Treatment with Adalimumab (Humira®) and Intravenous Immunoglobulin Improves Pregnancy Rates in Women Undergoing IVF*

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Problem
The purpose of this study was to investigate whether treatment with TNF-α inhibitors and/or intravenous immunoglobulin (IVIG) increases *in vitro* fertilization (IVF) success rates among young (<38 years) women with infertility and T helper 1/T helper 2 cytokine elevation.

Method of study
Seventy-five sub-fertile women with Th1/Th2 cytokine elevation were divided into four groups: Group I: Forty-one patients using both IVIG and Adalimumab (Humira®), Group II: Twenty-three patients using IVIG, Group III: Six patients using Humira®, and Group IV: Five patients using no IVIG or Humira®.

Results
The implantation rate (number of gestational sacs per embryo transferred, with an average of two embryos transferred by cycle) was 59% (50/85), 47% (21/45), 31% (4/13) and 0% (0/9) for groups I, II, III and IV respectively. The clinical pregnancy rate (fetal heart activity per IVF cycle started) was 80% (33/41), 57% (13/23), 50% (3/6) and 0% (0/5) and the live birth rate was 73% (30/41), 52% (12/23), 50% (3/6) and 0% (0/5) respectively. There was a significant improvement in implantation, clinical pregnancy and live birth rates for group I versus group IV (P = 0.0007, 0.0009, and 0.003, respectively) and for group II versus group IV (P = 0.009, 0.04 and 0.05, respectively).

Conclusion
The use of a TNF-α inhibitor and IVIG significantly improves IVF outcome in young infertile women with Th1/Th2 cytokine elevation.

Introduction

Repeated *in vitro* fertilization (IVF) failure remains an emotionally and financially distressing problem facing many couples seeking reproductive assistance. Data published by the Human Fertilization and Embryology Authority (HFEA) for the United Kingdom indicate that less than one-in-four attempted IVF cycles result in live birth (http://www.hfea.gov.uk/en/1215.html#Latest_annual_figures. Last updated 7 April 2008). Amongst the causes of infertility cited by the HFEA (http://www.hfea.gov.uk/en/406.html#Treatment_and_success. Last updated 7 April 2008), no identifiable cause was found amongst 23% of the couples. An immunologic basis for these unexplained losses has been suspected for many years.[1]

Tolerance of the fetal hemi-allograft has engaged the attention of reproductive immunologists since the time of Medawar.[2] A wide array of theories has since been invoked, but those proposing a T helper-2 cytokine bias in successful conception have received particular attention. Following Mossman's classification of helper T cells by cytokine profile, Wegmann observed protection of the fetus from maternal immune attack was characterized by a Th2 cytokine bias at the materno–fetal interface.[3,4] Hill first reported a shift towards a Th1 cytokine bias in women with recurrent pregnancy loss upon activation of peripheral blood lymphocytes with a trophoblast cell line (JEG-3).[5] The Th1/Th2 paradigm proposing a shift in the cytokine profile from Th2 to Th1 pre-dominance might play a significant role in recurrent miscarriage and implantation failure.[6]

Therapeutic intervention limiting the availability of Th1 cytokines, in particular TNF-α, might shift the Th1/Th2 ratio away from Th1 pre-dominance in patients contemplating pregnancy with unexplained failure. TNF-α blockers have been proposed to effect a shift in Th1-Th2 balance.[7,8] Flow cytometric quantification of *in vitro* activated CD4 T cells expressing Th1 and Th2 cytokines have been used in these studies to select candidates and monitor therapeutic intervention. The objective of this study was to evaluate whether the use of a TNF-α inhibitor with intravenous immunoglobulin could improve IVF success rates in young infertile women with Th1/Th2 elevation.

