4 synthesis while their interactive reaction is still kept into question for pregnancy achievement. For this reason, while our previous work[40] was based on the implementation of PBMC immunotherapy for RIF patients and proving its efficiency in tripling the clinical outcomes, the present work was more focused on demonstrating the correlation between LPL

and immunotherapy success highlighting the interactive reaction between luteal P4 synthesis by CL and immune system.

Materials And Methods

Ethical Standards

The study was approved by the ethics committee, (Comité d'Ethique pour la Recherche Biomédicale-Faculty of Medicine and Pharmacy, University Mohammed V, Rabat, Morocco) and patients provided written informed consent after being presented with the terms and issues of the study. 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.

Table 1: Comparison of the patient's characteristics

	PBMC-test (n=31)	Control (n=39)	p-value
Age of the partner	42.81 ±8.48	42.67 ± 5.58	0.93 (ns)
Age of the patient	35.81 ± 4.89	36 ± 4.71	0.82 (ns)
Number of RIF	4.39 ± 1.76	4.26 ± 1.96	0.77 (ns)
AMH (ng/ml)	2.61 ± 1.87	2.03 ± 1.35	0.13 (ns)
Estradiol (pg/ml)	32.87 ± 23.04	33.39 ± 16.51	0.91 (ns)
Progesterone (ng/ml)	0.48 ± 0.22	0.41 ± 0.23	0.21 (ns)
Endometrial thickness (mm)	9.32 ± 1.62	9.18 ± 2.4	0.12 (ns)
Number of oocytes per patient	9.13 ± 4.1	8.26 ± 5.27	0.45 (ns)
Maturation rate (%)	(192/283) 68%	(232/322) 72%	0.78 (ns)
Cleavage rate (%)	(131/134) 98%	(172/180) 96%	0.80 (ns)
Good quality embryos rate (A+B)	(63/131) 48%	(97/172) 56%	0.78 (ns)
Total number of embryos transferred	67	79	0.42 (ns)
Number of embryos transferred per patient	2.16 ± 0.78	2.03 ± 0.63	0.42 (ns)

Results are expressed as n, n(%) or mean ±standard deviation (SD). A statistic significant difference is considered when $P<0.05$ (n). $P\geq0.05$ is not significant (ns). AMH, estradiol and progesterone were

measured on day 2 of the cycle and the endometrial thickness was evaluated in day of oocyte retrieval. Cleavage rate was calculated relatively to embryos at day 3 by 2 pronucleus.

Patients' selection and study design

This was a prospective randomized study over two years conducted in an African fertility center including 70 couples who attended IVF program with at least 3 RIF without female age limit while 48 patients of them were less than 40 years old. In the selected couples, women had unremarkable clinical history and comparable clinical features and embryological outcomes (Table 1). All women received the same antagonist ovarian stimulation protocol (40) to minimize the effect of other parameters. Indeed, the whole lot was divided into two groups; the treated group with PBMC immunotherapy (PBMC-test, n=31) and the control group without treatment (Control, n=39).

IVF procedures

Embryos produced by ICSI40 were cultured up to day 3. Adequate embryo quality (good quality embryos; A+B) was defined based on the presence of uniformly sized and shaped blastomeres and fragmentation lower or equal to 10%. One or two good-quality embryos were transferred in utero using a Frydman catheter (CCD Laboratories, Paris, France). The implantation success (observation of the embryo sac) was assessed by ultrasound imaging and calculated relative to the number of transferred embryos. Clinical pregnancy was confirmed by ultrasound imaging 6-8 weeks after embryo transfer and calculated relative to the number of transferred cycles. The miscarriage ratio was calculated relative to the number of clinical pregnancies after the first trimester. Each couple went through a single ICSI cycle during this study.

PBMC immunotherapy and LPL assay

After the antagonist ovarian stimulation protocol, a blood sample is scheduled on the day of ovulation induction to isolate PBMCs using a separation protocol based on Ficoll. PBMCs are well prepared after culture for 72 h and then transferred to the patient in utero two days before embryo transfer as it was elucidated by Madkour et al. (40). After embryo transfer, patients receive oral Utrogestan (200 mg×2/day) for luteal support.

