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Immunomodulatory effects of hydroxychloroquine on Th1/Th2 balance in women with repeated implantation failure

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ABSTRACT

Background: Cellular immune abnormalities such as the imbalance between T-helper (Th) 1 and Th2 cytokines have been implicated as potentially modifiable causes of idiopathic repeated implantation failures (RIF). The purpose of this study was to investigate the effects of hydroxychloroquine on IL-10 and TNF-α secretion, expression of T-bet and GATA-3 transcription factors and cellular localization of TNF-α, IFN-γ, IL-10 and IL-4 in endometrial cells in women with RIF.

Materials and methods: A total of 17 women with a history of RIF and elevated TNFα/IL-10 ratio (TNFα/IL-10 > = 30.6) were included in the study. The serum levels of TNFα and IL-10, the expression of transcription factors related to Th1 and Th2 cells and the immune-reactivity of TNFα, IFN-γ as Th1 related cytokines and IL-10, IL-4 as Th2 related cytokines in endometrial tissues were evaluated by ELISA, real-time PCR, and fluorescent immunohistochemistry respectively. All, evaluations were done both before and after treatment with hydroxychloroquine (400 mg/orally per day).

Results: Hydroxychloroquine treatment significantly decreased ($p < 0.0001$) serum level of TNF-α and significantly increased serum level of IL-10 ($p < 0.0001$). T-bet, the Th1 transcription factor, expression was down-regulated and GATA-3, the Th2 transcription factor, expression was up-regulated. IL-10 and IL-4 fluorescent immunoreactivities significantly increased ($p < 0.05$ and $p < 0.001$ respectively) and TNFα and IFN-γ fluorescent immunoreactivities significantly decreased ($p < 0.05$) in endometrial tissue in women with RIF after treatment in comparison with before treatment.

Conclusion: Hydroxychloroquine administration in women with RIF With a high TNF-α/IL-10 ratio during the implantation window can decrease this ratio and seems to be an effective therapeutic strategy in RIF caused by cellular immune abnormalities through a shift in Th2 responses.

1. Introduction

Despite the rapid development and widespread clinical application of in vitro fertilization (IVF) for patients with infertility during the past decades [1], the rate of successful implantation after embryo transfer (ET) is low (10%–15%) and the repeated implantation failure (RIF) still remains a major challenges in human reproduction [2]. RIF is defined as lack of pregnancy after at least 3 times of fresh or frozen embryo transfer (FET) to the uterus [3]. In recent years, it has been suggested that RIF is associated with Innate or Acquired Immunity disorders and Immunological factors can cause failure in the implantation through reducing endometrial receptivity [2,4,5]. In pregnancy, the immunological system plays an important role both in ensuring normal pregnancy development and in the development of complications. A pregnancy is successful when the balance among Th1, Th2, Th17 cytokines and Tregulatory cells works properly [6]. During implantation

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and at the beginning of pregnancy, dynamic changes occur in the immune responses of the female reproductive system, affect the implantation of the fetus and the progression of pregnancy [7]. It has been suggested that Th1 cytokines such as TNF-α and IFN-γ have adverse effects on pregnancy, while Th2 cytokines such as IL-4 and IL-10 are important in successful pregnancy. Therefore, the increase in Th1/Th2 ratios may increase cytotoxicity against embryo and lead to implantation failure. The balance of Th1 and Th2 has a potential to become a useful marker for detecting immunological rejection between the uterine endometrium of the infertile women and transferred embryos in Assisted reproductive technology (ART). Moreover, this ratio can be used for choosing the treatment strategy for the patients for RIF [8,9]. Many studies have demonstrated that elevated anti-inflammatory cytokines such as IL-10 and IL-4 and decreased pro-inflammatory cytokines such as TNF-α and IFN-γ lead to better pregnancy outcomes and the precise balance between these cytokines in endometrial tissue is necessary to achieve good reproductive results. According to the previous studies the increased level of TNF-α and decreased level of IL-10 have been reported in women with RIF after IVF/ET implantation and the low levels of TNF-α cytokine in serum can be considered as a valuable predictive for an IVF with a successful pregnancy [2,5,10,11].

Therefore the suppression and regulation of the immune system by using immunomodulator or immunosuppressive agents are one of the key ways to reduce RIF [1,5,12,13]. Hydroxychloroquine, an antimalarial drug is widely used in patients with various autoimmune diseases, particularly systemic lupus erythematosus (SLE). This drug has anti-inflammatory and immune-regulatory properties: such as inhibiting phospholipase activity, stabilizing lysosomal membranes, blocking the production of several pro-inflammatory cytokines such as TNF-α, IL-17, IL-6, IFN-α, and IFN-γ and, in addition, decreasing complement-dependent antigen-antibody reactions and increased T-regulatory cells (T-reg) [14–15]. Accordingly, in the current study, the effects of hydroxychloroquine on immune abnormalities associated with RIF were investigated and the pro-inflammatory and anti-inflammatory cytokines were evaluated. To our knowledge, this is the first study to investigate the potentiality of hydroxychloroquine treatment to create a shift toward Th2-type responses which are useful for a successful implantation in patients with RIF.