Materials and methods

Between May 2003 and December 2006, all patients with an elevated Th1/Th2 cytokine ratio (a TNF-α:IL-10 ratio above 30.6 and/or the IFN-γ:IL-10 ratio above 20.5) prior to their index IVF cycle at the Assisted Reproduction and Gynaecology Center (ARGC) in London were identified. From this group, patients were selected if they had (i) a female age <38 years, (ii) a negative Heaf test (tuberculin skin test using 100,000 units/mL concentrated tuberculin p.p.d.) which is a contraindication to using Adalimumab (Humira®; Abbott Laboratories, North Chicago, IL, USA) (iii) an index cycle with good embryo development (≥5 day three embryos with ≥5 cells). In cases where patients had more than a single eligible IVF cycle, only the first cycle was included in the sample. Using these inclusion criteria, 75 eligible treatment cycles were included in this study.

Cycles were divided into four groups by treatment regimen: Group I: Forty-one patients treated with both IVIG and TNF inhibitor, Adalimumab (Humira®), Group II: Twenty-three patients treated with IVIG but no Humira®, and Group III: Six patients treated with Humira® but no IVIG, and Group IV: Five patients treated with neither IVIG nor Humira®. Selection of treatment was based on severity of laboratory abnormalities, clinical findings and patient willingness to accept treatment (See further for protocol details). All patients signed standard clinical IVF consent forms plus detailed consent forms (one for Humira® and one for IVIG) explaining the nature of the medication, the possible risks and the lack of proof for clear evidence-based determination of efficacy.

Humira® Therapy

Patients who were treated with Humira® underwent an initial two injections of 40 mg each separated by a 2-week interval. Approximately 2–3 weeks following the second injection, a second Th1/Th2 assay was performed. If the initial elevation persisted, an additional two injections were started approximately 3–4 weeks following the second injection. The embryo transfer was done a mean of 2.1 ± 1.3 months (median: 2.1 months; range: 0.1–5.6 months) following the last injection of Humira®.

Intravenous Immunoglobulin Therapy

Intravenous immunoglobulin (IVIG) was administered at 400 mg/kg body weight at least once during the IVF cycle with Natural Killer (NK) Cell Assay cytotoxicity (50:1 effector: target cell killing ratio) of >15%, $CD56^+/CD3^-$ over 12% and/or $CD19^+/CD5^+$ level over 10%. Additional IVIG was given during the first trimester of pregnancy, if these levels were still elevated following (repeated) monthly NK assay assessment.

Clexane®

All patients included in this study were treated with low molecular weight heparin, enoxaparin or Clexane® during their treatment cycles (Sanofi-Aventis, Paris, France) at a dose of 20 mg daily, started preconceptionally. All patients also took supplementary low dose aspirin 75 mg/daily.

IVF Procedure and Outcome Measures

The study was limited to fresh IVF cycles only. No more than two embryos were transferred during each procedure except for four cycles in 2003 and 2004 when three embryos were transferred. *Pregnancy* was defined as a serum beta HCG result ≥10 on day 10–12 post embryo transfer. *Clinical pregnancy* was defined as fetal heart activity detected by transvaginal ultrasound scanning approximately 2 weeks after a positive pregnancy test. *Implantation* was defined as an intrauterine gestational sac detected by transvaginal ultrasound scanning. All pregnancies were followed until delivery.

Pre-conceptional Testing Parameters

All women completed immunologic testing. Testing was performed at the Rosalind Franklin Clinical Laboratory in Chicago. Blood samples were drawn preconceptionally.

Flow cytometry

Flow cytometry was performed on peripheral blood mononuclear cells to assess percentage of NK cells ($CD56^+/CD3^-$) and $CD19^+/CD5^+$ B cells. A percentage of $CD56^+/CD3^-$ lymphocytes greater than 12% was defined as elevated.[9,10] The percentage of $CD19^+$/CD5+ cells was calculated among $CD19^+$B cells. A percentage >10% was defined as elevated.[9,11]

NK cytoxoxicity

Natural killer cytotoxicity was assessed by flow cytometry where labeled K562 target cells were incubated with isolated patient mononuclear cells and propidium iodide which stains dead cells. Target cell killing was assessed by quantification of dually labeled cells. NK cytotoxicity was tested at an effector to target ratio of 50:1. Cytotoxicity was regarded as increased when target cell killing exceeded 15%.[12–14]

