Immunotherapy to improve pregnancy outcome in women with abnormal natural killer cell levels/activity and recurrent miscarriage or implantation failure: a systematic review and meta-analysis

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Abstract

There is a trend towards offering immunotherapy to women with unexplained reproductive failure based on abnormal Natural Killer (NK) cell levels. Previous systematic reviews evaluating immunotherapy usage have not focused on women with abnormal level of NK cells. To address the gap in literature, this systematic review aims to evaluate the efficacy of immunotherapy to improve pregnancy outcome in women with recurrent miscarriage (RM) or implantation failure (RIF) specifically selected based on abnormal levels and/or activity of NK cells. Six databases were searched for peer-reviewed studies following PRISMA guidelines. Risk of bias assessment was conducted using RoB2 for randomized controlled trials (RCT) and ROBINS-I for non-RCT. Of 1025 studies identified, seven studies on intravenous immunoglobulin (IVIG) (four), prednisolone (one), etanercept (one) and intralipid (one) were included. Meta-analysis of the non-RCT IVIG studies (557 participants; 312 intervention, 245 controls) showed livebirth in favour of intervention (RR 2.57; 95% CI= 1.79-3.69; $p<0.05$), however there were significant heterogeneity ($I^2=62\%$) and moderate to severe risk of bias in these studies. Individual RCTs reported improved livebirth outcome in etanercept, intralipid and prednisolone and this was significant in the former two ($p<0.05$). In conclusion, there may be some benefit of immunotherapy, but paucity of high quality evidence means that it is not possible to support the use of immunotherapy even when selected based on abnormal NK cell level/activity. Further research with application of scientifically validated immunological biomarkers in well-planned large scale RCTs will determine whether immunotherapy is beneficial in this subpopulation of women.

Keywords:

Recurrent miscarriage; Recurrent implantation failure; Natural killer cells; NK cells cytotoxicity; Immunotherapy; IVIG

1. Introduction

Reproductive failure causes significant emotional distress that has a major psychological impact on couples. In the past two decades, there has been much research in the role of Natural Killer (NK) cells in poor reproductive outcomes, such as recurrent miscarriage (RM) or recurrent implantation failure (RIF). Despite controversial evidence, many reproductive clinics have been offering immunotherapy to these groups of patients on the basis of abnormal levels and/or activity of NK cells.(Moffett and Shreeve, 2015)

RM affects 1-2% of couples trying to conceive and is defined as either loss of two or more consecutive pregnancies (American Society for Reproductive Medicine, 2020) or three or more pregnancies (RCOG *Green-top Guideline No. 17*, 2014) before 24 weeks of pregnancy. There are known aetiologies underlying RM (previous history of miscarriage, thrombophilia, antiphospholipid syndrome, genetic cause, anatomical malformation, endocrine pathology, infection) but in 50% of the cases no parental pathology is identified.(Rai and Regan, 2006) A proportion of unexplained RM is accounted for by fetal aneuploidy in up to 39% of the cases.(van den Berg *et al.*, 2012) After excluding this, the remaining proportion of unexplained RM have genuine pathological conditions not detected by routine investigations such as sperm abnormalities, poor endometrial receptivity or immunological dysfunction.(Saravolos and Regan, 2014)

RIF affects 10% of patients undergoing *in vitro* fertilization (IVF).(Bashiri *et al.*, 2018) Presently, the most complete working definition of RIF is “failure to achieve clinical pregnancy after transfer of at least four good quality embryos in a minimum of three fresh/frozen cycles in a woman who is less than 40 years old”.(Coughlan *et al.*, 2014) The underlying causes for RIF may include poor embryo quality, sperm or oocyte quality, tubal or uterine anomaly (hydrosalpinges, uterine polyps, adhesions, congenital uterine anomalies, intramural fibroids), infection (chronic endometritis), thrombophilic conditions (antiphospholipid syndrome) or immunological dysfunction.

The term “reproductive failure” was coined on the basis of studies investigating molecular mechanisms of RIF and RM that suggested a common pathogenic pathway.(Makrigiannakis *et al.*, 2011) In order to achieve successful blastocyst implantation in the maternal decidua, followed by deep placentation, a complex interplay of hormonal and immunological factors

are involved to ensure optimum endometrial receptivity. Thus, immune dysregulation is postulated to contribute to defective implantation and deep placentation resulting in problems in pregnancy such as recurrent miscarriage, pre-eclampsia, intrauterine growth restriction (IUGR) and preterm labour collectively termed the “Great Obstetrical Syndromes”.(Brosens *et al.*, 2011)

NK cells account for up to 70% of total immune cells in the endometrium during the luteal phase of the menstrual cycle and in the decidua in the first trimester of pregnancy. They have been suggested to play a critical role in the success of pregnancy. Uterine NK cells (uNK) have long been known to differ from peripheral blood NK cells (pNK) in both phenotype and function: uNK are less cytotoxic than their peripheral counterparts (King *et al.*, 1989; Koopman *et al.*, 2003) and produce factors that mediate placental implantation and tissue remodelling (Hanna *et al.*, 2006; Lash *et al.*, 2006). Immunogenetic evidence points to a relationship between specific combination of maternal activating killer-like immunoglobulin receptors (KIR) on decidual NK cells and fetal HLA-C haplotype being a key determinant factor of successful embryo implantation, suggesting that uNK activation promotes successful placentation.(Hiby *et al.*, 2004, 2010; Nakimuli *et al.*, 2015)

