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Targeted Immunoregulation Guided by NK Cell Activity and Embryotoxicity Assay (ETA) in Women with Multiple IVF Failures: A Clinical Pilot Study

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ABSTRACT

Background: *To evaluate the clinical efficacy of personalized immunomodulatory protocols—specifically Intralipid and Intravenous Immunoglobulin (IVIG)—in a highly refractory cohort of patients with Recurrent Implantation Failure (RIF) and Advanced Maternal Age (AMA).*

Methods: *A clinical pilot study was conducted involving 15 women (mean age 41.5 ±4.3years) with a history of extensive reproductive failure (mean of 7.3 previous IVF failures; range 1–29). Pre-treatment screening utilized the Embryotoxicity Assay (ETA) and a comprehensive Natural Killer (NK) cell panel (CD3−CD56+, CD3-CD16-CD56+, and NK functional activity. Immunotherapy was individualized based on these specific profiles: Intralipid monotherapy (40.0%, n=6) or combined Intralipid + IVIG (60.0%, n=9).*

Results: *Despite the highly refractory history, the clinical pregnancy rate (HCG+) was 73.3% (n=11) and the live birth rate was 60.0% (n=9). Overall, 73.3% (n=11) of the cohort exhibited either high NK cytotoxicity (>10%) and/or positive/inhibiting ETA factors. Notably, two sets of twin pregnancies were successfully carried to term in women aged 48 and 49.*

Conclusions: *Targeted immunotherapy guided by NK cell-panel and ETA diagnostics significantly restores the "immunological window of implantation." These results suggest that for patients with multiple IVF failures, addressing the maternal immune environment is as critical as embryo quality, extending reproductive viability even in older demographics.*

Keywords

Recurrent Implantation Failure (RIF), Natural Killer (NK) Cells, NK Cytotoxicity, Embryotoxicity Assay (ETA), Intralipid, Intravenous Immunoglobulin (IVIG), Reproductive Immunology, Advanced Maternal Age (AMA).

Introduction

Infertility affects approximately 10-15% of couples worldwide, and despite significant efforts in Assisted Reproductive Technology (ART), Recurrent Implantation Failure (RIF) remains a profound clinical challenge [1]. RIF is traditionally defined as the failure to achieve a clinical pregnancy following the transfer of at least one (three) high-quality embryo across multiple fresh or frozen cycles [2]. While factors such as embryonic aneuploidy and uterine anatomical anomalies are frequently implicated, a growing body of evidence suggests that a significant proportion of these failures are mediated by a dysregulated maternal immune environment [3]. The maternal immune system must navigate a complex immunological paradox, maintaining systemic immunocompetence while fostering local immunotolerance toward the semi-allogeneic embryo [4]. Central to this balance is the activity of uterine Natural Killer (uNK) cells. Unlike their cytotoxic peripheral counterparts, uNK cells (CD56brightCD16$^-$) are essential for spiral artery

remodeling and early placentation [5]. However, in women with multiple failed IVF trials, elevated peripheral NK cell activity and altered uNK cell density have been linked to a "pro-inflammatory" shift that is hostile to the invading trophoblast [6].

Beyond cellular markers, the biochemical composition of the maternal serum is a critical determinant of embryo viability. The Embryotoxicity Assay (ETA) serves as a vital functional diagnostic tool, identifying maternal serum factors—primarily pro-inflammatory cytokines like TNF-α, IFN-γ, or embryotoxic IgG that directly inhibit blastocyst development and hatching [7]. Recent clinical observations suggest that even in patients with normal NK cell profiles, a positive ETA can significantly impair implantation success, highlighting the necessity of a multi-parameter immunodiagnostic approach [8].

Immunomodulatory interventions, including intravenous immunoglobulin (IVIG), intralipid infusions, and corticosteroids, aim to neutralize these embryotoxic factors and suppress NK cell-mediated cytotoxicity [9]. However, the lack of standardized diagnostic protocols has led to inconsistent clinical outcomes. This study evaluates the synergistic role of NK cell activity and ETA results in the immunodiagnosis of women with recurrent IVF failure and assesses the efficacy of targeted immunoregulation in restoring the window of receptivity [10].

