Original Article

Contradictory Effect of Lymphocyte Therapy and Prednisolone Therapy on $CD3^+CD8^+CD56^+$ Natural Killer T Population in Women with Recurrent Spontaneous Abortion

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Abstract

Background: Natural killer T (NKT) cells are influential immune cells in pregnancy failures, including recurrent spontaneous abortion (RSA). Different approaches are used for these disorders due to their effects on maternal immunomodulation. **Aims:** In the present study, we compared the effects of two typical immunotherapies (lymphocyte immunotherapy [LIT] and low-dose prednisolone) on $CD3^+CD56^+CD16^+$ and $CD3^+CD56^+CD8^+$ cells as two distinct subsets of NKT cells in Women with RSA. **Settings and Design:** This study was a comparative cohort study conducted from 2021 to 2022. One hundred and five women with RSA were distributed into three treatment groups randomly. **Materials and Methods:** Fifty women in the group of low-dose prednisolone therapy, fifty women in the LIT group and five women without any treatment as the control group were included in the study. NK and NKT cell subsets were assessed using flow cytometry. Furthermore, the concentration of interferon-gamma (IFN-γ), transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10) was measured quantitatively using the enzyme-linked immunosorbent assay technique. **Statistical Analysis Used:** Normality and comparisons between study groups were performed by non-parametric unpaired Mann–Whitney, Kruskal–Wallis rank sum test, and one-way ANOVA. **Results:** The percentage of $CD56^{dim}$ NK cells was increased after prednisolone therapy, while this population significantly decreased in the LIT group. In contrast to the LIT group, the administration of prednisolone increased $CD3^+CD8^+CD56^+$ NKT cells ($P < 0.0001$), which is helpful for pregnancy. The effect of the investigated treatment approaches on the population of peripheral $CD3^+CD56^+CD16^+$ NKT cells of women with RSA was not adequately significant. The same situation was also observed regarding the serum level of IFN-γ. However, a significant decrease in serum levels of IL-10 and TGF-β was observed after prednisolone therapy. **Conclusion:** The lower capability of LIT in changing the population of NKT cells compared to prednisolone therapy may be due to its mechanism of action, which is related to the production of blocking antibodies. These treatment approaches had different effects on NKT cells, indicating that NKT cell population and function can be affected using LIT and prednisolone therapy distinctly. In addition, prednisolone therapy and LIT in women with normal serum levels of IFN-γ have no harmful effects in changing the production of this critical cytokine.

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Introduction

Recurrent spontaneous abortion (RSA) is defined as three or more consecutive unexpected miscarriages before 20 weeks of gestation[1] and is a common disorder in obstetrics and gynaecology.[2] It has several causes, including chromosomal and anatomical abnormalities, infections, and autoimmunity. However, in 50% of cases, RSA occurs without precise reason. In these women, immunological disorders affect foeto-maternal immune tolerance disruption.[3]

Infertility is also an increasing gynaecological problem. Fifteen per cent of couples in developed countries are infertile.[4] *In vitro* fertilisation (IVF) is an advanced therapeutic technique that has become an accessible treatment in different countries.[5] However, the IVF cycle universal success rates were reported <50%. Recurrent implantation failure (RIF) is defined as three or more pregnancy losses due to implantation failure in the IVF cycle.[6] The role of the maternal immune responses has been confirmed in the embryo implantation process, and there is an immunologic basis for unexplained failures.[7] RSA and RIF could occur separately, but some women with poor obstetrical outcomes may experience both RSA and RIF.[8] RSA and RIF may have several common characteristics. Maternal immune response to the semi-allogeneic or allogeneic embryo is an influential factor in a significant ratio of cases of both types.[3]

All treatment approaches are used for RSA and RIF due to their effects on maternal immunomodulation. Various immunotherapies may be considered one of the treatment options to improve uterine receptivity, enhance implantation, prevent early miscarriages and increase pregnancy outcomes in these women, including intravenous immunoglobulin, lymphocyte immunotherapy (LIT), intrauterine infusion of granulocyte colony-stimulating factor and peripheral blood mononuclear cells (PBMCs), anticoagulants and oral administration of aspirin and glucocorticoids.[9] Unfortunately, the confirmed effectiveness of these immunomodulators remains unclear; therefore, a combination of these immunotherapy methods is used more efficiently in treating RSA and RIF. One of the ways to evaluate the efficacy of each of the immunotherapies is the effect of each one on the immunological factors involved in early miscarriage or implantation failure. Natural killer (NK) cells, NKT cells, and their subsets play a critical role in implantation failures, RSA and infertility.[10-13] Therefore, in the present study, we compared the effects of two typical immunotherapies for RSA on NK and NKT cell subsets.

Materials and Methods

This comparative cohort study was conducted from April 2021 to February 2022. One hundred and five women eligible to enter the study were distributed into three treatment groups randomly with a volume of 6. The participants were fully aware of the received treatment. Furthermore, the laboratory technician has sampled and numbered samples (without announcing the patient's profile). Iran's ethical committee approved the protocol (IRCT registration number: IRCT20210115050041N2). The patients (105 women) were recruited from women referred to the immunology clinic of Alzahra Hospital in Isfahan, one of the principal centres of recurrent miscarriage and infertility. The Principles of Helsinki have been followed while handling human subjects. The sample size was determined based on statistical analysis of comparable studies.

