



Impact of prednisone in patients with repeated embryo implantation failures: Beneficial or deleterious?

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ABSTRACT

Introduction: Corticotherapy is the leading medication worldwide for patients with history of repeated implantation failures (RIF) after IVF/ICSI. Nevertheless, we still do not know its local mechanism of action, hence its precise indication. Our objective is to document the impact of prednisone on the endometrial expression of immune biomarkers (CD56 cells count, IL-18/TWEAK, IL-15/Fn-14 mRNA ratio) at the time of uterine receptivity in a RIF population.

Materials and method: An endometrial biopsy was realized in the mid luteal phase for immune profiling: IL-15/Fn-14 and IL-18/TWEAK mRNA ratios were determined by quantitative RT-PCR and CD56 mobilization per IHC. Fifty-five patients with a RIF history were diagnosed to have local over-immune activation [high IL-18/TWEAK mRNA ratio, and/or high IL-15/Fn-14 mRNA ratio] likely to impair the implantation process. They underwent a second immune profiling with supplementation of prednisone. A paired comparison of the immune profile before and under prednisone was performed in the subset of patients subsequently pregnant under prednisone.

Finding: In 54.5% of the cases, both immune biomarkers were normalized and in 16.5%, only one was normalized under prednisone. In 29% we observed a paradoxical increase of both immune biomarkers. The IL-18/TWEAK mRNA ratio reflecting the Th-1/Th-2 local equilibrium was significantly reduced (0.29 versus 0.10, $p = .004$), through very significant increase of TWEAK expression, in patients who were subsequently pregnant under prednisone.

Conclusion: Testing the response to prednisone in a RIF context may be very useful. Less than half of RIF patients with immune deregulation may be prednisone responders and would benefit from its administration.

1. Introduction

70 to 80% of transferred embryos fail to implant after IVF/ICSI (in vitro fertilization/intracytoplasmic sperm injection). Defining the most adequate treatment able to increase the implantation rate of transferred embryos is one of the most important challenge of reproductive medicine. Personalization of treatment – especially in the painful context of unexplained and repeated implantation failures (RIF) after IVF/ICSI, is a major issue in routine. Many physicians need and claim precise indication for potentially active immunotherapy based on a clear scientific understanding convinced that their duty is to find personalized solutions for their patients (Sacks, 2015). Several fundamental scientists, on their part, highlight that efficacies of most, if not all,

immunologic therapies are unproven (Wong et al., 2014) and often prescribed on the false basis of the necessity of local down regulation of natural killer (NK) cells activity. Uterine and decidual NK cells are viewed by immunotherapists solely as a danger for the foeto placental unit and thus mostly, if not always, as killer cells (Moffett and Shreeve 2015; Robertson et al., 2016).

In fact, a controlled activation of key elements in the adaptive as well as innate immune system is central for the embryo implantation and for an adequate placentation. Local endometrial and immune cells should promote a local immune tolerance like status regarding the embryo while promoting simultaneously local angiogenesis and immunotrophism to let him grow and survive. A uterine immune disequilibrium may have deleterious consequences on subsequent embryo

Abbreviations: P, prednisone; RIF, repeated embryo implantation failure; IL, interleukin; uNK cells, uterine natural killer cells

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implantation.

By assessing the effect of prednisone on endometrial immune biomarkers [interleukin (IL)-15, IL-18, Tumor necrosis factor-like weak inducer of apoptosis (TWEAK), fibroblast growth factor-inducible molecule (Fn-14)], we aim to give new insights on the mechanism of action of corticoids in the endometrium at the time of uterine receptivity in this very specific context of RIF. Our objective is to define the specific set of patients that would benefit from corticosteroids to successfully implant. In the recent years, it became clear that several, distinct immune mechanisms may lead to such a deleterious uterine immune over-activation. In theory, each specific mechanism inducing immune over-activation requires a specifically targeted treatment (Coulam and Acacio 2012). In a Th1-dominant environment, uterine NK (uNK) cells may become killer cells able to target trophoblast cells as they would do for non-self and hence to reject them. Thus, a proper balance is required for successful implantation as well as pregnancy success (Wegmann et al., 1993; Chaouat, 2007). Key regulatory elements are dendritic cells and regulatory T cells (Aluvihare et al., 2004; Hanna et al., 2006; Blois et al., 2011). The absence or a down-regulation of Fox-P3 + cells have been correlated with infertility (Jasper et al., 2006). Uterine dendritic cells, for their own, may differentiate into deleterious DC-1 cells rather than the more helpful DC-2 cells (Tirado-Gonzalez et al., 2012), and T cells may differentiate into deleterious Th17 lymphocytes rather than into the Treg cells required for pregnancy (Sharma, 2014). Uterine immune over-activation may also result from a local hyper-activation of complement, mostly through the mannose-binding lectin pathway, as recently suggested in a murine abortive model (Girardi et al., 2006; Petitbarat et al., 2015; Chaouat, 2016).