In the course of our study, the included patients underwent the LPL analysis on day 15 after embryo transfer to reflect the P4 synthesis by CL after implantation using the serum for the first pregnancy test

for β-hCG assay. Indeed, the LPL analysis was assessed using the immunological technique of electro-chemiluminescence (ECLIA, Roche, Mannheim, Germany) at the LABOMAC center.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) or percentage of the total. Data were analyzed with the Student's t-test for comparison of mean values or with the chi-squared test for comparison of percentages, and r-correlations using Statistical Package, version 6.0 (Statistica); $p < 0.05$ shows significant differences. Then, the mean values of each parameter's results were evaluated to calculate the study power with the post-hoc test using the G*Power software (version 3.0.10).

Results

Clinical outcomes were doubly improved after immunotherapy including implantation and clinical pregnancy in Control versus PBMC-test for patients with at least 3 RIF (10% and 21% vs 24% and 45%) while the effect of PBMC on miscarriage rate was non-significant (75% vs 21%; p=0.06). On the other hand, this strategy shows an increase over double in LPL (9ng/ml for PBMC-test vs 4ng/ml for Control) showing a significant correlation with clinical pregnancy rate for PBMC treated patients (Table 2). Moreover, the LPL was not influenced by RIF number with a non-significant r correlation (r=-0.36).

Table 2: Clinical outcomes and luteal progesterone level after PBMC immunotherapy for patients with at least 3 RIF

Clinical outcomes	PBMC-test (n=31)	Control (n=39)	P-value	Power 1-β
Implantation rate (%)	(16/67) **24%**	(8/79) **10%**	**0.02 (s)**	**89%**
Clinical pregnancy rate (%)	(14/31) **45%**	(8/39) **21%**	**0.03 (s)**	**71%**
Miscarriage rate (%)	(3/14) **21%**	(6/8) **75%**	0.06 (ns)	**99%**
LPL (ng/ml)	**9.32 ± 5.70**	**3.80 ± 3.65**	**0.00001 (s)**	**99%**
r-correlation (LPL to clinical pregnancy)	**0.28 (p=0.01)**	0.16 (p=0.32)	-	-

Results are expressed as n, n(%) or mean ±standard deviation (SD). A statistic significant difference is considered when $P<0.05$ (n). $P\geq0.05$ is not significant (ns). Power 1-β (β is error type II) is calculated basing on difference of mean values between two groups (PBMC-test vs Control) with α= 0.05 (α is error

type I). Power value is considered highly important when it is above 80%. r-correlation was calculated relatively to LPL depending on clinical pregnancy rate in each group of PBMC-test and Control, and it is considered significant when P<0.05 and non significant when p≥0.05.The implantation rate is expressed as the ratio between the number of embryonic sacs and the total number of transferred embryos; the miscarriage rate is expressed relative to the number of clinical pregnancies. LPL (Luteal progesterone level) was measured at day 15 after embryo transfer.

Discussion

Despite there being no clear differential clinical diagnostic to differ between RIF and RPL, over 75% of pregnancy failures are due to implantation failures.[54] Whatever controversies regarding RIF and RPL clinical definition, hypothetically in our previous study[40] it was suggested that RIF is due to pro-inflammatory (Th1) deficiency while RPL is due to Th1 persistence inhibiting the anti-inflammatory (Th2) release. Therefore, in this current study following the results of our previous work40 PBMC immunotherapy was efficient for RIF patients since their second implantation failure to double their chance to conceive. Nevertheless, we are not the only team who are prescribing this kind of treatment to patients with RIF or generally with IVF failures. Yoshioka et al. were the pioneers in this approach application while the research teams' followers could involve some technical modifications in PBMC preparation protocol.[41] Some could prove the efficiency of hCG supplementation on PBMC culture for 72h[40,42] or trying to minimize latter to 24h44,45,55 while others were more focused on the efficiency of CRH43. All these technical adaptations have occurred to enhance at maximum the function of PBMC and their cytokines secretions to activate thereafter the maternal immune system into endometrium after an intrauterine administration and be ready for embryo implantation. Indeed, as expected, the implantation rate after PBMC immunotherapy was over double compared to control (24% vs 10%; table 2) and the result was similar to other studies with interval 21-25% for treated patients.[40- 42,44,45]