Study population

A total of 105 women with investigated RSA were randomly distributed into three study groups. Fifty women were in the group of low-dose prednisolone therapy, fifty women in the group of LIT and five women without any treatment as controls. Inclusion criteria were women between the ages of 25 and 40 years, natural fertility history, two or more spontaneous abortions before 12 weeks of gestation, and anti-paternal cytotoxic antibody titre <10%. Women were excluded if they had any of the following exclusion criteria: pregnancy, smoking and alcohol consumption, body mass index (BMI) more than 30 kg/m^2, certain autoimmune diseases such as systemic lupus erythematosus and anti-phospholipid antibody,[14] acute infection with TORCH agents including Toxoplasma, hepatitis C virus, Rubella, cytomegalovirus and herpes simplex virus, previous treatment with corticosteroids and immunotherapy. All women participating in this study had written informed consent and ethical approval was obtained from the ethical committee of Isfahan University of Medical Sciences (IR.MUI.MED.REC.1399.878).

Ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood samples were collected from each participant before and after interventions for flow cytometry

assessment. In addition, whole blood was collected and sera were separated using a centrifuge and stored at −80°C for cytokines measurement. The treatment duration was 2 months, and the control group followed for 2 months without any medication.

Intervention

Group 1 (prednisolone therapy)

Fifty women with RSA who were administered 5 mg prednisolone (one tablet per day), and low-dose aspirin (80 mg, one tablet daily) were included in this group.

Group 2 (lymphocyte immunotherapy)

This group included 50 women with RSA who had three-time paternal lymphocyte transfers within 2 months. They also were administered low-dose aspirin (80 mg, one tablet daily).

Lymphocyte immunotherapy protocol

Fifty mL anti-coagulated venous blood was obtained from the husband's (paternal cells). PBMCs were isolated using density-gradient centrifugation with Ficoll–Hypaque. After twice washing, cells were irradiant and resuspended in sterile normal saline (4 mL). Finally, the paternal cell suspension was transferred through four intra-dermal injections in the arms and legs (by a physician).

Group 3 (recurrent spontaneous abortion controls)

This group included five women with RSA who were administered low-dose aspirin (80 mg, one tablet daily).

The intervention started at the same time in all groups for 2 months. Immediately after this interval, women were referred to a laboratory for sampling.

Flow cytometry

Two millilitre of fresh EDTA anti-coagulated venous blood were collected and stained directly with the monoclonal antibodies, including anti-human CD3 (FITC, Clone: UCHT1, Biolegend, USA), anti-human CD56 (PerCP, Clone: 5.1H11, Biolegend, USA), anti-human CD16 (PE, Clone: 3G8, Biolegend, USA) and anti-human CD8 (PE, Clone: SK1, Biolegend, USA) according to the manufacturer recommendations. IgG Isotype controls were used to determine non-specific staining. All prepared samples were analysed by flow cytometry using a FACSCaliber (BD Biosciences San Jose, CA, USA) with Cell-Quest research (Becton Dickinson) software for the primary analysis.

$CD3^+CD56^+CD8^+$cells and $CD3^+CD56^+CD16^{+/-}$cells were considered as NKT cell subsets. $CD56^{bright}$ and $CD56^{dim}$ were gated as two subsets of NK cells ($CD3^-CD56^+CD16+$). Furthermore, $CD3^+CD8^+$T cell percentages were measured. Figures 1 and 2a represent the gating strategy for NK and NKT cell subsets.

Determination of serum levels of cytokines by enzyme-linked immunosorbent assay

Transforming growth factor-beta (TGF-β), interleukin-10 (IL-10) and interferon-gamma (IFN-γ) serum concentrations were measured using the enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. All measurements were performed in duplicate.

Statistical analysis

Data were analysed using GraphPad Prism V9.0 (MacKiev Company, USA, Boston) software. Normality and comparisons between study groups were performed by non-parametric unpaired Mann–Whitney, Kruskal–Wallis rank sum test and one-way ANOVA. GraphPad Prism V9.0 and FlowJo V10.0 (Ashland, Oregon) software were used for graphical analyses and flow cytometry analysis data, respectively. Data were reported as mean ± standard error of mean, and a $P < 0.05$ was considered a statistically significant difference.

Results

All participants were matched in age, BMI and the number of previous IVF failures and miscarriages. The clinical and demographic characteristics of participants are summarised in Table 1. During the first sampling, the percentages of NK and NKT cells were estimated [Table 2], and the patients with a significant difference were omitted. Finally, 105 women were divided randomly into three study groups [Figure 1]. The control group in all measurements had no significant differences before and after intervention time [Table 3].

Decrease of $CD3^+CD8^+$ T cells in prednisolone therapy group

The percentage of $CD3^+CD8^+$ T cells was decreased in both study groups [Figure 2a and b]. As shown in Figure 2c, prednisolone therapy decreased $CD3^+CD8^+$ T cells percentages significantly in comparison to LIT ($P < 0.05$).