Since distinct mechanisms may lead to over-immune activation, distinct drugs may also be required to control the specific diagnosed local deregulation. Corticotherapy is the leading medication worldwide for RIF, but we still lack precise indications for its use based on objective testing (Lunghi et al., 2010; Boomsma et al., 2012). All Authors suggest the necessity to precisely define the subgroup that would benefit from corticoids.

In this retrospective study, the endometrial immune profile of 55 RIF patients was evaluated in the mid-luteal phase and all were diagnosed as having – according to our criteria- an endometrial over-immune activation potentially leading to a rejection like process of the embryos or an improper engagement in a trophic/angiogenic pathway. This immune status being likely to explain their RIF history. The next subsequent step was to identify the most adequate treatment able to correct the observed uterine over-immune activation.

Here, we investigate the place of prednisone as a potential candidate.

Corticoids are often the first line of immunotherapy prescribed in the painful context of RIF or repeated miscarriages for their potent and broad-spectrum anti-inflammatory and immune-suppressive properties (Franchimont, 2004; Rhen and Cidlowski 2005). In theory, the objective of such prescription is to prevent an engagement of the uterus in a non-receptive state causing a rejection like process.

In the present cohort, we observe the variations of our immune endometrial biomarkers before and under prednisone to understand its impact. Variations of immune biomarkers before and under P are compared among the 55 RIF patients tested and among the selected group who normalized their immune parameters under corticoids and became at the next subsequent embryo transfer pregnant under corticoids. This group is defined as responder to corticoids.

2. Materials and methods

2.1. Protocol approval and patient consent

The Institutional Review Board of St Louis Hospital approved this study. Patient undergoing an endometrial biopsy provided their written informed consent allowing uterine immune analysis and a prospective

follow-up. All patients included in the assisted reproductive therapy program gave their informed consent before any fertility treatment (IVF/ICSI/Frozen Embryo transfer).

2.2. Study design

Fifty-five patients with a history of RIF underwent an endometrial immune profiling before and under prednisone between 2012 and 2014. Inclusion criteria were the absence of embryo implantation despite the transfer of at least 6 day-3 embryos or at least 4- day 5 embryos and an immune profiling under prednisone.

The mean age of patients included was 37 years old (28–43). They all, according to the inclusion criteria, previously failed to implant to IVF/ICSI despite a mean of 3 (1–7) oocytes retrieval and repeated transfer of a mean of 8 fresh or freeze-thawed embryos (4–35). They all had a negative check-up regarding their karyotype, autoimmunity, thyroid equilibrium and thrombophilia.

The basal exploration was set up in the mid-luteal phase of a cycle (monitored natural cycle or mock cycle). An endometrial biopsy was realized by aspiration with a Cormier pipelle. As previously described in detail (Lédée et al., 2016), after histological dating of an endometrial biopsy sample to confirm the mid-luteal phase, RNA was extracted with the RNeasy Plus kit (Qiagen, Courtabeuf, France), according to the manufacturer's instructions. The RNA was reverse-transcribed into cDNA with the first-strand cDNA synthesis kit for RT-PCR (Roche Diagnostic, Meylan, France). IL-15/Fn-14 and IL-18/TWEAK mRNA ratios were determined by quantitative RT-PCR with the Light Cycler 480 SYBR Green I Master mix (Roche Diagnostic), and uNK cells were counted after CD56 + immunohistochemistry.