Furthermore, an adequate decidualized endometrium developing receptive phenotype is not the only controlling trend for implantation establishment, but this uterine receptivity is under hormonal control involving the CL to produce P4 and estrogen.[35] Indeed, the reproductive system is a complex environment requiring the molecular communicative system to transfer the information between CL, uterus and immune cells before embryo presence and during its implantation and pregnancy. Although macrophages, NK, LT are present in ovarian luteal cells and uterine tissues, they are presenting with a general view an indispensable communicative system that could be dysregulated during the cascades of dialogues aforementioned.[13,35,56,57] Indeed, removal of macrophages CD11b+ in mice results in implantation failure occurred during the initial steps of embryo attachment and the ensuing decidual response, due essentially to insufficient P4 production by CL while women LPL under 10ng/ml could induce spontaneous miscarriage.3,56 Those conclusions allowed us to wonder about PBMC

immunotherapy efficiency and bypassing its effect on clinical outcomes after IVF process to be focused on the evaluation of the effect on the endogenous secretion of luteal P4 knowing that it is the main factor of pregnancy maintain.

Nevertheless, it was commonly accepted that insufficient P4 production causing miscarriages could be solved simply by an exogenous P4 administration to regulate the inflammatory mediators of pregnancy and even for patients undergoing IVF process in fresh or frozen cycles to improve clinical outcomes before embryo transfer.[49,58,59] Indeed, P4 presents an anti-inflammatory action enhancing Th2 cytokines production especially IL6, IL8 and some chemokines CXCL2 essential for macrophages recruitment to maintain pregnancy.[60,61] Thus, several molecular studies could have more interest in the P4 effect on immunity during pregnancy highlighting its particular anti-inflammatory action by reducing IL1-β-driven COX-2 via GR/MKP-1 with repression of p65 and c-Jun phosphorylation and inhibition of transcription of NF-KB to maintain uterine quiescence essential for pregnancy maintain.[14-17,61-65]

Moreover, the P4 action is dependent on CD8+ cells stimulating the differentiation of LTreg through the TGF and Foxp3 to promote immune balance toward Th2.[66-68] On the other hand, P4 production could be reduced via depletion of macrophages which are responsible for the formation of the dense capillary network necessary for corpus luteum maturation.[56] At one level, the P4 would act on the cells to make them produce the PIBF, considered the important protector of pregnancy after stimulation of Th2, highlighting the relationship "P4- immune-cells cytokines." The PIBF one hand blocks Th1 and system uNK and, secondly, stimulates the production of Th2-type cytokines knowing that the cells activated in the presence of PIBF produce more IL10, IL3 and IL4.[48,69]

All this knowledge helped us to develop our hypothesis about the interactive reaction between LPL issued from CL and PBMC administrated into endometrium to assure embryo implantation and pregnancy maintenance. Immune cells are indispensable for normal P4 production in the early pregnancy stage while its deficiency is incompatible with IVF success. Indeed, PBMC could provide the required elements certainly Th1 cytokines to activate endometrial immune cells necessary for implantation and inciting lymphocytes to produce PIBF essential for homing Th1/Th2 balance toward Th2 in one side. On the other side, Th1 cytokines and macrophages in CL could be activated to enhance their pro-angiogenic activity to produce VEGF as a key element for LPL increase which is essential for clinical pregnancy maintain (Figure 1).

Figure 1: Hypothesis of interactive effect of PBMC immunotherapy and luteal progesterone level for implantation and pregnancy success.

CL: Corpus Luteum; LPL: Luteal progesterone level; I. S (1): Immune System (Pro-inflammatory); I. S (2): Immune System (Anti-inflammatory); PBMC: Peripheral Blood Mononuclear Cells; End:

Endometrium, Emb: embryo; Vasc: Vascularization; PIBF: Progesterone induced blocked factor; VEGF: Vascular Epidermal Growth Factor, Treg: Lymphocyte T regulator.