Natural killer T cell subsets' changes

In the present study, we investigated $CD3^+CD8^+CD56^+$cells [Figure 3a] and $CD3^+CD16^+CD56^+$cells [Figure 4a] as two subsets of NKT cells. We found that prednisolone therapy caused to increase in the percentage of $CD3^+CD8^+CD56^+$cells ($P < 0.0001$) [Figure 3b], while LIT decreased this cell population significantly

Table 1: Clinical and demographical characteristics of women with recurrent spontaneous abortion enrolled in this study

	Mean±SEM			*P*
	Prednisolone therapy (*n*=50)	LIT (*n*=50)	Controls (*n*=5)	
Age (year)	32.46±0.5794	31.92±0.5568	28.86±1.455	0.0953 (ns)
BMI (kg/m^2)	26.10±0.2672	26.36±0.2716	25.86±0.7817	0.8647 (ns)
Previous IVF failures	0.760±0.1444	0.800±0.1714	0.800±0.5831	0.9536 (ns)
Previous miscarriages	3.760±0.2242	3.080±0.1662	3.00±0.3162	0.0587 (ns)

P >0.05 = ns (no significant), *P* < 0.05 = * (significant), *P* < 0.01 = **, *P* < 0.001 = ***, *P* < 0.0001= ****. LIT=Lymphocyte immunotherapy, BMI=Body mass index, IVF=*In vitro* fertilisation, SEM=Standard error of mean

Table 2: The percentage of natural killer, natural killer T and T cell subsets in the first sampling before intervention

	ean±SEM			*P*
	Prednisolone therapy (*n*=50)	LIT (*n*=50)	Controls (*n*=5)	
$CD3^+CD8^+CD56^+$ cells	2.814±0.5582	2.111±0.1802	2.274±0.3251	0.0.4725 (ns)
$CD3^+CD56^+CD16^+$ cells	3.274±0.1323	3.937±0.2782	3.224±0.267	0.0830 (ns)
$CD3^-CD56^+CD16^+$ cells	17.04±0.6165	19.23±1.397	19.45±1.38	0.3270 (ns)
$CD3^+CD8^+$ cells	26.00±1.525	27.13±1.369	26.43±2.746	0.9705 (ns)

P >0.05 = ns (no significant), *P* < 0.05 = * (significant), *P* < 0.01 = **, *P* < 0.001 = ***, *P* < 0.0001= ****. LIT=Lymphocyte immunotherapy, SEM=Standard error of mean

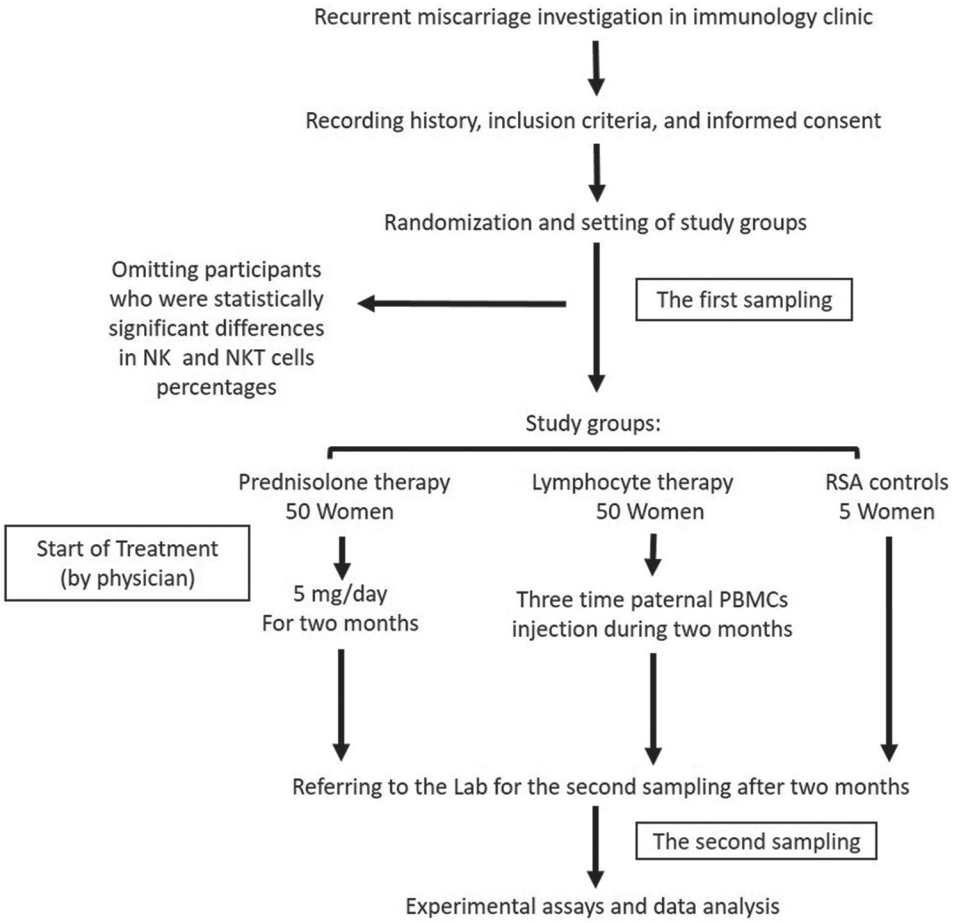

Figure 1: An overview of the steps of selection, grouping and follow-up of the patients in the trial. NKT = Natural killer T, RSA = Recurrent spontaneous abortion, PBMCs = Peripheral blood mononuclear cells

(*P* < 0.0001) [Figure 3c]. Furthermore, the percentage of $CD3^+CD8^+CD56^+$ NKT cell subset was increased in women treated with low-dose prednisolone compared to other groups [Figure 3d].