The association of three biomarkers defines the uterine immune profiling. The norm has been previously defined in a fertile cohort (Lédée et al., 2016):

- The IL-18/TWEAK mRNA ratio, which reflects the local immune-regulated Th1/Th2 balance and local angiogenesis.
- The IL-15/Fn-14 mRNA ratio, which reflects uNK cell maturation. The number of CD56 positive cells.

All 55 patients were diagnosed as having – according to our criteria- an over-immune activation. Over-immune activated profile was characterized by high IL-18/TWEAK mRNA ratio, and/or high IL-15/Fn-14 mRNA ratio.

A second immune profiling was performed in the same conditions but with supplementation of corticoids [prednisone (20 mg orally, each morning)] from day-3 of the cycle to the day of the uterine evaluation.

2.3. Statistical analysis

Variations of immune biomarkers were observed (CD56 cells Count, IL-18/TWEAK and IL-15/Fn-14 mRNA ratio) before and under prednisone among the cohort (55 patients) with the paired *t*-test and with the Wilcoxon test among the selected pregnant patients under prednisone (10 patients).

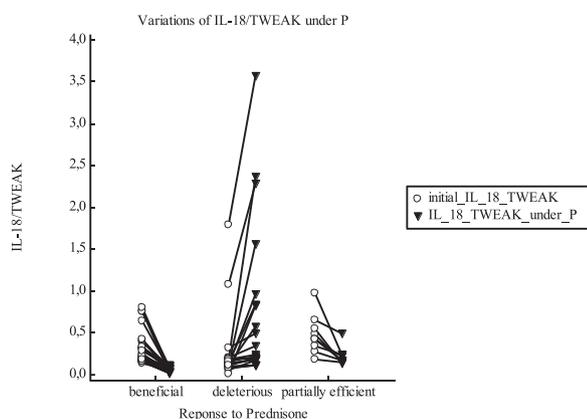
A *p* value below 0.05 was considered as significant.

3. Results

3.1. Comparison of immune biomarkers before and under prednisone in over-activated RIF patients

Before any intervention, among the 55 patients evaluated:

- 87.5% (48/55) had an IL-18/TWEAK mRNA ratio over the upper-threshold signing a Th-1 disequilibrium able to activate uNK cells in killer cells
- 34.5% (19/55) had an IL-15/Fn-14 ratio over the upper-threshold



Graph 1. Variations of IL-18/TWEAK mRNA expression in response to prednisone.

signing a possible deleterious activation of all the immune cells (Activation of NK receptor, T reg cells in Th-17 cells, Dendritic cells DC-2)

- 5% (3/55) had a high CD56 cell count.

Under P, we observed a normalization of initially elevated IL-18/TWEAK in 52% (25/48) of the patients, a normalization of initially elevated IL-15/Fn-14 in 73.5% (14/19) and a normalization of the CD56 positive cells count in only 1 out of 3 cases.

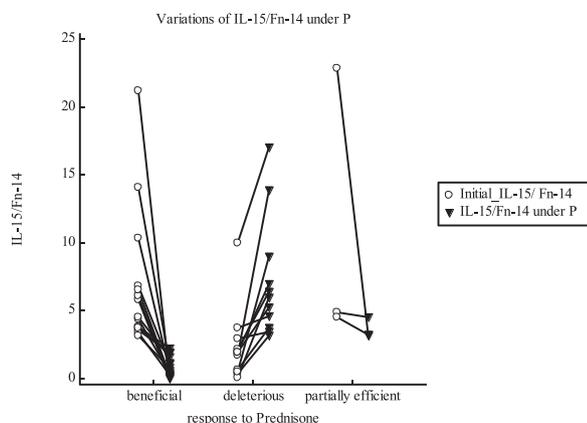
In 5.5% (30/55) of the cases, both IL-18/TWEAK and IL-15/Fn-14 were normalized under prednisone.

On the opposite, in 29% (16/55), the immune profile under prednisone was worse than initially with a paradoxical increase of either IL-18/TWEAK or IL-15/Fn-14 under prednisone regarding their basal value. In 16.5% (9/55), either IL-18/TWEAK or IL-15/Fn-14 were still over the superior threshold of activation under P.