The picture based on studies of the uNK in reproductive failure themselves is less clear, but cumulative evidence does not support the theory of NK cells effecting its action by killing trophoblast cells.(Moffett and Colucci, 2014) If we take the view that uNK promote placentation, low uNK numbers or activity might be expected to be harmful because defective placentation is associated with problems in pregnancy, including miscarriage.(Brosens *et al.*, 2011) In support of this hypothesis, two studies reported a reduction in NK cells with a uterine CD56^{bright}CD16⁻ phenotype in infertile patients, and a concomitant increase in NK cells with a circulating CD56^{dim}CD16⁺ phenotype (Lachapelle *et al.*, 1996; Fukui *et al.*, 1999), but another study reported the contrary (Matteo *et al.*, 2007). On the other hand, it has also been suggested that high uNK numbers may also be deleterious, since uNK density correlates with increased angiogenesis and this could be associated with early establishment of placental circulation, leading to pregnancy failure secondary to oxidative stress.(Quenby *et al.*, 2009) In support of this hypothesis, studies have shown a correlation between raised levels of uNK and reproductive failure.(Tuckerman *et al.*, 2007; Venkatakrishnan *et al.*, 2013; Chen *et al.*, 2017)

There is an abundance of clinical studies on peripheral NK cells, but these have been controversial at best with some reporting significantly raised pNK in RIF and RM (Yamada *et al.*, 2003; King *et al.*, 2010; Sacks *et al.*, 2012), and others reporting no correlation (Thum *et al.*, 2004). Further, two systematic reviews (Tang *et al.*, 2011; Seshadri and Sunkara, 2014) concluded that further research was required before recommending measurement of NK cells as a diagnostic tool for infertility and RM.

Immunotherapies used to treat women with RM and RIF reported in the literature include prednisolone, intravenous immunoglobulin (IVIG), intralipid, granulocyte-macrophage colony stimulating factor, anti-TNF alpha, and lymphocyte immunization therapy. Polanski *et al.* (2014) conducted a systematic review in infertile women undergoing assisted reproductive technology (ART) with elevated NK cells, and found some benefit of immunotherapy on ART outcome. However due to paucity of high quality evidence, the authors did not support the use of immunotherapy. The latest Cochrane Database Systematic Review (CDSR) of twenty three high quality trials concluded that immunotherapy provided no significant benefit over placebo to increase livebirth rate in RM. (Wong and Porter, 2014) This was supported by another more recent systematic review by Achilli *et al.* (2018) which concluded that there was no role for immunotherapy to improve pregnancy outcomes in women with RM or infertile women. However, the studies included in both of these systematic reviews were not specific to patients with abnormal levels of NK cells.

There is a current gap in the literature for a systematic review specifically reporting on efficacy of immunotherapy in the subpopulation of women with RM or RIF found to have abnormal NK cell level and/or activity. A review focusing on this question would shed some light on claims that the reason for the mixed and inconclusive results that have been reported in RM and RIF patients is because not all of these studies included women with dysregulated NK cells, and therefore only a subpopulation of patients would have been expected to benefit. In view of this, we aimed to perform a systematic review to evaluate : i) the effect of immunotherapy on pregnancy outcomes in women with RIF or RM and abnormal NK cells level and/or activity compared to no intervention or placebo; ii) the effect of immunotherapy on NK cell level and/or activity; iii) maternal and fetal adverse outcomes after immunotherapy.

2. Methodology

2.1 Protocol Registration

The protocol for this review has been registered with the International Prospective Review of Systematic Reviews (PROSPERO). The registration number is CRD42019155346.

2.2 Eligibility Criteria

Studies were included in our review if they fulfilled the following criteria: i) RCT, case-controlled or cohort studies that reported livebirth outcomes; ii) population of interest were women with RM or RIF with confirmed abnormal level of NK cells level and/or activity iii) intervention in the form of immunotherapy before or after confirmation of pregnancy; iv) presence of a control group that received placebo or no intervention; and. No restriction was specified on the definition of abnormal NK cells as there is no current consensus within the scientific community regarding reference range of NK cells.

All English language peer-reviewed human studies that fulfilled our inclusion criteria were included. We excluded conference abstracts, studies with no control groups or control groups with no raised NK cells. A full list of inclusion and exclusion criteria can be found in Supplementary Table 1 and studies excluded after full-text screening in Supplementary Table 2.

2.3 Information Sources and Search Strategy

A combination of Medical Subject Headings (MeSH) terms and keywords was used to generate subsets of citation for NK Cells ("Natural killer cells" or "NK cells" OR "Natural killer T cells" OR "uNK" OR "dNK" OR "pNK" OR "CD56"), immunotherapy ('immunotherapy OR 'prednisolone' or 'steroids' or 'intravenous fat emulsion' OR 'intralipid' OR 'tumour necrosis factor' OR 'intravenous immunoglobulin' OR IVIG), RM ('Spontaneous abortion" OR 'habitual abortion' OR 'miscarriage' OR 'pregnancy loss' OR 'fetal loss' OR 'RM') and RIF ('implantation failure OR RIF). The full search strategy is listed in Supplementary Table 3.

Electronic bases searched included MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials (CENTRAL), Web of Science and Google Scholar, clinicaltrials.gov and

the World Health Organization International Trials Registry Platform. To ensure literature saturation, hand searching of references and citations from similar systematic reviews and primary papers was performed which yielded 31 papers. The citations were uploaded to Covidence (2020) for screening and synthesis.

2.4 Selection Process

The selection process was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA, 2009) guidelines by two independent reviewers (EW and AD). After an initial search and de-duplication, a total of 1025 studies were identified for title and abstract screening. Full text screening for eligibility was conducted for 25 studies, and 18 studies were excluded resulting in 7 studies fulfilling our inclusion criteria. An overview of the screening process is displayed in the PRISMA flow diagram (Figure 1). If any clarification was needed regarding eligibility of the study, the author of the paper was contacted. Any disagreements were resolved by consensus or consulting the senior author.