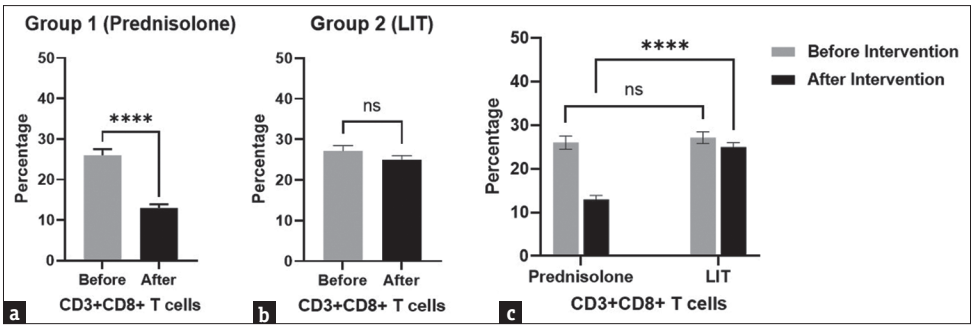

Figure 2: Percentage of $CD3^+CD8^+$ T cells in group 1 ($n = 50$) and group 2 ($n = 50$). (a and b) represent the percentage of $CD3^+CD8^+$ T cells after prednisolone therapy and lymphocyte immunotherapy (LIT). Prednisolone therapy, in comparison to LIT, significantly decreased the percentage of $CD3^+CD8^+$ T cells (c). $P > 0.05$ = ns (no significant), $P < 0.05$ = * (significant), $P < 0.01$ = **, $P < 0.001$ = ***, $P < 0.0001$= ****. LIT = Lymphocyte immunotherapy

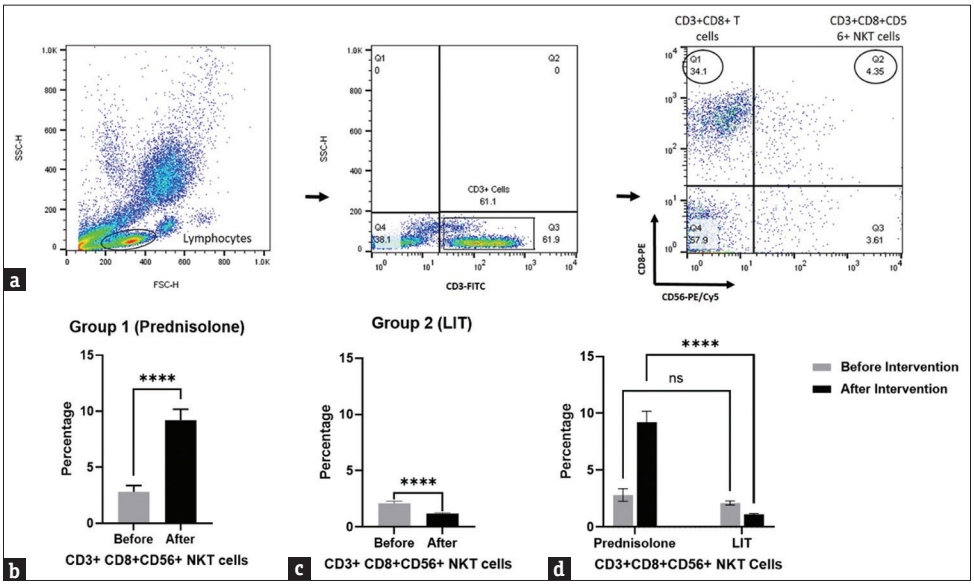

Figure 3: The strategy of gating in flow cytometry plots (a). Total lymphocytes were selected from peripheral blood events against forward and side scatter parameters. $CD3^+CD8^+CD56^+$ and $CD56^{dim/bright}$ cells were analysed by gating on lymphocytes. The cells positive for CD3 were selected and afterward displayed on a plot of CD8 against CD56 expression. The upper left and upper right parts of the quadrants show $CD3^+CD8^+$T cells and $CD3^+CD8^+CD56^+$cells, respectively. Distinct treatment approaches had different effects on the percentage of $CD3^+CD16^+CD56^+$ Natural killer T (NKT) cells, such that prednisolone therapy (b) Increased and lymphocyte immunotherapy (LIT) (c) Decreased them. This subset of NKT cells was significantly decreased during prednisolone therapy in comparison to LIT (d). $P > 0.05$ = ns (no significant), $P < 0.05$ = * (significant), $P < 0.01$ = **, $P < 0.001$ = ***, $P < 0.0001$= ****. LIT = Lymphocyte immunotherapy, NKT = Natural killer T

Table 3: Natural killer T, natural killer and T-cell subsets and cytokine concentration in the control group

	Mean±SEM		*P*
	Before intervention	After intervention	
$CD3^+CD8^+CD56^+$ cells	2.712±0.1887	2.744±0.2488	0.9209 (ns)
$CD3^+CD56^+CD16^+$ cells	3.366±0.1957	3.192±0.2262	0.5768 (ns)
$CD3^-CD56^+CD16^+$ cells	17.52±1.093	18.87±0.8860	0.3655 (ns)
$CD3^+CD8^+$ cells	24.77±2.055	24.66±0.9765	0.9640 (ns)
Concentration of TGF-β	209.7±26.44	229.0±17.23	0.5595 (ns)
Concentration of IFN-γ	0.950±0.013	0.956±0.005	0.6800 (ns)
Concentration of IL-10	30.70±5.067	29.66±3.337	0.8689 (ns)

$P > 0.05$ = ns (no significant), $P < 0.05$ = * (significant), $P < 0.01$ = **, $P < 0.001$ = ***, $P < 0.0001$= ****. TGF-β=Transforming growth factor beta, IFN-γ=Interferon gamma, IL-10=Interleukin 10, SEM=Standard error of mean

Prednisolone therapy and lymphocyte immunotherapy decreased $CD16^+CD56^+$ natural killer cell percentages

As represented in Figure 4, both treatment approaches (prednisolone therapy and LIT) decreased the percentage of $CD16^+CD56^+$ NK cells [Figure 4b]. This decrease was significantly more in the prednisolone-treated group [Figure 4c].