Th1:Th2 Cytokine Assay

Mitogen stimulation of peripheral blood mononuclear cells in the presence of a secretion inhibitor (Brefeldin A) was followed by membrane permeabilization and combination staining for T-helper surface markers and anti-cytokine markers. The Th1/Th2 intracellular cytokine ratio was assessed by measuring the ratio of TNF-alpha-expressing cells to IL-10-expressing cells (TNF-α:IL-10); and interferon gamma-expressing cells to IL-10-expressing cells (IFN-γ:IL-10). A TNF-α:IL-10 ratio above 30.6 and/or the IFN-γ:IL-10 ratio above 20.5 exceeded one standard deviation (S.D.) in non-pregnant women of reproductive years by the testing laboratory. Only two of the 75 women included in our study had been previously given a diagnosis of non-specific autoimmune disease.

Statistical Analysis

Statistical analysis of success rates was performed using Fisher's exact test (Graphpad Software® Inc., La Jolla, CA, USA). Statistical analysis of variance for patient characteristics, IVF parameters and immune test results was performed using a one-way ANOVA calculator (Danielsoper.com online Statistics Calculators, version 2.0, http://www.danielsoper.com/statcalc/).

Results

All patients included in this study had an elevated Th1/Th2 cytokine ratio exceeding one standard deviation above the mean for non-pregnant women without a history of IVF failure [TNF-α:IL-10 ratio above 30.6 (normal range 13.2–30.6) and/or the IFN-γ:IL-10 ratio above 20.5 (normal range 5.8–20.5). Patients in the treatment groups were comparable in relation to baseline and IVF characteristics however, Groups II & IV exhibited less severe Th1/Th2 and CD56 abnormality pre-conceptionally (Table I). Groups I and III using Humira® experienced significant average Th1/Th2 decrease with immunotherapy. Group I (Humira+IVIG) experienced an average TNF-α:IL-10 decrease of 15.7 ± 11.1 S.D. (from 41.5 ± 8.5 to 25.8 ± 8.0; $P < 0.0001$) and IFN-γ:IL-10 decrease of 5.4 ± 8.9 (from 16.8 ± 8.1 to 11.4 ± 7.0; $P < 0.002$). Group III (Humira only) experienced an average TNF-α:IL-10 decrease of 19.5 ± 9.7 (from 42.0 ± 7.1 to 22.5 ± 3.4; $P = 0.0001$) and IFN-γ:IL-10 decrease of 3.7 ± 3.6 (from 11.8 ± 4.1 to 8.1 ± 3.2; $P = 0.11$). Follow-up Th1/Th2 testing was not performed on Group II (IVIG only) so there was no follow-up cytokine information available for this group.

The implantation rate (number of gestational sacs per embryo transferred, with an average of two embryos transferred by cycle) was 59% (50/85) in Group I, 47% (21/45) in Group II, 31% (4/13) in