2.5 Data Collection and Data Items

Data was extracted independently into a spreadsheet by EW and AD after study parameters were agreed by all the authors. Information selected included author details, year of publication, country of study, study aim and primary outcome, sample size, definition of reproductive failure (RM or RIF), source and method of measurement of NK cells, reference range used, intervention details (type, frequency of administration and dose of drug), follow up length, pregnancy outcome (livebirths, miscarriage rate, clinical pregnancy rate), adverse fetal and maternal outcomes and NK cell level/activity after intervention.

2.6 Risk of Bias

Risk-of-bias tool for randomized trials (RoB 2) was used to assess the quality of randomized controlled trials (RCT). (Higgins *et al.*, 2011) The methodological quality of non-randomized studies of the effects of intervention (NRSI) was assessed using ROBINS-I tool. (Sterne *et al.*, 2016) Quality assessment for each study was performed independently by EW and AD. Any disagreement was resolved by consulting the third author (TBM).

2.7 Summary Measures and Synthesis of Results

For every considered outcome, risk ratio with confidence interval of 95% was used as the summary measure. P value of <0.05% was considered significant. When appropriate, meta-analysis was performed using Review Manager (RevMan) version 5.3 (2014). A fixed effect

model was used for $I^2 < 50\%$ and in the presence of significant heterogeneity with $I^2 > 50\%$, a random effect model was used.

3. RESULTS

3.1 Study and Patient Characteristics

Seven studies were selected for analysis. Three studies were RCTs for prednisolone (Tang *et al.*, 2013), intralipid (Dakhly *et al.*, 2016) and etanercept (Fu *et al.*, 2019). The remaining four studies were non-randomised studies on IVIG (Ramos-Medina *et al.*, 2014; Chernyshov *et al.*, 2016; Ahmadi *et al.*, 2017, 2019).

Differences were identified in the definition of RM and RIF, mode of conception, type and regime of intervention, and follow up protocols. One study measured uNK (Tang *et al.*, 2013), whereas the other six studies used pNK cell frequency and/or cytotoxicity. There was variation in timing, method and reference range in NK cell measurement. Some studies also included measurement of other immunological parameters alongside NK cells e.g. serum TNF- α (Fu *et al.*, 2019), NKT-lymphocytes (Chernyshov *et al.*, 2016) and Th1/2 cytokine expression (Ahmadi *et al.*, 2017). The study characteristics are summarized in Table 1.

Patient characteristics including mean age, number of previous miscarriages or implantation failure, fetal karyotyping, number of patients in each arm and level of NK cells pre-intervention are summarised in Table 2.

3.2 Meta-Analysis of Pregnancy Outcomes

Only the studies on IVIG were pooled for meta-analyses whereas outcomes for the studies on prednisolone, etanercept and intralipid were reported individually.

3.2.1 Effect of Immunotherapy on Livebirth

The result pooled from IVIG studies that included 557 women (312 intervention and 245 control) showed a risk ratio favouring the group that received intervention (RR 2.57; 95% confidence interval 1.79, 3.69; $p < 0.05$; Figure 2). There was significant heterogeneity ($I^2 =$

62%) in the overall meta-analysis. Test for subgroup differences based on RM or RIF showed no significant difference ($p=0.63$).

Each of the RCT studies on prednisolone, etanercept and intralipid showed livebirth outcomes favouring intervention (RR 1.50, 1.24, 1.68; Figure 3), with statistical significance in the intralipid and etanercept studies ($p<0.05$) but not prednisolone ($p>0.05$).

3.2.2 Effect of Immunotherapy on Clinical Pregnancy Rate

Two studies reported on clinical pregnancy rate (Figure 4). Both studies showed risk ratios of 1.22 (intralipid) and 3.58 (IVIG) in favour of immunotherapy, but only the study on IVIG reported significant difference ($p<0.0001$).

3.2.3 Effect of Immunotherapy on Miscarriage

Meta-analysis of the IVIG studies revealed that intervention resulted in lower miscarriage rate compared to the control group (RR = 0.24; 95% CI, 0.07, 0.75; $p=0.01$; Figure 5) but the studies demonstrated significant heterogeneity ($I^2=83\%$). Subgroup analysis by RM and RIF showed a statistically significant difference between the two groups ($p=0.003$).

Similarly, individually reported miscarriage rates in the RCTs showed the same trend (RR 0.67 prednisolone; 0.63 intralipid; 0.38 etanercept; Figure 6) although this was significant only in the etanercept study ($p=0.004$).

3.3 Effect of Immunotherapy on NK cell level/activity

Overall, the three studies that monitored NK cells after intervention found a decrease in pNK level and activity. Ahmadi *et al.* (2019) reported a significant decrease ($p<0.05$) in the percentage of NK cell frequency (from $16.89 \pm 3.91\%$ to $12.02 \pm 3.45\%$) and NK cell cytolytic activity against K562 cells (from $19.13 \pm 4.91\%$ to $14.16 \pm 3.95\%$) in the treated group but no significant changes in the control group. They also reported a significant increase in inhibitory NK Cell receptors (KIR2DL1, KIR2DL2, KIR2DL3 and NKG2A) at positive pregnancy test and after 32 weeks, and a significant decrease in activating NK cell receptors (KIR2DS1, KIR2DS4, NKG2C) after 32 weeks in the treated RM group ($p<0.05$).

Similarly, Ramos-Medina *et al.* (2014) reported a general decrease in the percentage of pNK in women with RM and RIF who received IVIG therapy. There was also significant difference in pNK level at baseline and third trimester in treatment group compared to healthy controls ($p < 0.05$).