In a total gating of $CD3^-CD56^+$cells, we observed that prednisolone therapy and LIT have different effects on $CD56^{dim}$ cells. The percentage of these cells was increased after prednisolone therapy while significantly decreased in group 2 (LIT) compared to group 1 (prednisolone therapy). The initial subpopulation of $CD3^-CD56^{bright}$ cells had a statistically significant difference between groups 1 and

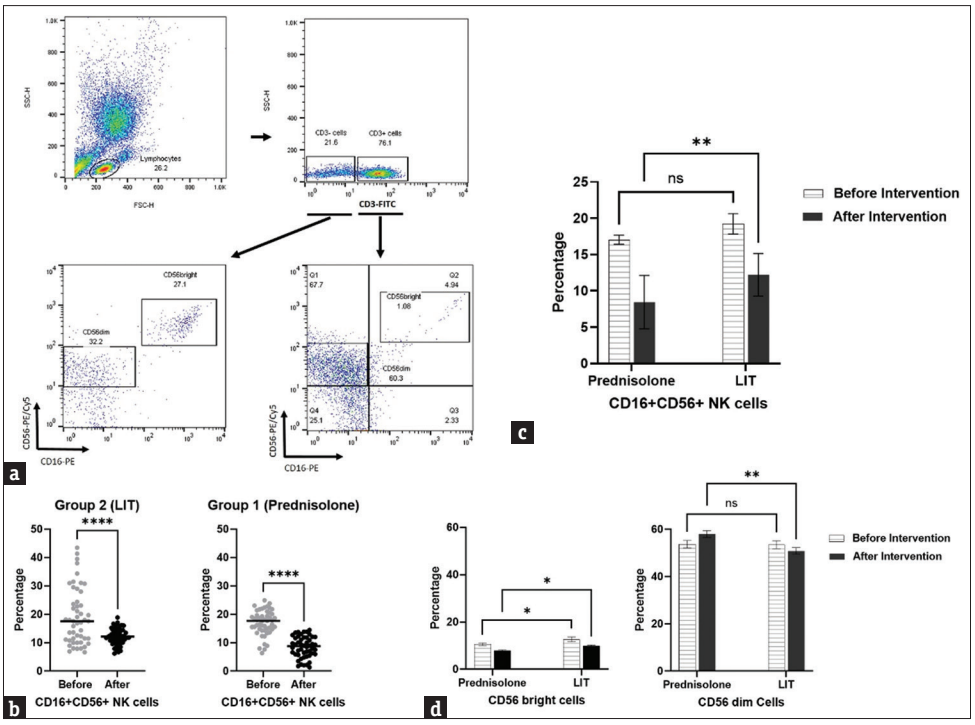

Figure 4: Changes of $CD3^{-}CD16^{+}CD56^{+}$ Natural killer (NK) cells during interventions. The strategy of gating in flow cytometry plots (a). Cells negative and positive for CD3 were selected separately, and then cells were measured CD56 against CD16 in distinct plots. $CD56^{dim}$ and $CD56^{bright}$ cells were measured in each $CD3^{+}$ and $CD3^{-}$ cell population. The percentage of $CD16^{+}CD56^{+}$ NK cells decreased after prednisolone therapy ($n = 50$) and lymphocyte immunotherapy (LIT) ($n = 50$) (b). In a comparison of two treatment approaches, prednisolone decreased the percentage of $CD16^{+}CD56^{+}$ NK cells more than LIT (c), while LIT decreased significantly $CD3^{-}CD56^{dim}$ cells (d). $P > 0.05$ = ns (no significant), $P < 0.05$ = * (significant), $P < 0.01$ = **, $P < 0.001$ = ***, $P < 0.0001$= ****. LIT = Lymphocyte immunotherapy, NK = Natural killer

2. Subsequently, this difference affected $CD56^{bright}$ cell changes after intervention [Figure 4d].

Prednisolone therapy and lymphocyte immunotherapy affects the serum levels of cytokines

In the present study, the serum levels of IFN-γ, IL-10 and TGF-β were measured by the ELISA method. IFN-γ, as a type I cytokine, is produced by NK and T cells to stimulate the inflammatory responses, and IL-10 and TGF-β, as two anti-inflammatory cytokines, are involved in immunomodulation during pregnancy.[15] In both groups of study, no significant differences were observed in serum levels of IFN-γ. However, we observed a significant decrease in serum levels of IL-10 and TGF-β after prednisolone therapy. In group 2 (LIT), the serum levels of these cytokines decreased, but a decline in serum concentration of TGF-β was not statistically significant after intervention [Figure 5].