Graphs 1 and 2 illustrate the variations of respectively IL-18/TWEAK and IL-15/Fn-14 in response to P with a clustering of the response according to the final interpretation (beneficial, deleterious, partial response)

According to our criteria, prednisone was evaluated as beneficial in 54.5% (30/55) of the cases, deleterious in 29% (16/55) and only partially efficient in 16.5% (9/55) in the observed cohort.

After the immune profiling under prednisone, according to the observed variations of immune biomarkers, we recommended to change the immunotherapy at the next embryo transfer for 25 patients (45.4%). 30 patients underwent their next embryo transfer under prednisone.



Graph 2. Variations of IL-15/Fn-14 mRNA expression in response to prednisone.

3.2. Comparison of immune biomarkers before and under treatment in the selected group who gave birth or not under prednisone

Over the 30 patients, a subset of 10 patients gave birth as the next embryo transfer under prednisone and were defined as the group who proved to be responder to corticoids while 20 did not get pregnant. We performed a paired comparison of their immune profile before and under P.

In the pregnant group, IL-18/TWEAK mRNA ratio which we believe is one of the biomarkers defining an over-immune activation was significantly reduced ($p = .002$). The mechanism was likely not the decrease of IL-18 ($p = .69$) but the significant increase of TWEAK ($p = .006$).

In patients who did not get pregnant, we observed a significant decrease of IL-18/TWEAK and IL-15/Fn-14 through a significant decrease of the expression of both IL-15 and IL-18 but with no significant variation regarding TWEAK.

Table 1 illustrates the variations of the immune biomarkers under corticoids in the selected group defined as responders with a clustering of patients successfully pregnant or not under corticoids.

4. Discussion

“One fits not all” seems, as previously reported by numerous authors, the true adage. A pre-selection of patients has been made and excluded per-se RIF patients with a documented low immune activation in which corticoids would be perceived as deleterious (Ledee et al., 2016, 2017). Despite this selection, we here observed a clear degradation of immune parameters under corticoids in 29% of the observed cases. On the opposite, corticoids prescription seems to have been potentially useful in 54.5% of RIF patients in over-immune activation. As stated in the introduction, we believe that there are multiple pathways leading to uterine immune deregulation. It is therefore likely that this split between two groups reflects the underlying involvement of 2 or more different immunoregulatory circuits, one positively affected by prednisone treatment, the other(s), on the opposite, showing amplification of the deregulation. This emphasizes, in our opinion, the need to search for more markers, allowing to define the P resistant (or negatively affected) circuits, and the one(s) for which prednisone treatment is efficient. Nevertheless, it remains that 54.5% of the patients with an initial diagnosis of over-immune activation according to our criteria show what we define as a positive improvement after prednisone treatment.

Identifying how corticoids act locally may help to define their indication. It is noteworthy that the sub-group who became pregnant under corticoids, the IL-18/TWEAK mRNA ratio was significantly decreased. We previously documented that local high IL-18/TWEAK endometrial expression reflected a deleterious Th-1 dominant environment and was associated with cytotoxic activation of innate immune cells (Petitbarat et al., 2011).

But surprisingly, under prednisone we did not observe a significant decrease of IL-18 or IL-15 in patients who got pregnant but in patients who did not. In contrast, we observed a significant increase of the TWEAK mRNA expression in patients who did get pregnant while it was not observed in the ones who did not get pregnant.

The significant increase of TWEAK appears as a potential immune mechanism able to explain the control the local cytotoxicity.

TWEAK is a cytokine of the TNF ligand superfamily expressed by many types of leucocytes including monocytes, dendritic and uNK cells (Chicheportiche et al., 2000; Maecker et al., 2005; Winkles, 2008). TWEAK and its receptor Fn-14 are involved in preventing local cytotoxicity and counterbalancing the cytotoxic function of uNK cells during gestation in favor of the constructive angiogenic/immunotrophic pathways in the human endometrium (Petitbarat et al., 2009).