Table I Characteristics of the Four Study Groups

	Group I (Humira+IVIG)	Group II (IVIG)	Group III (Humira)	Group IV (No treatment)	*P value (See comments below)
Number of cycles	41	23	6	5	
Baseline characteristics					
Maternal age (years)	33.5 ± 2.8	34.1 ± 3.2	34.7 ± 3.3	32.0 ± 1.9	0.40
Duration of infertility (years)	3.4 ± 1.4	4.2 ± 2.4	4.0 ± 1.4	2.3 ± 1.2	0.39
No. prior IVF failures	1.7 ± 1.8	0.8 ± 1.2	1.2 ± 1.6	1.6 ± 1.7	0.20
Previous pregnancies					
No. prior miscarriages	0.6 ± 0.9	0.4 ± 0.8	1.3 ± 1.4	None	0.08
No. prior live births	0.2 ± 0.6	0.2 ± 0.5	0.2 ± 0.4	0.4 ± 0.5	0.89
Average IVF parameters for index cycle					
Mean (S.D.) no. eggs	16.3 ± 4.9	15.8 ± 7.1	18.5 ± 5.4	20.0 ± 8.6	0.44
Mean (S.D.) no. 2PN embryos	10.8 ± 4.2	10.2 ± 34	11.7 ± 6.0	10.2 ± 0.8	0.84
Mean (S.D.) number Day 3 embryos ≥5 cells	8.8 ± 4.1	7.7 ± 2.7	10.8 ± 5.9	7.0 ± 1.4	0.24
Mean no. embryos replaced	2.1 ± 0.3	2.0 ± 0.2	2.2 ± 0.4	1.8 ± 0.4	0.07
Day of embryo transfer					
Day 3	3	1	0	0	
Day 5	38	22	6	5	
Immune test results					
TNF-α:IL-10	41.5 ± 8.5	36.2 ± 7.1	42.0 ± 7.1	33.3 ± 3.6	0.02
IFN-γ: IL-10	16.8 ± 8.1	11.0 ± 4.0	11.8 ± 4.1	7.0 ± 5.1	0.001
NK 50:1 cytotoxicity, %	16.9 ± 6.4	15.6 ± 6.9	11.8 ± 2.9	11.9 ± 2.4	0.14
CD 56^+, %	7.5 ± 4.7	10.4 ± 7.1	4.0 ± 1.8	3.6 ± 2.1	0.012
$CD19^+/CD5^+$, %	10.6 ± 8.2	11.3 ± 6.5	10.0 ± 7.5	7.2 ± 2.3	0.73
Success rates					
Implantation rate (No. gestational sacs per embryo transferred)	59% (50/85)	47% (21/45)	31% (4/13)	0%(0/9)	0.0007**
Clinical pregnancy rate (Fetal heart activity per IVF cycle)	80% (33/41)	57% (13/23)	50% (3/6)	0% (0/5)	0.0009**
Live birth rate	73% (30/41)	52% (12/23)	50% (3/6)	0% (0/5)	0.003**

All patients demonstrate pre-conceptional Th1/Th2 ratio elevation (TNF-α:IL-10 > 30.6 and/or IFNλ:IL-10 > 20.5), age <38 years and good IVF response at time of cycle (≥5 Day 3 embryos ≥5 cells).

*****Statistical analysis of variance for patient characteristics, IVF parameters and immune test results was performed using a one-way anova calculator (Danielsoper.com Statistics Calculators, version 2.0).

**Statistical analysis of success rates was performed using Fisher's exact test (Graphpad Software®). Groups I and IV were compared in this table.

Group III and 0% (0/9) in Group IV. The clinical pregnancy rate (fetal heart activity per IVF cycle started) was 80% (33/41) in Group I, 57% (13/23) in Group II, 50% (3/6) in Group III and 0% (0/5) in Group IV. The live birth rate was 73% (30/41) in Group I, 52% (12/23) in Group II, 50% (3/6) in Group III and 0% (0/5) in Group IV. There was a significant improvement in implantation, clinical pregnancy and live birth rates for Group II (IVIG) versus Group IV ($P = 0.009$, 0.04 and 0.05, respectively) and there was significant improvement in implantation rate, clinical pregnancy rate and live birth rate for Group I (Humira +IVIG) versus Group IV ($P = 0.0007$, 0.0009, and 0.003, respectively). There was a significant improvement in clinical pregnancy rate for Group I (Humira +IVIG) versus Group II (IVIG; $P < 0.05$), however the increase in implantation rate and in live birth rate did not reach significance comparing these two treatment groups ($P = 0.20$, 0.11, respectively). Also, the improvement in implantation, clinical pregnancy and live birth rates between Groups I and III and in Groups III and IV did not reach significance ($P = 0.075$, 0.10 and 0.34 and $P = 0.18$, 0.18 and 0.12, respectively).