As presented in Figure 6, there were no significant differences between prednisolone therapy and LIT in serum levels of IFN-γ and IL-10. However, we observed that prednisolone therapy decreased serum levels of TGF-β significantly ($P < 0.05$) in comparison to LIT.

Discussion

In recent decades, various treatment approaches have been introduced and investigated to reduce the occurrence of RSA and RIF. In the meantime, immunotherapies based on regulating or suppressing the maternal immune responses have been more efficient. LIT and oral administration of prednisolone are among the most common treatment approaches. In the present trial, we investigated the minimum functional dose of prednisolone in the reproductive field and the time before trying to get pregnant. All participants in this study used low-dose aspirin; therefore, according to the results of the control group, the immunomodulatory effect of aspirin was considered negligible in all study groups. We observed that the administration of low-dose (5 mg daily) prednisolone for 2 months in non-pregnant women with RSA caused to decrease of two primarily involved immune cells in miscarriage, including $CD3^{+}CD8^{+}$ T and $CD16^{+}CD56^{+}$ NK cells.

Glucocorticoids can exert a range of positive effects on outcome improvement during the first trimester of pregnancy.[14] Prednisolone is one of the glucocorticoids. It has powerful anti-inflammatory effects[16] and administers orally in short-term low doses in the reproductive field to improve foeto-maternal tolerance.[17]

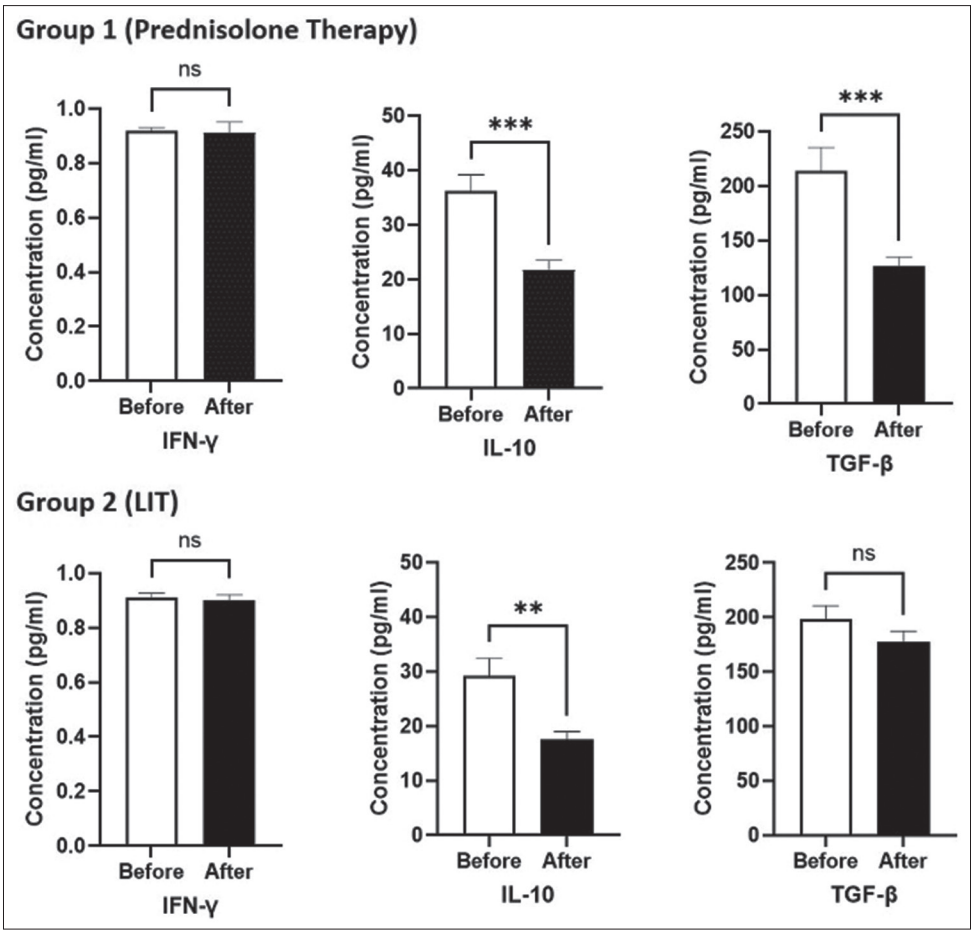

Figure 5: The serum concentration of cytokines was measured before and after intervention in groups 1 and 2. There were no significant differences in interferon-gamma concentration (pg/mL) in both groups. Interleukin-10 concentration was decreased significantly after treatment in both groups, but the decline of transforming growth factor-beta was statistically significant in group 1 (prednisolone therapy). P >0.05 = ns (no significant), P < 0.05 = * (significant), P < 0.01 = **, P < 0.001 = ***, P < 0.0001= ****. IFN-γ = Interferon-gamma, IL-10 = Interleukin-10, TGF-β = Transforming growth factor-beta, LIT = Lymphocyte immunotherapy