Previous authors described TWEAK/Fn-14 as the Yin Yang of the

Table 1

Evolution of immune uterine biomarkers under prednisone among patients who succeed to give birth.

| Immune Profile among RIF patients responder to prednisone | Pregnant Patients (n = 10) | | p value | Patients not pregnant (n = 20) | | P value |
|---|----------------------------|------------------------|--------------|--------------------------------|------------------------|---------------|
| | Initial value | value under prednisone | | Initial value | value under prednisone | |
| CD56 cells count | 26.7 [7–69] | 54 [12–105] | 0.23 | 53 [21–105] | 48 [5–105] | 0.67 |
| IL-18/TWEAK | 0.30 [0.20–0.41] | 0.10 [0.05–0.11] | 0.002 | 0.20 [0.08–0.80] | 0.07 [0.02–0.35] | 0.0001 |
| IL-15/Fn-14 | 1.11 [0.47–2,3] | 0.53 [0.08–1,9] | 0.3 | 2.61 [0.08–14] | 1,02 [0.02–4,5] | 0.02 |
| Normalized IL-18 | 0.48 [0.07–0.70] | 0.32 [0.18–0.45] | 0.69 | 0.60 [0.04–3,91] | 0.22 [0.009–0.87] | 0.0009 |
| Normalized IL-15 | 0.62 [0.29–2] | 0.29 [0.06–1,7] | 0.23 | 1.65 [0.16–6,82] | 0.58 [0.003–3.8] | 0.0071 |
| Normalized TWEAK | 1.5 [0.19–2,6] | 3.5 [2,5–5,8] | 0.006 | 2,63 [0.11–12,6] | 3.72 [0,45–10,88] | 0.47 |
| Normalized Fn-14 | 0.55 [0.24–1,9] | 0.68 [0.41–1,7] | 1 | 0.81 [0.05–2,63] | 0.64 [0.11–2.21] | 0,67 |

innate immunity (Bell E. 2006). TWEAK/Fn-14 attenuates the transition from innate to adaptive immunity.

The role of TWEAK during embryo implantation has been studied in two distinct abortive models. In the murine abortive model CBA/J x DBA/2 compared to a control model CBA x BALB/c, our team showed that TWEAK offers protection against the deleterious effects of a Th-1-dominant (TNF-rich) environment during implantation and thus increases embryo survival (Mas et al., 2008). Recently, Qi et al. (2016) used the murine LPS-induced model of abortion to show that in such a mating, the LPS stimulation induces a downregulation of TWEAK with simultaneous up-regulation of Fn-14 in the uNK cells. Such TWEAK downregulation may contribute to the disruption of decidual homeostasis by altering the uNK cell cytotoxicity. Decreased levels of TWEAK resulted in an increased number of TNF-alpha producing NKG2D-positive cells possibly involved in subsequent fetal loss (Qi et al., 2016). Prednisone may contribute to a better control of the local cytotoxicity by increasing local secretion of TWEAK. Interestingly, recently soluble TWEAK has been shown to be lower in patient with preeclampsia compared to control (Yildirim et al., 2016).

Prednisone action has been mainly explored on target belonging to the adaptive immunity and in the blood. In the clinical context of RIF, potential benefit of prednisone may not be the consequence of the classical anti-inflammatory and immune-suppressive prednisone properties because of the particularities of the immune composition in the uterus.

Functions of prednisone regarding circulating NK cells in a IL-15 supplemented environment has been extensively studied (Perez et al., 2005; Moustaki et al., 2011). Authors described a synergistic effect between corticoids and IL-15 on promoting NK cells expansion and functions. prednisone were described as positive regulators of IL-15-mediated effects on peripheral NK cells. To our knowledge, impact of prednisone on the repertory of receptor of uNK cells which are some distinct cells in phenotype and function compared to circulating NK (Manaster and Mandelboim, 2010) has never been done.

To conclude, we here propose that prednisone acts positively in 54% of the cases retained after screening on uNK cells cytotoxicity in the specific context of RIF. Only a normalization of the immune biomarkers under corticoids may attest of the efficacy of corticoids. In case of no response, other therapies may be tested with the same method to document their ability to normalize the uterine immune profile before any attempt. The observed effect of prednisone on immune biomarkers was the significant increase of TWEAK able to locally decrease the amount of TNF-alpha producing cells possibly involved in repeated implantation failures. Further studies should document uNK phenotype and their repertory of receptor under prednisone.

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Competing interest

None.

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