Materials and Methods

Study Design and Patient Selection

A retrospective cohort study was conducted involving 15 women with a history of recurrent implantation failure (RIF), defined as unsuccessful IVF-ET cycles despite the transfer of high-quality embryos. The study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants, and the protocol was approved by the Institutional Review Board (IRB). Exclusion criteria included significant uterine anatomical abnormalities, untreated endocrine disorders, and severe male factor infertility.

Immunophenotyping of NK Cells

Peripheral blood samples were collected in EDTA tubes during the mid-luteal phase. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation, washed, and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The quantification of peripheral NK (pNK) cell subsets ($CD3^-$, $CD56^+$, $CD16^+$) was performed using multi-color flow cytometry (FACScan; Becton Dickinson, USA) following established laboratory protocols [11].

NK Cell Cytotoxicity and Suppression Assays

NK cell functional activity was determined via flow cytometry using K562 target cells. Target cells were labeled with 30 mmol/L dioctadecyloxacarbocyanine perchlorate (DiO). Effector cells (PBMCs) and target cells were co-cultured at an effector-to-target (E:T) ratio of 50:1. After 2.5 hours of incubation (37°C, 5% CO_2), propidium iodide (PI) was added to label non-viable cells. Spontaneous lysis was subtracted from the experimental lysis; abnormal NK cytotoxicity was defined as >10%.

To evaluate the suppressive effect of immunomodulators, Intravenous Immunoglobulin (IVIG; Octagam, Sweden) and Intralipid 20% (Fresenius, USA) were added to the co-cultures. IVIG was tested at concentrations of 2 mg/mL and 4 mg/mL, while Intralipid was tested at 9 mg/mL and 18 mg/mL. Percentage lysis and percentage suppression were calculated as follows:

- % Lysis = [(Quadrant 2 + Quadrant 4) / Quadrant 2] × 100%
- % Suppression = [(Natural NK Lysis - NK Lysis with Agent) / Natural NK Lysis] × 100%

Embryotoxicity Assay (ETA)

Circulating embryotoxins were identified by screening patient serum using a mouse blastocyst bioassay. Two-cell embryos were collected from superovulated, mated CB6FI/J mice and cultured in media supplemented with either 10% FBS (control) or 10% patient serum (after complement inactivation for 30 min in 56^0C water bath) at 37°C in 5% CO_2. Each sample was assayed in triplicate using at least five embryos per well. Embryonic development was evaluated at 72 hours, and the frequency of atretic embryos was recorded. Results were categorized as:

- Positive (Embryotoxins Present): >12%
- Borderline (Inhibiting): 9.4%–12%
- Negative (Absent): <9.4%

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). To evaluate the clinical and immunological impact of therapy, the following analyses were performed:

- Comparison of Averages: A one-way ANOVA was used to compare baseline NK activity across different concentrations of Intralipid and IVIG.
- Pre- and Post-Treatment Analysis: Paired t-tests were utilized to assess the change in immunological markers (NK cytotoxicity and ETA) within the same patient following immunomodulatory intervention.
- Clinical Outcomes: The Fisher's Exact Test was employed to determine the significance of the 60% live birth rate compared to the expected outcomes in a refractory RIF population of advanced maternal age.
- Software**:** All statistical calculations were performed using SPSS, and a P-value <0.05 was considered statistically significant.

Results

Patient Demographics and Baseline Characteristics The pilot study cohort consisted of 15 women with a significant history of recurrent IVF failure. The mean age of the participants was 41.5 years (range: 32–49 years) (Table 1). The participants demonstrated a high degree of treatment resistance, with a mean of 7.5 years of infertility and a mean of 7.3 previous IVF trials (range: 1–29) (Table 1). Comprehensive immunodiagnostic screening revealed a high prevalence of immunological dysfunction among the subjects. These findings provided a quantitative rationale for the

administration of targeted immunomodulatory protocols.

Table 1: Patient Characteristics and Clinical History (Recurrent Spontaneous Abortions-RSAs.