It should also be noted that within the high cytokine Humira® plus IVIG group (Group I), the

patients with a history of at least two IVF failures (mean 3.6 ± 1.4) experienced a 100% (16/16) clinical pregnancy rate and an 88% (14/16) live birth rate with the Humira plus IVIG protocol. This is a remarkably high IVF success rate for a population with such a poor prior IVF history.

Discussion

We report data from an observational study showing that the use of a TNF-α inhibitor with intravenous immunoglobulin improves IVF success rates in young (<38 years) infertility patients with pre-conceptional Th1/Th2 elevation. These interventions were based on the Th1/ Th2 paradigm developed over the past two decades. Etanercept (Enbrel®; Amgen, Thousand Oaks, CA, USA) is a soluble TNF-α receptor that works in a similar way to Humira® by binding TNF-α. Etanercept has been used pre-conceptionally in women suffering from recurrent implantation failure and who had an elevation in their peripheral blood Th1/Th2 ratio.[15,16] Successful use of IVIG to treat recurrent IVF and implantation failure has been described.[17–20] Low-dose heparin may have an anti-inflammatory effect beneficial in patients with elevated Th1/Th2 ratios. Clark showed that Th1 cytokines trigger inflammatory as well as thrombotic processes within utero-placental blood vessels.[21] Moreover, Clark showed that such cytokines activated by danger signals can lead to activation of coagulation through effects upon endothelium which, in turn, lead to activation of FGL2, a prothrombinase implicated in human recurrent miscarriage.[22] Winger and Reed[23] suggest that elements of the coagulation cascade may aggravate the effects of inflammatory cytokines.

Recently, recognition of the importance of the innate immune system has challenged the pre-eminence of the Th1/Th2 paradigm.[24] The decidual NK cell plays a unique role in blood vessel remodeling at the implantation site.[25] Moreover, the inflammatory nature of implantation runs directly against the Th2 dominance envisioned in the Th1/Th2 paradigm. Interferon γ, a traditional Th1 cytokine, is an important NK activator needed for vascular bed remodeling.[26] Moreover, it has also been suggested that use of anti-inflammatory agents at the time of implantation might actually interfere with the appropriate orchestration of the implantation site cytokine milieu which did not appear to occur in this study.[27]

The decidual NK cell differs from its peripheral blood counterparts. Like the peripheral blood CD56 bright NK cell, they function as cytokine producers; however, like the CD56 dim peripheral blood NK cell, they are also armed with cytotoxic granules. The dual potential of the decidual NK cell permits it to serve separate roles in pregnancy. Under normal conditions, decidual NK cells can produce factors supportive of placentation. However, under other conditions, like infection or chronic disease, these same NK cells may be activated to function as cytotoxic cells contributing to pregnancy loss.

Unlike T cells that inherit a single antigen receptor, immature NK cells randomly inherit a complement of receptors that act together in determining activation status upon encounter with fetal cell-associated MHC. The balance between activating and inhibitory receptors engaging target cell ligands determines the NK cell activation state. Like T cells, self-reactive NK cells are eliminated prior to maturation. No such truce, however, prevails between fetal MHC and the maternal complement of NK receptors. Decidual NK cells become activated upon their encounter with fetal MHC. Hiby found women with highly suppressive NK receptor haplotypes are more likely to develop pre-eclampsia than those of the general population. Diminished decidual NK cell activation expected in these women may result in diminished pro-angiogenic activity.[28]

While the Hiby data might suggest inhibitory interactions have a negative effect on placentation, other investigators have found the opposite association in recurrent miscarriage.[29,30] Recognition of distinct and different roles in which the decidual NK cell participates may resolve this apparent paradox. Activation pathways leading to cytokine production or cytotoxic activity are separable. Activation of the decidual NK cell into a pro-angiogenic, cytokine-producing or a cytotoxic phenotype occurs along separable and distinct pathways.[31–33] The addition of a 'danger signal' to the decidual environment may alter the phenotype of the decidual NK cell. Infection may transform the placental supportive decidual NK cell into a cytotoxic phenotype terminating pregnancy.[34] It would appear that pre-conception 'maternal-only' factors may provide an additional signal capable of redirecting the decidual NK cell activation pathway.