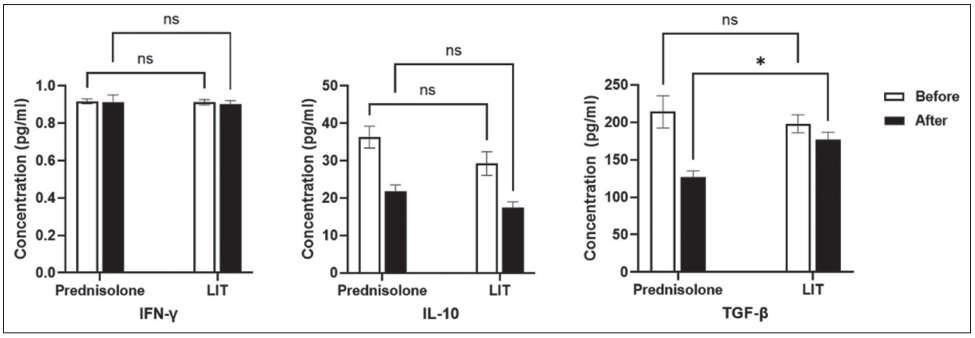

Figure 6: There were no statistically significant differences in interferon-gamma and interleukin-10 concentrations (pg/mL) between prednisolone therapy and lymphocyte immunotherapy (LIT). Transforming growth factor-beta concentration decreased significantly more in prednisolone therapy in comparison to LIT. P >0.05 = ns (no significant), P < 0.05 = * (significant), P < 0.01 = **, P < 0.001 = ***, P < 0.0001= ****. IFN-γ = Interferon-gamma, IL-10 = Interleukin-10, TGF-β = Transforming growth factor-beta, LIT = Lymphocyte immunotherapy

Prednisolone can suppress the activity of several types of immune cells, including NK and T cells.[14,18] The binding of prednisolone to the glucocorticoid receptor causes to inhibit the action of the transcription regulator Nuclear factor kappa B.[19] Many clinical trials and meta-analyses have investigated the effects of oral administration of prednisolone on women with RSA and RIF.[17,20] However, their findings have controversy. Notably, the prescribed dose and duration of prednisolone administration have been different among various studies. Glucocorticoids have some possible adverse effects, including arterial hypertension, diabetes, prematurity and low birth weight, whereas pregnancy adverse effects are uncommon with low prednisone doses (<10 mg/day).[21,22]

Another treatment approach that we investigated was LIT. Historically, Mowbray *et al*. conducted the first study estimated the effectiveness of LIT in couples with RSA.[23] In current years, multiple studies have been conducted to investigate the efficacy of this treatment method on women with RSA. The meta-analysis of the two most relevant studies (Cochrane and Liu *et al*.)[24,25] and the data recently published by Cavalcante *et al*.[26] have indicated a positive impact of LIT on the number of live births in women with RSA; however, various factors are involved in LIT efficacy. In addition to the heterogeneity of patients with RSA, other factors, such as selection criteria for LIT candidates, the number of applied lymphocytes, the lymphocyte preparation procedure and its concentration, the route of administration and the frequency of immunisation, are influential in the efficacy of LIT.[24,27-29]

According to related studies, LIT can affect the maternal immune system through several mechanisms, such as inhibition of NK cell activity,[30-34] production of anti-T-cell receptor (TCR) idiotypic antibodies,[34,35] T-cell suppression,[30,35,36] and production of mixed lymphocyte reaction blocking antibodies.[37-40] Based on our findings, transfusion of 100 million inactivated paternal lymphocytes per dose to mothers with RSA before getting pregnant decreased $CD8^+$T and $CD16^+CD56^+$ NK cells.

NK cells have a critical role in the processes of reproduction,[10,13] and they are associated with implantation failures, recurrent abortions and infertility because of their cytotoxicity.[11,12,41] NK cells divide into two principal cell subsets, $CD16^+CD56^{dim}$ and $CD16^{low/-}$ $CD56^{bright}$ NK cells. Approximately 80% of uterine NK (uNK) and 10% of peripheral NK cells are $CD56^{bright}$.[42,43] Hence, the phenotype of uNK cells is similar to a small subgroup of peripheral blood NK cells. The intensity of CD56 surface marker expression and the lack of two typical NK cell markers (CD16 and CD57) distinguish uterine from peripheral NK cells. Chrysoula and Linda have indicated that the decidual NK (dNK) cells can recruit into the uterus and decidua from peripheral blood and subsequently differentiate into uNK under the influence of chemokines.[44] Accordingly, the investigation of peripheral NK cells can indirectly help to apprehend the immune status of the uterus to study the mechanisms of recurrent abortion.[45] The increasing $CD16^+CD56^{dim}$ NK cells in the endometrium and peripheral blood are considered risk factors for recurrent abortion.[12,41,46] Considering previous studies, uNK cell density in women with RSA and RIF has positively correlated with endometrial angiogenesis and uterine artery blood flow.[47,48]

uNK cells express both glucocorticoid and oestrogen receptor beta receptors.[49] Therefore, prednisolone can be used as a steroid to influence these cells.[50] In the present study, we observed that oral administration of prednisolone decreased $CD16^+CD56^+$ NK cells. However, it led to an increase in the percentage of peripheral blood $CD56^{dim}$ NK cells. In the meantime, LIT reduced the $CD56^{dim}$ subset and $CD16^+CD56^+$ NK cells. $CD56^{bright}$ NK cells primarily secrete cytokine, whereas $CD16^+CD56^{dim}$ NK cells are mainly responsible for cytotoxicity.[51] dNK cells are mainly $CD56^{bright}$ and represent the dominant maternal immune cells in the decidua. They are involved in foeto-maternal immune tolerance and the remodelling of spiral uterine arteries.[52] $CD56^{bright}$ NK cells are $CD16^{dim}$ or $CD16^-$ and the increase in $CD56^+CD16^+$ NK cells may lead to pregnancy loss.[53,54] In this study, according to the inclusion criteria of the women participating in the trial, there were no significant differences in the initial population of NK cells, $CD8^+$ T cells and the serum levels of the investigated cytokines. While in subsequent analysis, we found that the $CD56^{bright}$ subset of NK cells was not identical in the studied groups. Therefore, even though the results have shown the effectiveness of treatment approaches on $CD56^{dim}$ NK cells, we could not debate these results on $CD56^{bright}$ NK cells.