After intrauterine administration PBMC, the Th1 / Th2 balance towards Th1 tends to ensure ignition shift by secreting pro-inflammatory cytokines and several growth factors primarily VEGF inducing vascularization in one hand into endometrium to prepare for embryo invasion embryo and in the other hand into CL in order to increase luteal progesterone level (LPL). All these immune-endocrine factors are limited in closest communication circle with mutual interactive modulation to ensure the embryo implantation. Thereafter, an increased LPL can induce immunomodulation by promoting T cells differentiation into Treg and secreting PIBF as an immunosuppressor factor that promotes the Th1/Th2 balance to Th2 anti-inflammatory system ensuring immunotolerance of allograft “embryo”. Thus, Th2 cytokines secretion involved in CL maturation can eventually to increase more LPL required for pregnancy maintain.

Indeed, our doubled clinical outcomes including implantation and clinical pregnancy rates could have a more evident explanation especially when LPL showed a high increase in PBMC-test compared to control (9ng/ml vs 4ng/ml) with positive correlation relatively to clinical pregnancy just for treated RIF patients (r=0.28) (Table 2). However, 4 ng/ml of LPL was not correlated to clinical pregnancy rates for RIF patients in the control group (r=0.16). This observation allowed us to conclude that certainly even with P4 importance to maintain clinical pregnancy but it could have occurred. Moreover, it explained why P4 supplementation treatments kept into question their efficiency for RIF, RPL and RM patients despite the maternal immune system being dysregulated led toward Th1 or Th2.[39,47-49] On the opposite side, when this latter is probably trying to turn back its balance via PBMC immunotherapy which acted not only into endometrium but also in CL to produce more the P4, LPL became correlated to clinical pregnancy. Furthermore, luteal P4 would regulate the uterine level synthesis of CSF-1, a cytokine essential for the vascularization of the endometrium and to maintain pregnancy by increasing just before implantation to achieve a peak tripled to day 15 of pregnancy.[56,70] Our results show a 63% increase in the synthesis of the luteal progesterone in pregnant patients treated with PBMC which joins perfectly the observed effect of PBMC on clinical pregnancy rate in our study. It seems that this effect is mediated by Th2 cells secreting IL4 and IL10 able to optimize the recruitment of leukocytes for VEGF secretion in CL.[13] The latter is better-vascularized release P4 production.[71]

In addition, several teams have shown the critical role of macrophages and GM-CSF in the implantation process. Thus, a decrease in GM-CSF type 2 (CSF2) produced by macrophages directly induces the decrease in LPL until 20% during pregnancy.[72] Among mice CSF1- showed a decrease of LPL by 50%[73] and 75% among mice TGFβ1- while TGFβ1 is CSF2 regulator[74]. This probably explains that the included patients in our study, who could become pregnant through this PBMC immunotherapy, were presenting

CSF1 decrease, while non-pregnant patients even after treatment, still have a TGFβ1 decrease, thus requiring a different therapeutic strategy, especially for patients since their second implantation failure.

Furthermore, based on our hypothesis (Figure 1), the PBMC effect could not be certainly over the early pregnancy stage to balance the maternal immune system and LPL while P4 will be placental and the ongoing pregnancy until delivery will be more influenced by the fetus and genetic reproductive function. Maybe, for this reason, PBMC immunotherapy is less effective to avoid miscarriages as shown in our study with a non-significant difference in miscarriage rate (21% for PBMC-test and 75% for control; Table 2) and confirmed by others.[40,44]

The paramount function of PBMC is to provide trophic support for the endometrium to be decidualized and for the formation of a dense vascular network in CL to produce more P4 that is essential for pregnancy maintain. However, perturbations of immune-endothelial cell crosstalk within the ovary during the peri-conceptional period are likely to be pivotal in luteal insufficiency in women. This issue could provide more therapeutic trends to enhance luteal function through the targeting of the immune system.

Conclusion

This immunotherapeutic strategy based on PBMC intrauterine administration suggests that embryo implantation is controlled by maternal immune cells in utero and this treatment is showed its efficiency for RIF patients doubling their clinical outcomes with a significant increase of LPL. This issue demonstrated that immunotherapy had a positive effect on luteal P4 synthesis during implantation which acted dually on homing the maternal immune system into endometrium to maintain pregnancy. This non-invasive and much less expensive treatment than the multiplication of IVF attempts could be proposed as part of ART to patients since their second implantation failure or even for patients with RPL or RM who are directly redirected to be treated with P4 supplementation or other anti-inflammatory treatments. Nevertheless, this issue needs eventual researches and clinical investigations.

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