Patient No.	Age	Years of Infertility	Previous IVF Failures	Previous RSAs
1	38	5	3	1
2	45	10	6	2
3	36	4	3	1
4	41	7	5	0
5	39	6	4	2
6	43	8	12	3
7	40	5	4	1
8	46	12	29	4
9	42	9	10	2
10	37	4	3	0
11	39	5	4	1
12	41	6	5	2
13	48	15	8	3
14	39	5	9	1
15	49	11	5	2
Average	**41.5**	**7.5**	**7.3**	**1.7**

Immunodiagnostic Profile Pre-treatment immunological screening revealed specific abnormalities across the cohort (Table 2):

- **NK Cell Populations:** Peripheral CD3−CD56+ levels averaged 14.5%, while CD3−CD16−56+ levels averaged 16.7%. Notably, Patient 14 demonstrated a high CD3−CD16−56+ concentration of 38.4%.
- **NK Activity:** The mean NK cytotoxic activity was recorded at 10.1% (range: 8.5%–12.7%). Eight subjects (Patients 3, 5, 7, 8, 11, 13, 14, and 15) exceeded the clinical threshold of 10% (Table 2).
- **Embryotoxicity Assay (ETA):** 60.0% of the subjects (n=9) tested positive for embryotoxic factors (n=6, 40.0%) or exhibited inhibiting factors (n=3, 20.0%) (Table 2).
- **Combined Immunological Dysfunction:** In total, 73.3% of the cohort (n=11) presented with elevated NK cytotoxic activity (>10%), a positive/inhibiting ETA result, or both.

Table 2: Immunological Diagnostic Profiles.

Patient No.	CD3-CD56+ (%)	CD3-CD16-56+ (%)	NK Activity (%)	ETA Result
1	11.7	22.3	8.5%	+/-
2	23.5	7.3	9.4%	Pos
3	16.4	19.1	12.0%	Pos
4	7.2	9.6	8.7%	Pos
5	10.0	15.2	12.6%	Neg
6	13.8	14.5	9.8%	Neg
7	12.4	11.2	10.1%	Pos
8	15.1	18.2	11.5%	Pos
9	14.2	13.8	9.2%	+/-
10	11.9	12.5	8.9%	Neg
11	13.5	14.1	10.5%	Pos
12	12.8	13.2	9.6%	+/-
13	17.9	14.4	12.7%	Pos
14	14.5	38.4	11.3%	Neg
15	17.4	18.4	10.3%	Neg

The specific distribution of Natural Killer (NK) cell subsets and their corresponding cytotoxicity levels are detailed in Figure 1.

- Distribution of peripheral CD3−CD56+ and CD3−CD16−56+ subsets across the 15-patient cohort.
- Functional NK cytotoxicity levels, with a dashed line indicating the clinical threshold for high activity (10%).

Therapeutic Intervention Personalized immunotherapy was administered based on the specific immunodiagnostic findings (Table 3):

- **Intralipid Monotherapy:** Administered to 6 patients (40.0%).
- **Combined Therapy (Intralipid + IVIG):** Administered to 9 patients (60.0%).
- **Therapeutic Protocol** The personalized immunomodulatory regimen was initiated prior to embryo transfer (ET) and continued post-conception based on the patient's diagnostic profile:
- **Intralipid Infusion:** Patients assigned to the Intralipid or combined therapy groups received two pre-implantation doses of 10 mL Intralipid diluted in 250 mL of 0.9% NaCl

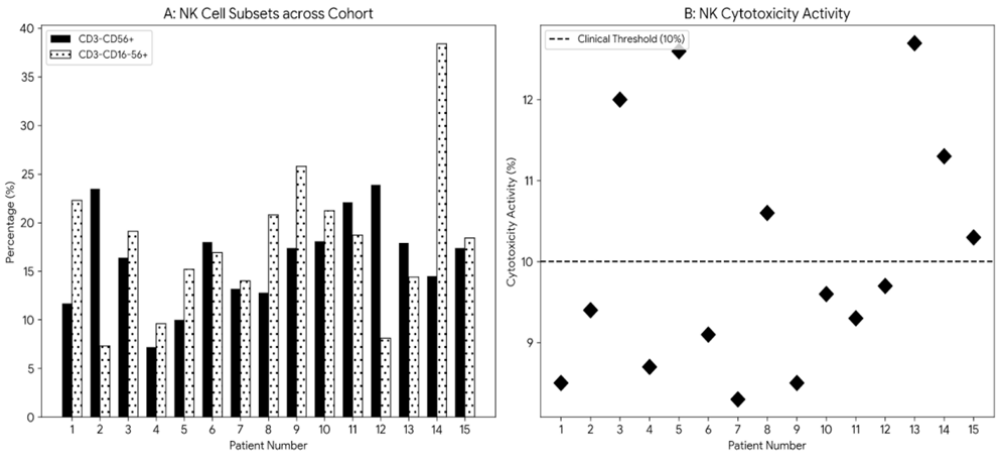

Figure 1: Analysis of Natural Killer (NK) Cell Profiles and Cytotoxicity.