Fu *et al.* (2019) found a significant decrease ($p < 0.05$) in percentage of pNK cell activity and serum TNF- α level in the RM group treated with etanercept as compared to placebo from weeks 4 to 10 gestation.

3.4 Adverse outcomes

One study on IVIG reported significantly higher adverse outcomes in the untreated RM group when compared to the treated RM group, specifically gestational diabetes mellitus (GDM) (10%; 3%), preterm deliveries (22%; 12%) and pre-eclampsia (13%; 5%).(Ahmadi *et al.* 2019) However, these differences were not significant when compared to the healthy pregnant group. The remaining three studies on IVIG did not report on adverse outcomes.

In the study on prednisolone, there was a case each of preterm delivery, mild jaundice, hypoglycaemia and mild hearing problem in the intervention group. In the non-intervention group, one case of renal tract abnormality was reported. Mean birthweight was noted to be similar in both treated and untreated groups, and there were no incidences of IUGR, macrosomia, gestational hypertension, pre-eclampsia or GDM.(Tang *et al.*, 2013)

In the etanercept treatment group, there was no significant difference in birth weight and treatment side effects. One case each of atrial septal defect requiring intervention and inguinal hernia were reported in the intervention group.(Fu *et al.*, 2019)

No adverse effects were reported in the study using intralipid.(Dakhly *et al.*, 2016)

3.5 Risk of Bias

The assessment of the RCT studies using ROB-2 tool is reported in Table 3 and observational studies using ROBINS-I reported in Table 4. Overall, the observational studies are deemed to

have moderate to serious risk of bias whereas risk of bias for the RCTs were deemed to be either 'low' or 'with some concerns'.

4. DISCUSSION

This is the first systematic review and meta-analysis to consider the impact of immunotherapy in women with RM/RIF who were specifically selected for abnormal NK cells level and/or activity. Additionally, we have included four new studies that were not reported in previous systematic reviews on immunotherapy for RM/RIF. (Wong and Porter, 2014; Polanski *et al.* 2014; Achilli *et al.*, 2018) All seven studies included in the review reported favourable pregnancy outcomes for patients who received immunotherapy. Three studies also reported reduction in pNK cell level and/or activity in patients who received intervention. However, four out of the seven studies were non-randomized studies and were found to have a moderate to serious risk of bias in the study design. There were also variation in patient characteristics, type of NK cell measured, reference range of NK cell, timing of NK cell measurement and immunotherapy protocol.

4.1 Patient Characteristics

There were a few patient characteristics contributing to clinical heterogeneity among the studies. First is the difference in definition of RM, whereby Fu *et al* (2019) defined RM differently (> 4 previous RM) compared to the other RM studies (≥ 3). This may have significance as a higher number of previous miscarriages is known to be a poor prognostic factor for future pregnancy outcomes.(Clifford *et al.*, 1997) Second is the absence of fetal karyotype reporting in most studies. After known causes of RM are ruled out, abnormal fetal karyotype accounts for a significant proportion of unexplained RM(van den Berg *et al.*, 2012), revealing this as a potential source of confounding factor. Third, the studies have different inclusion criteria with regard to previous successful pregnancies. A recent large prospective observational study reported significantly lower pNK frequency in women with secondary RM compared to primary RM which may be explained by feto-maternal microchimerism resulting in dysfunctional immune reaction towards a newly implanting embryo in subsequent pregnancies.(Toth *et al.*, 2019) Finally, the studies that administered

IVF treatment did not fully report on other potential sources of confounding variables such as embryo grade or day of embryo transfer(Ramos-Medina *et al.*, 2014; Chernyshov *et al.*, 2016; Dakhly *et al.*, 2016).

4.2 NK Cell Measurement

Another major source of heterogeneity was in the measurement of NK cells, specifically the type, method, timing and reference range. A majority of our included studies measured pNK frequency by flow cytometry and only one study evaluated uNK by immunohistochemistry. There are a few theories about the correlation between peripheral and uterine NK cells but there is insufficient scientific evidence to reach a firm conclusion. Clinical studies revealed conflicting evidence with some studies not showing any correlation in infertility(Laird *et al.*, 2011) and RM(Kuon *et al.*, 2017) and other reporting a weak but significant correlation(Toth *et al.*, 2019). A meta-analysis by Seshadri *et al.* (2014) reported significantly higher pNK number and percentage in women with RM compared to controls, but this finding was not reflected in studies on uNK in RM. Thus measurement of pNK level may or may not be indicative of immunological abnormality in the uterus.

Next is the difference between studies in timing of baseline NK cells measurement: some measured during luteal phase and others after positive pregnancy test. Variation may occur in pNK level during different phases of menstrual cycle(Sulke *et al.*, 1985), and in uNK level from one cycle to another(Mariee *et al.*, 2012). There is also recent evidence that pregnancy can induce phenotypic and functional changes to pNK.(Le Gars *et al.*, 2019)

Further, there is the unresolved debate on what constitutes normal reference range for pNK cell levels. The cut-off pNK percentage of >12% is widely used in infertility patients after a landmark study reporting higher conception rates in previous IVF failure with pNK <12%.(Beer *et al.*, 1996) However, some studies published after that reported 12% to be in the normal range and that pNK of >18% was more specific for RM patients.(Eidukaite *et al.*, 2004) Although three out of six of our included studies used pNK cut-off of >12% of total lymphocytes, the rest did not specify a reference range. Additionally, some of our included studies concomitantly analysed other immunological parameters such as NK cell cytotoxicity, NKT lymphocytes and serum TNF alpha level, for which reference ranges have not been validated by any large scale studies.