The two investigated treatment approaches (prednisolone therapy and LIT) have different mechanisms of action despite their comparable results in creating foeto-maternal tolerance and maternal immune response modulation. This functional difference between these two treatment approaches was also well-observed in our study. The effect of prednisolone in reducing the population of $CD8^+$ T and NK cells was more influential than that of LIT and caused to increase in $CD3^+CD8^+CD56^+$ NKT cells.

NKT cells have been considered another effective immune cell in foeto-maternal tolerance maintenance during early pregnancy. NKT cells express a TCR complex (CD3) and NK receptors (CD56), which include a minor subpopulation of human decidual immune cells.[55] NKT cells increase in the decidua during early pregnancy and have the unique potential to produce large amounts of cytokines (IFN-γ and IL-4) to control the T helper cell function at the maternal-foetal interface.[56-58] Notably, the dNKT cells produce much more IFN-γ than peripheral NKT cells.[59] We studied $CD3^+CD56^+CD16^+$ and $CD3^+CD8^+CD56^+$cells as two distinct subsets of NKT cells. Several studies have shown that the $CD3^+CD56^+CD16^+$ NKT cell subset was a principal source of pro-inflammatory mediators, and their increase was associated with post-implantation

pregnancy loss and pregnancy loss after IVF[60-62] whereas some studies have reported that $CD3^+CD8^+CD56^+$ NKT cells may have a regulatory function.[63-65] Meggyes *et al*. have indicated the activation and pre-inflammatory or regulatory function of NKT cells can depend on their microenvironment in the uterus and periphery; therefore, this cell population is under exact control during pregnancy.[66]

The effect of the investigated treatment approaches on the population of peripheral $CD3^+CD56^+CD16^+$ NKT cells of women with RSA was not adequately significant. The same situation was also observed regarding the serum level of IFN-γ. It was noted that dNKT cells have a more significant potential to produce this cytokine, so local investigation of IFN-γ may be more efficient. In contrast to the LIT group, the administration of prednisolone increased $CD3^+CD8^+CD56^+$ NKT cells which are helpful for pregnancy. The lower capability of LIT in reducing T and NK cells compared to prednisolone therapy may be due to its mechanism of action, which is because of stimulating the production of blocking antibodies. Therefore, immune cells that depend on antibodies for their function, such as $CD56^{dim}$ cells, have shown a further decrease after LIT. In the present study, we found that prednisolone increased $CD56^{dim}$ cell subsets. The increase in this subset of NK cells may be caused by the disruption of the balance of NK cell subsets due to the overall suppression of NK cells by prednisolone.

The balance between inflammatory and anti-inflammatory cytokines is crucial in implantation success and preventing miscarriage.[67] In early pregnancy, IFN-γ is mainly produced by Th1 cells.[68] The presence of IFN-γ in implantation and the first trimester is crucial,[69] and then, its level in serum and tissue will be regulated.[68] The excessive increase or decrease of this inflammatory cytokine can increase the risk of miscarriage. Inagaki *et al*. demonstrated that the IFN-γ concentration in the uterine cavity fluid decreases in women with RIF.[70] As mentioned, one of the sources that produce this cytokine is NKT cells.[59] Of course, their local secretion is more in the decidua than in the blood. In our trial, the investigated treatment approaches did not significantly alter the serum level of this cytokine. Therefore, these treatment approaches in women with normal serum levels of IFN-γ have no harmful effects in changing the production of this cytokine.

Nevertheless, in the case of two anti-inflammatory cytokines, including TGF-β and IL-10, LIT and prednisolone therapy caused a decrease in the serum levels. These cytokines are secreted mainly by Th2 cells.[68] The suppression of Th cells by these interventions may result in the reduction of TGF-β and IL-10 serum levels. The effect of prednisolone in reducing the serum level of cytokines possibly was because of its general immunosuppression.

In the case of anti-inflammatory cytokines, the effect of LIT on the cells producing anti-inflammatory cytokines was less in comparison to prednisolone therapy. However, according to our expectations, LIT could increase the production of these two cytokines.

Conclusion

Based on the findings of the present study, prednisolone therapy and LIT can have positive effects in reducing the risk of implantation failure and foetal loss through the reduction of T and NK cell populations in women with RSA and RIF. On the other hand, this inhibitory effect is more in the case of prednisolone therapy, and the minimum dose used shows these effects well. LIT, with a different mechanism of action than prednisolone therapy, has less impact in declining the population of NKT cells. But despite this, it did not cause a general suppression of maternal immune cells and a decrease in the production of various cytokines.

These treatment approaches had different effects on NKT cells, which indicate that NKT cell population and function can be affected using LIT and prednisolone therapy. However, for a more detailed examination of the functional and phenotypic changes in NKT subsets, their status in the endometrium and decidua should be further investigated.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement

Data is available upon request from the corresponding author.

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