The Th1/Th2 ratio of peripheral blood T-helper cells was used in this study to identify candidates for immunotherapy. Numerous investigators have found that a high pre-conceptional Th1/Th2 ratio has been

associated with subsequent recurrent implantation failure and miscarriage.[35,36] High pre-conceptional NK peripheral blood numbers have also been associated with subsequent miscarriage.[9,10] The success reported in this study with the use of TNF-α inhibitors either alone or in combination with intravenous immunoglobulin suggests improvement in both implantation and live birth rates with immunotherapy (Table I).

Variation of the time period between the last dose of Humira® and embryo transfer may have provided us with insight as to its mechanism of action. As the interval progressively increased in length, one might have expected its efficacy to diminish, were its action an effect upon the orchestration of the cytokine network at the implantation site, but diminished efficacy was not observed. Humira® has a half-life of approximately 2 weeks.[37] Because the drug has no direct pharmacologic effect, acting only to deplete TNF-α, the drug has no biologic effect when its concentration is insufficient to decrease the level of active cytokine. In a significant patient cohort, there was a washout period of 5–6 half-lives (time period between the last dose of Humira® and embryo transfer 90–120 days; 12 patients). In this group, any safety issue regarding the use of a TNF-α inhibitor becomes moot. Moreover, concerns regarding adverse effects of the drug on the cytokine network at the implantation site required for successful implantation, likewise, become moot.

What then can explain the positive effects on implantation success demonstrated by these data? Direct effects on the endometrium are improbable because of monthly shedding and regeneration. A systemic or regional effect, therefore, seems probable. An effect at the level of a long-lived, pathogenic cell population or network responsive to TNF-α might be suspected. Interaction of a stable, proinflammatory cell population with uterine NK cells might provide a danger signal directing activation along the cytotoxic pathway. Candidate long-lived cell populations might include those discussed by Saito[38] and Winger.[39] Moreover, in an observational study, Clark found that patients with elevated numbers of NKT cells were most likely to respond to IVIG therapy.[40] Further, dendritic cells may participate in long-lived cell networks. Blois suggests the ultimate maturation state of dendritic cells may determine tolerance or rejection of fetal expressed antigens.[41] TNF-α drives dendritic cells to their fully mature, immunogenic state. Finally, Th17 cells have recently been recognized as a unique lineage of T-helper cells that mediate inflammatory responses.[42] Cellular networks involving paracrine and autocrine secretion of TNF-α might serve to stabilize such long-lived cell populations or networks and be amenable to anti-TNF-α therapy. Further study directed toward defining these cell populations is indicated.

Our study is the first to examine the success rate for an IVF subgroup defined by a pre-conceptional Th1/Th2 ratio elevation (TNF-α:IL-10 > 30.6 and/or IFNλ:IL-10 > 20.5), age <38 years, good IVF response at time of cycle (≥5 Day 3 embryos ≥5 cells). No other center has examined success rates for a similarly selected IVF group. We have essentially eliminated all the non-immune causes of infertility (by eliminating patients with poor eggs, older age, etc). In addition, we have required that all patients have a pre-conceptional cytokine ratio at least one standard deviation above the mean for a fertile population. The dramatic nature of these results emphasizes a need to further analyze this data. With more peer discussion and follow-up research, better understanding about the mechanism can be gained. Despite its small size and pilot nature, the data is significant and should be discussed and shared in a public format. In conclusion, our observations suggest that immune treatment can offer hope to a subgroup of young patients suffering unexplained infertility. We suggest that these observations merit further examination in larger clinical trials.

Details of Ethics Approval

Retrospective analysis of clinical data. All patients signed standard clinical IVF consent forms plus detailed consent forms (one for Humira® and one for IVIG) explaining the nature of the medicine, the possible risks and the lack of proof for clear evidence-based determination of efficacy.

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