Similarly, there is a variation in the cut-off value used to define abnormal uNK level reported in literature. Tang *et al.* (2013) used uNK density of >5% of total stromal cells as definition of abnormal. This was determined by a previous study where 5% represented the upper end of interquartile range of a sample consisting of eighteen fertile women.(Quenby *et al.*, 2005) Further studies suggested a cut-off value of 13.8% using 90th percentile as the upper limit of normal.(Tuckerman *et al.*, 2007) In summary, what constitutes an abnormal uNK level is still a contentious issue which require standardisation of measurement protocol to establish a normal range before it can be used as a diagnostic tool and guide to immunotherapy.(Lash *et al.*, 2016)

4.3 Immunotherapy

The basis for offering immunotherapy in RM/RIF originates from the theory that the maternal immune system can negatively impact fetal trophoblasts due to an imbalance in T helper cells (Th1/Th2) subsets, impairment of T regulatory function or an increase in pro-inflammatory Th17 function which culminates in excessive activated uNK.(Bansal, *et al.* 2010; 2012) Here, we have explored the potential mechanisms of each immunotherapy with a discussion of the clinical evidence for their efficacy.

It was postulated that IVIG has immunosuppressive properties, reducing pro-inflammatory Th1 cell number and cytokine production relative to Th2(Graphou *et al.*, 2003; Yamada *et al.*, 2003), and inhibition of B cell autoantibody production(Coulam and Acacio, 2012). Our included studies also reported reduction in frequency of pNK, NK cell cytolytic activity, and increase in inhibitory NK cell receptors with a simultaneous decrease in activating NK cell receptors. Pregnancy outcome was significantly improved in our meta-analysis, in keeping with other previous meta-analyses on women with RIF(Clark *et al.*, 2006; Wade *et al.*, 2017; Abdolmohammadi-Vahid *et al.*, 2019) and RM(Stricker *et al.*, 2002; Hutton *et al.*, 2007; Najafi, 2013). On the other hand, there were systematic reviews that has reported no significant benefit in IVIG usage (Egerup *et al.*, 2015) including the most recent CDSR (Wong and Porter, 2014). Since this latter review, a further RCT on IVIG use in women with secondary RM also did not show any improvement in livebirth outcome.(Christiansen *et al.*, 2015)

Intralipid has been reported to suppress pNK cytotoxicity on K562 target cells(Roussev et al., 2007) and to promote the invasive capacity of JEG-3 cells after treatment of peripheral blood mononuclear cells with intralipid (Meng et al., 2016). Previous RCTs on intralipid without NK cell measurement reported benefit although one study did not reach statistical significance. (Al-Zebeidi et al., 2019; El-khayat et al., 2015; Singh et al., 2019) In comparison, the RCT included in this review also reported a significant improvement in livebirth rates in women with RM. Contradicting these findings, a large retrospective study on women with RM and raised NK cells using historical controls from a previous study did not report any significant improvement of livebirth outcomes.(Martini *et al.*, 2018) It has also been suggested that intralipid is less effective when used during pregnancy in women with RM and high luteal phase pNK level (19%).(Kuon *et al.*, 2017)

Glucocorticoids are able to suppress T cells by inhibition of the transcription factor NFkB(Hermoso and Cidlowski, 2003), reduce uNK count(Quenby *et al.*, 2005) and reduce mature endometrial blood vessels with resulting avoidance of oxidative stress(Lash et al., 2011). Two meta-analyses showed that glucocorticoids conferred benefit for women with idiopathic RM, although one found significant heterogeneity across the studies.(Dan *et al.*, 2015; Mekinian *et al.*, 2016) A more recent retrospective study revealed a significant reduction in uNK density but no significant difference in livebirth outcomes with prednisolone usage.(Cooper *et al.*, 2019) Our included study was conducted as a feasibility trial thus it was underpowered to show any significant difference in livebirth outcomes. Interestingly, meta-analyses on glucocorticoid usage in infertile women also did not show any benefit.(Boomsma *et al.*, 2012; Dan *et al.*, 2015)

Anti-TNF α is thought to reduce Th1 cytokines including TNF-alpha and IFN-gamma.(Clark, 2010) The mechanism through which high circulating TNF- α levels lead to reproductive failure was suggested to be related to the upregulation of prothrombinase fgl2.(Clark and Daya, 1998) This generates thrombin leading to thrombotic and pro-inflammatory changes resulting in impaired blood vessel development.(Heinz and Loos, 1983) Two observational studies on women with RM reported improved livebirth rates with adalimumab in combination with IVIG (Winger *et al.*, 2009) and etanercept alone (Jerzak *et al.*, 2012). These findings were in keeping with Fu *et al.* (2019) who reported improved livebirth rate and reduction of pNK and serum TNF-alpha level.

Overall, the studies that we found have a wide divergence in immunotherapy usage. Besides the different types of immunotherapy, there is also the contentious issue of timing and duration of administration the immunotherapy. Some of the studies administered treatment pre-conception and others post-conception. It was suggested that the most vulnerable stage for immune mediated damage occurs shortly after fertilization and implantation(Bulmer *et al.*, 2010; Zhang *et al.*, 2011), hence any benefit of immunomodulation is likely to be highest at this stage. Additionally, there was no standardization of dosage and duration of treatment, some extending treatment until end of first trimester and others until 36 week. In our opinion, the usage of immunotherapy for RM and/or RIF beyond first trimester is not evidence-based and continued use in second or third trimester may result in unknown adverse outcomes to mother or fetus.