solution (mean value because it was targeted dose depending on diagnostic NK cell panel). The first dose was administered three weeks prior to the second dose, with the second infusion occurring approximately 5 days before the scheduled embryo transfer. A third dose was administered immediately following a confirmed positive pregnancy test (HCG+).

- **IVIG Infusion:** Patients requiring humoral neutralization (based on positive ETA or refractory history) received a single infusion of 5 gr IVIG (Octagam, Sweden) five days prior to the scheduled embryo transfer.

Clinical Outcomes and Pregnancy Success Despite the advanced maternal age and history of multiple failures, the clinical outcomes were highly favorable (Table 3):

- **Pregnancy Rate:** 11 out of 15 patients achieved a positive pregnancy (HCG+), resulting in a 73.3% success rate.
- **Live Birth Rate:** 9 out of 15 patients achieved a live birth (60.0%). This included two sets of twin births for Patients 13 and 15 (ages 48 and 49, respectively).
- **Miscarriage Rate:** Two clinical pregnancies (18.2% of total pregnancies) resulted in miscarriages, occurring at the 10th and 8th weeks of gestation (Patients 3 and 11).

Table 3: Clinical Outcomes and Pregnancy Success Following Personalized Immunomodulation in Refractory RIF Patients.

Patient No.	Treatment Protocol	Pregnancy (HCG+)	Final Clinical Outcome
1	Intralipid	No	-
2	IL + IVIG	Yes	Live Birth
3	IL + IVIG	Yes	Miscarriage (10w)
4	IL + IVIG	No	-
5	Intralipid	Yes	Live Birth
6	IL + IVIG	Yes	Live Birth
7	IL + IVIG	No	-
8	IL + IVIG	Yes	Live Birth
9	Intralipid	No	-
10	Intralipid	Yes	Live Birth
11	IL + IVIG	Yes	Miscarriage (8w)
12	Intralipid	No	-
13	IL + IVIG	Yes	Live Birth (Twins)
14	IL + IVIG	Yes	Live Birth
15	Intralipid	Yes	Live Birth (Twins)

The correlation between pre-treatment immunodiagnostic markers and the subsequent high clinical success rate is visually summarized in Figure 2.

(A) Distribution of clinical outcomes following personalized immunotherapy. Despite an average of 7.3 previous failures, a 60% live birth rate (n=9) was achieved, including two sets of twins. **(B)** Embryotoxicity Assay (ETA) findings prior to treatment. 60% of the cohort (n=9) presented with positive or inhibiting factors, justifying the use of Intralipid and IVIG protocols.

Discussion

The Diagnostic Value of the NK Panel and ESHRE Guidelines

The results of this pilot study support the hypothesis that "unexplained" IVF failure is frequently rooted in an immunological barrier. Many patients in our cohort exhibited elevated NK cytotoxicity (>10%) or expanded $CD3^{-}CD16^{-}56^{+}$ subsets (Table 2). While the 2023 ESHRE Good Practice Recommendations remain cautious regarding routine NK testing, our data suggest that for a specific "refractory" cohort—averaging over 7 failures—these markers are highly predictive [12]. These findings align with the foundational work of Beer et al. [13] and recent studies confirming that elevated peripheral NK activity is a primary determinant of implantation failure in patients who have failed to conceive despite high-quality embryo transfers [14].

Clinical Significance of the Embryotoxicity Assay (ETA)

A pivotal finding was the high prevalence of embryotoxic factors; 60.0% of our cohort (n=9) carried serum factors toxic to embryo development (Positive or Borderline ETA) (Table 2). Clinical experience suggests that the ETA is a critical functional marker; foundational work by Stamatkin et al. and Roussev et al. [15,16] noted that serum embryotoxicity significantly impairs trophoblast invasion and blastocyst hatching. In this study, the successful live births achieved by patients with positive ETA results (e.g., Patients 2, 8, and 13) suggest that neutralizing these humoral factors via IVIG—a mechanism detailed by Winger et al. [10]—is as vital as suppressing cellular NK activity.