4.4 Adverse Outcomes

IVIg is usually well tolerated in pregnancy but maternal side effects of headache, skin rash and predisposition to thrombosis in antiphospholipid syndrome without significant risk of congenital malformation have been reported(Ramírez *et al.*, 2014; Christiansen *et al.*, 2015). More recently, a twenty year follow up of IVIg used during spontaneous pregnancy reported no changes in thrombotic state or development of new autoimmune disease in the mother, and development of allergies in 23% and non-autoimmune thyroid pathologies in 8% of children.(Triggianese *et al.*, 2020) Ahmadi et al. (2019) observed lower rate of pre-eclampsia and preterm deliveries in the IVIg group. This finding was replicated in other studies with elevated pNK level(Reed *et al.*, 2017) and antiphospholipid syndrome(Valensise *et al.*, 1995) which may be attributed to improved placentation.

An important concern regarding side effects of corticosteroids in early pregnancy is development of orofacial cleft palate. Individual studies reported no evidence of this(Nelson-Piercy *et al.*, 2001; Gur *et al.*, 2004) but a meta-analysis(Park-Wyllie *et al.*, 2000) concluded that although there was no significant increase in major congenital malformation, the odds ratio risk of oral cleft palate was 3.4 with the use of prednisolone.

Data for safety of anti-TNF alpha in pregnancy is extrapolated from studies of its use in inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis. In two meta-analyses (Marchioni *et al.*, 2013; Khan *et al.*, 2014), no congenital malformation were

reported. However, in a prospective observational cohort study across 9 countries, 5% major birth defects were reported in the exposed compared to control group (OR 2.2) including atrial septal defect.(Weber-Schoendorfer *et al.*, 2015)

Studies on intralipid usage in early pregnancy so far have not reported on any maternal or fetal adverse outcome(Meng *et al.*, 2016; Lédée *et al.*, 2018; Al-Zebeidi *et al.*, 2019; Singh *et al.*, 2019).

4.5 Strengths and Limitations

The strengths in our systematic review lie in our extensive search of six different databases, as well as application of our strict inclusion criteria. There were many studies found on immunotherapy usage, but narrowing down the selection to studies that specifically reported on NK cell measurement yielded a surprisingly small number of studies. Nonetheless, supplementation of the results of our electronic search with hand-searching the reference list of included studies and similar reviews allows confidence that the conclusions arising from this review are based on all available evidence. Additionally, we have excluded conference abstracts due to insufficient information to facilitate a robust risk of bias assessment.

Although this review included both RCTs and non-RCTs, meta-analysis was only conducted when data was sufficiently homogenous in terms of study design and comparator. We have also conducted subgroup analysis of RM or RIF to identify possible sources of heterogeneity. Further subgroup analyses based on primary/secondary pregnancy failure, timing of administration and type of conception would have been useful but not practical to conduct due to the small number of studies.

There were a few limitations of the studies evaluated in this systematic review. Firstly, a majority of the studies found were observational studies on IVIG and these studies have moderate to high risk of bias due to study design. The only RCT identified on steroid use was designed as a feasibility trial which was insufficiently powered to come to a valid conclusion about its effect on pregnancy outcome. Additionally, we have only identified one RCT each for use of anti-TNF α and intralipid, therefore the evidence is sparse for each type of intervention.

Secondly, six of the seven studies used pNK as the preferred means to identify raised NK cell levels. Although this is a more convenient method of testing, there is no firm evidence of a relationship between pNK and uNK levels. Similarly, evaluating an “improvement” in pNK levels or cytotoxicity after immunotherapy may not reflect any change in uNK activity. Furthermore, how uNK influence pregnancy outcomes is still not clear and requires further research.

Thirdly, although a majority of the studies used pNK of >12% as the cut off for an abnormal NK cell level, some studies used it in combination with other immunological parameters. This introduced further heterogeneity that may impact the results. Standardised criteria of NK cell levels and/or activity should be agreed before employing this test on patients with RM and/or RIF to diagnose immune dysregulation prior to prescribing immunotherapy.

Fourthly, there is no agreement on the best type of immunotherapy to be used, and among each individual immunotherapy there are differences in dose, preparation and timing of administration. Existing studies produce conflicting data on the best regimen to use for different subsets of patients with reproductive failure; therefore consensus needs to be made.

Finally, there is insufficient safety data on the different forms of immunotherapy in early pregnancy. The main concern is on the impact on embryo development and the only data that is available thus far is based on observational studies for other clinical indications. Moreover, the cost of some of these therapies can be extortionate hence its usage in the general population can introduce an ethical dilemma.

5. Conclusion

This systematic review found potential benefit in offering immunotherapy in a carefully selected subpopulation of women with immunological dysfunction. However, the paucity of high quality evidence means that it is not possible to support the use of immunotherapy in patients with RM or RIF even when selected on the basis of abnormal NK cell level or activity. Further basic science research is needed, with collaboration between reproductive immunologists and clinicians to elucidate the immunological role of NK cells in reproductive failure. This will aid in the identification of more specific biomarkers to predict the

population of women who will respond to immunotherapy. The application of scientifically validated immunological biomarkers in well-planned RCTs will determine whether immunotherapy is beneficial in a subgroup of women with RM and RIF.

CONFLICT OF INTEREST

Declarations of interest: none

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Tables

Table 1: Study characteristics

Author, year, country	Study Design; Inclusion Criteria	Aim of Study	NK Cell Measurement: Method of analysis; Timing; Reference range of abnormal level	Type and regime of intervention (Intervention; Control)	Primary Outcome
Tang et al(2013), UK	Feasibility randomized controlled trial; ≥ 3 consecutive RM, <40 years old, raised uterine NK cells, negative for all known causes of RM	To assess whether women with RM would attend for a screening visit of endometrial biopsy and if screen positive, return when pregnant for randomization	Immunohistochemistry to identify uNK cells with antibody CD56; Measured 7-9 days after urinary LH surge in a menstrual cycle; Reference range >5% of percentage of uNK to total stromal cells	Prednisolone 20 mg/day from 4-6weeks of pregnancy for 6 weeks, then 10 mg/day for 1 week then 5 mg/d for 1 week; Control group received placebo tablets	Trial recruitment occurred at planned rate despite 85% of women said they prefer active treatment
Ramos-Medina et al (2014), Spain	Retrospective cohort study; RRF* (including ≥ 3 RM or RIF after transfer of 2 good quality embryos in ≥ 3 fresh/frozen IVF cycles/ET or in ≥ 2 egg donations), peripheral blood NK or NKT-like cells expansion, negative for all known causes of RM	To report experience in IVIG therapy of a large cohort of women with RRF with expanded circulating NK and/or NKT-like cells	Multiparametric flow cytometry staining of peripheral blood NK (CD3-CD56+CD16+, CD3-CD56+CD16-) NKT-like cells (CD3+CD56+); Timing of measurement at "baseline" not otherwise specified; Reference range pNK > 12% or NKT cells >10% of total lymphocytes	IVIG 400 mg/kg every 3 weeks in 1st trimester, 200 mg/kg every 4 weeks until 35-36/40 for RM, within 24 hours before ET, Day 15, then every 3 weeks until 35-36/40 for RIF; Control groups no intervention	IVIG significantly improved live birth rates in RM and clinical pregnancy rate in RIF patients after multivariate logistic regression analysis adjusting for age, NK cell subsets and other therapies

Chernyshov et al (2016), Ukraine	Prospective cohort study; RIF in ≥ 3 previous IVF cycles with 2 high quality embryos,	To investigate multiple immune deviations predictive for IVF failure as possible additional markers for IVIG therapy.	Three-colour flow cytometry for lymphocyte subsets, and proportion of killed k562 target cells assessed by propidium iodide intake to evaluate cytotoxicity; Measured on day 17-23 of menstrual cycle; Reference range based on divergences outside 90% "corridor"†	IVIG 200-400 mg/kg; 1 week before embryo transfer; Control group no intervention	Three or more immune deviations may predict implantation failure to a greater degree than one or two immune deviations
Dakhly et al (2016), Egypt	Randomized Controlled Trial; Unexplained secondary infertility, ≥ 3 consecutive RM after either spontaneous pregnancy or IVF/ICSI and elevated peripheral blood NK Cells, negative for all known causes of RM	To investigate the efficacy of intralipid in women with RSA and elevated natural killer cell activity undergoing IVF/ICSI cycles (primary outcome is chemical pregnancy).	Flow cytometry for proportion of CD56+CD16+ peripheral NK cell; Reference range of abnormal $>12\%$ NK cells of total lymphocytes	Intralipid 2ml diluted at 20% in 250 ml saline infusion, on the day of oocyte retrieval, repeat within 1 week of PPT then every 2/52 until end of first trim; Control group received IV infusion of 250 ml saline solution	No significant difference in chemical pregnancy between intralipid and control group
Ahmadi et al (2017), Iran	Non-randomized controlled trial; RM ≥ 3 , cellular immune abnormalities including elevated frequency and cytotoxicity of CD3=CD56+ NK cells, negative for all known	To compare the possible effect of IVIG administration on Th1 and Th2 cell frequency, cytokine secretion, and expression of transcription factors between RM patients and control group.	Flow cytometry immunostaining for elevated CD3- CD56+ NK cell frequency, propidium iodide uptake for proportion of killed k562 target cells to evaluate cytotoxicity; Measured after first positive pregnancy test; Reference range not specified	IVIG 400 mg/kg, From pregnancy (confirmed by b-HCG), every 4 weeks until 28-32/40; Control group no intervention	A significant reduction in Th1 frequency, transcription factor expression and cytokine levels in IVIG treated group and significant increase in Th2 for above

	causes of RM				
Ahmadi et al (2019), Iran	Non-randomized controlled trial; ≥ 3 RM, age 18-40 years, one or more of peripheral blood NK cells elevation or cytotoxicity, negative for all known causes of RM	To evaluate the changes in natural killer cells' frequency and cytotoxicity in RM patients who received IVIG	Flow cytometry immunostaining for elevated CD3- CD56+ CD16+ NK cell frequency, propidium iodide uptake for proportion of killed k562 target cells, to evaluate cytotoxicity; Measured after first positive pregnancy test; Reference range $>12\%$ of total lymphocytes or target cell killing $>15\%$	IVIG 400 mg/kg every four weeks until 30-32 weeks gestation; Control group no intervention	IVIG significantly decreased the frequency and cytotoxicity of NK cells but untreated controls showed no significant difference
Fu et al (2019), Taiwan	Randomized controlled trial; ≤ 39 years, >4 previous miscarriages, serum TNF-alpha >5.14 pg/ml or increased NK cell activity, failure of conventional treatment for RSA, negative for all known causes of RSA	To evaluate the effectiveness of etanercept in treatment of refractory recurrent spontaneous abortion with innate immune disorders	Proportion of killed k562 target cells assessed by propidium iodide intake to evaluate NK cell activity; Timing of measurement during pregnancy, not otherwise specified; Reference range of abnormal NK cell level not specified	Etanercept 25 mg sc od from end of menstruation until 10 th week of gestation or to start of menstruation. Control group received saline s.c. of same volume. Both groups received heparin, aspirin, prednisolone and cyclosporine.	Livebirth in intervention group was significantly higher than placebo group

RRF, Recurrent Reproductive Failure; RM, Recurrent Miscarriage; RIF, Recurrent Implantation Failure; ET, Embryo transfer