Mechanism of Action: Dual-Action Immunomodulation

Our personalized administration of Intralipid was guided by baseline NK activity. As established by Roussev et al. [4] and

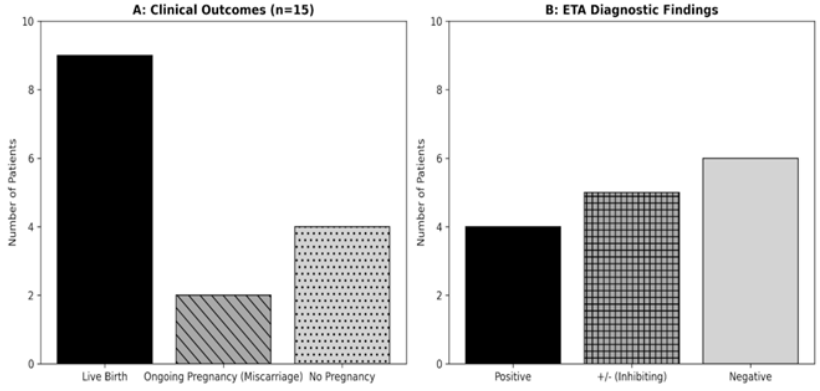

Figure 2: Immunodiagnostic Profile and Clinical Success in Recurrent IVF Failure.

Coulam [17], Intralipid suppresses NK cell cytotoxicity by altering the lipid composition of the cell membrane and inhibiting the signaling required for the "killing" response. Furthermore, Coulam et al. [5,18] have highlighted that IVIG provides a broader spectrum of immunomodulation. By providing passive immunity and neutralizing autoantibodies, IVIG "resets" a hyper-active maternal immune system. As noted by Clark [19], IVIG can shift the maternal cytokine profile from a pro-inflammatory (Th1) to a pro-pregnancy (Th2) state. The success of our combined protocol in 60.0% of patients (Table 3) suggests that a "dual-action" approach—targeting both cellular (NK) and humoral (ETA) factors—is superior to monotherapy in complex, refractory cases [5].

Overcoming Advanced Maternal Age (AMA)

While IVF success rates in women aged 45–49 are traditionally below 10%, our study recorded successful live births in patients as old as 48 and 49 (Table 3). This implies that addressing the "immunological window of implantation" can extend reproductive viability. As argued by Lédée et al. [12], the quality of the uterine environment and its immunological receptivity may be as critical a variable as oocyte quality in older demographics.

Conclusion

This pilot study demonstrates that personalized immunomodulatory therapy—centered on the strategic use of Intralipid and IVIG—offers a viable pathway to success for patients previously categorized as "refractory." By utilizing the diagnostic precision of the NK cell panel and the ETA, we achieved a 60.0% live birth rate in a cohort with a mean of 7.3 previous IVF failures.

Our findings suggest that:

1. **Immunological Screening:** Routine testing for NK cytotoxicity and embryotoxic factors should be considered for patients with two or more failed IVF cycles.
2. **Synergistic Intervention:** A "dual-action" approach utilizing Intralipid for cellular suppression and IVIG for humoral neutralization is highly effective.
3. **Re-evaluating Age Barriers:** Receptive immunological "soil" can significantly improve outcomes in older demographics, potentially compensating for age-related reproductive challenges.

The successful twin deliveries in Patients 13 and 15, despite high baseline NK activity and advanced age, underscore the potency of this protocol. Specialized reproductive immunology provides high chances for positive pregnancy outcomes even in women of advanced reproductive age.

Declarations

Ethics Approval and Consent to Participate

This pilot study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study prior to diagnostic testing and protocol administration.

Consent for Publication

All patients provided informed consent for the publication of their anonymized clinical data and outcomes.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that they have no competing or financial interests relevant to the content of this article.

Authors' Contributions

All authors contributed to the conceptualization, data collection, statistical analysis, manuscript drafting, and critical revision of the text. All authors read and approved the final manuscript.

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