† Elevated NK cytotoxicity ($>30\%$ of NK cytotoxicity at ratio target/effector cells 1:10 and $>40\%$ of NK cytotoxicity at ratio target/effector cells 1:20); CD56+ in T cells (CD3+) $>18\%$; CD158a+ in T cells (CD3+) $>5\%$; CD4+ T cells (CD3+) 30% ; NK cells (CD56+CD3-) $>21\%$; HLA DR+ in NK cells (CD56+CD3-) $>25\%$; CD8+ in NK cells (CD56+CD3-) $>64\%$; CD8+ in NK cells (CD56+CD3-) 65% ; CD158a+ in NK cells (CD56+CD3-) $<17\%$

Table 2 : Patient Characteristics

Author, Year	Number of previous RM/IVF cycles (Intervention; Control) [†]	Percentage (%) of confirmed normal karyotype in miscarriage (Intervention; Control) [†]	Number of patients (Intervention; Control)	Mean Age (years, intervention; control) [†]	NK cell level and/or cytotoxicity pre-intervention (%) (intervention; Control) [†]
Tang et al (2013) ⁵⁵	5 (3-14); 5 (3-15) [§]	67; 67	20;20	34 (25-39); 33 (27-39)	uNK level “higher” in recurrent miscarriage, no numerical data
Ramos-Medina et al (2014) ⁶¹	3.39 ± 1.37 (RM), 4.72 ± 2.36 (IVF) [‡]	Not reported	162 (82 RM, 80 RIF); 123 (39 RM, 84 RIF)	36.4 ± 3.6 RM, 37.5 ± 3.3 RIF*	13.35 ± 5.60 (RM), 12.88 ± 6.31 (RIF) [‡]
Chernyshov et al (2016) ⁶⁰	Not reported	Not reported	115; 123	33 ± 0.5; 32.9 ± 0.5	Not reported
Dakhly et al (2016) ⁵⁶	4.1 ± 0.9; 4.1 ± 0.9	Not reported	144; 152	35.5 ± 3.7; 36 ± 3.7	Not reported
Ahmadi et al (2017) ⁵⁸	3.3 ± 1.4; 3.2 ± 1.1	50; 71	31; 12	33.8 ± 3.6; 34.1 ± 3.4	Not reported
Ahmadi et al (2019) ⁵⁹	4.3 ± 1.3; 4.4 ± 1.2	60; 86	38; 40	34.8 ± 3.7; 33.1 ± 3.2	pNK level 16.65 ±3.74; 15.81 ±5.27 pNK cytotoxicity 18.56 ± 3.2; 18.28 ±5.61
Fu et al (2019) ⁵⁷	5.4 ± 0.3; 5.5 ± 0.4	77 [‡]	95; 93	34.5 ± 2.7; 34.0 ± 2.8	Not reported

[†] Mean +/- Standard deviation unless stated otherwise

[‡] Intervention and control group not reported separately

[§] Mean (Range)

Table 3 : Cochrane *risk-of-bias* tool for randomized trials (RoB 2)

Study ID	Randomization Process	Deviations from Intended Interventions		Missing Outcome data	Measurement of the Outcome	Selection of the reported result	Overall risk of bias
		Effect of assignment to intervention	Effect of adhering to intervention				
Tang et al (2013) ⁵⁵	Low	Low	Low	Low	Low	Low	Low [†]
Dakhly et al (2016) ⁵⁶	Low	Low	Low	Low	Low	Some concerns	Some concerns [‡]
Fu et al (2019) ⁵⁷	Low	Unclear	Low	Low	Low	Low	Low [†]

†The study is judged to be at low risk of bias for all domains for this result.

‡The study is judged to raise some concerns in at least one domain for this result, but not to be at high risk of bias for any domain.

Table 4 : Cochrane Risk of Bias in Non-Randomized Studies of Interventions (ROBINS-I) for observational studies

Study ID	Study Type	Pre-intervention		At intervention	Post-Intervention				Total Score
		Confounding Bias	Selection Bias	Classification Bias	Deviation Bias	Missing Data Bias	Measurement of Outcome Bias	Selective Reporting Bias	Overall risk of bias judgement
Ramos-Medina et al (2014) ⁶¹	Retrospective observational	Serious	NI	Low	NI	Serious	Low	Serious	Serious [‡]
Chernyshov et a. (2016) ⁶⁰	Retrospective observational	Serious	Low	Low	NI	Low	Low	Moderate	Serious [‡]
Ahmadi et al (2017) ⁵⁸	NRSI	Serious	NI	Low	NI	Low	Low	Serious	Serious [‡]

Ahmadi et al (2019) ⁵⁹	NRSI	Moderate	Low	Low	NI	Low	Low	Moderate	Moderate [†]
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NRSI, Non randomized study of intervention

[†]The study provides sound evidence for a non-randomized study but cannot be considered comparable to a well-performed randomized trial. The study is judged to be at low or moderate risk of bias for all domains.

[‡]The study has some important problems. The study is judged to be at serious risk of bias in at least one domain, but not at critical risk of bias in any domain.

Figure Legend

Figure 1: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart

Figure 2: Meta-analysis of effect of IVIG on livebirth

Figure 3: Effect of intralipid, etanercept and steroid on livebirth (non-pooled)

Figure 4: Effect of intralipid and IVIG on clinical pregnancy rate (non-pooled)

Figure 5: Meta-analysis of IVIG on miscarriage

Figure 6: Effect of intralipid, etanercept and prednisolone on miscarriage (non-pooled)

Figure 1

PRISMA diagram

Figure 2

Figure 3

Figure 5

Figure 6

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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