2. Materials and methods

2.1. Study population

The study was performed at the Infertility and IVF division of Shahid Motahari Hospital of Urmia University of Medical Sciences during the 24-month period from March 2017 to February 2018, in collaboration with the department of reproductive biology of Shahid Beheshti University of Medical Sciences. The study was approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU. MSP.REC.1396.160). an informed consent was obtained from all of the subjects. Immunological disorders in women with RIF were determined by detecting the TNF-α, IL-10 and TNF-α/IL-10 ratio of 30.6 or above was classified as an elevated Th1/Th2 ratio [9]. From 93 patients with a history of at least three repeated implantation failure 76 women with normal TNF-α/IL-10 ratio were excluded from the study and only 17 Infertile patients with impaired TNFα/IL-10 balance (TNFα/IL-10 > = 30.6) Were included in the study. Prior to inclusion, all women were examined for genetic, endocrine, anatomic and infectious abnormalities and confirmed as negative for these parameters. Demographic data of patients are summarized in Table 1.

2.2. Hydroxychloroquine treatment protocol

Patients with impaired TNF/IL-10 ratio were treated with 400 mg hydroxychloroquine daily for 16 consecutive days. The treatment was started from day 3 or 4 of the menstrual cycle and continued until the day 20 of the menstrual cycle.

Table 1
Age and clinical data of women with RIF which were included to study (n = 17).

Number of patients	17
Age (y)	33.6 ± 4.2
BMI (kg/m2)	24.7 ± 3.8
Infertility duration (y)	5.3 ± 2.6
Failed prior IVF cycles	4 ± 6.5
Number of embryo transferred	12.19 ± 3.58
TNF-α/IL-10 ratio (mean ± SD)	35.24 ± 2.3

2.3. Blood sampling

Peripheral blood from selected women were collected into sodium heparin VACUTAINER_ tubes (Becton Dickinson & Co, NewJersey USA), three times, in two separate menstrual cycles. The first sample was obtained in first Menstrual Cycle from all patients between days 5–10 in order to select the patients with the elevated TNFα/IL-10 ratio (TNFα/IL-10 > = 30.6). The second sample was obtained from selected women in day 21 of the same menstrual cycle (before treatment with hydroxychloroquine) and the third one was obtained at the same day in the next cycle (hydroxychloroquine treatment cycle) for post-treatment testing and for estimation of possible changes in the above parameters. The flowchart of the experimental study design is shown in Fig. 1.

2.4. Endometrial biopsy

Endometrial biopsies were collected twice from all patients. The First sampling was done before the treatment and the second was conducted after the treatment with hydroxychloroquine at day 21 of each menstrual cycle. Each endometrial sample was divided into two parts to examine the intracellular cytokines involved in the implantation by immunohistochemistry and their related genes by Real Time PCR.

2.5. Determination of TNF-α and IL-10 Cytokines release by enzyme linked immunosorbent assay (ELISA)

Secretion of TNF-α and IL-10 cytokines was analyzed by enzyme-linked immunosorbent assay (ELISA). Serum was prepared by centrifugation of coagulated blood tubes at 2000 *g* for 10 min at room temperature and stored in −70 °C. Samples were tested for IL-10 and TNF-α using a sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, USA). All sample conditions were measured in duplicate.

2.6. Expression analysis of T-bet and GATA-3 transcription factors by quantitative real-time polymerase chain reaction (qPCR)

The mRNA levels of T-bet and GATA-3 were determined by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. To evaluate the mRNA expression of transcription factors related to Th1,Th2 cells(T-bet, GATA-3), total RNA was isolated from endometrial tissue using RNX-PLUS Solution (SinaClon, Tehran, Iran) according to the manufacturer's procedure and Concentration of each RNA sample was measured using the Nanodrop 1000Spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized by using the AccuPower CycleScript RT PreMix (dN 6) kit (Bioneer Inc., Seoul, South Korea). SYBR Green method was employed for T-bet and GATA3 analyses. Quantitative PCR reactions were done in a 20 µL volume including 10 µl SYBR Green PCR master Mix 2X (Takara Bio, Otsu, Japan), 1.5 µL of forward and reverse primers, 2 µL of cDNA and 5 µL DEPC Treated Water following the manufacturer's instructions. After a

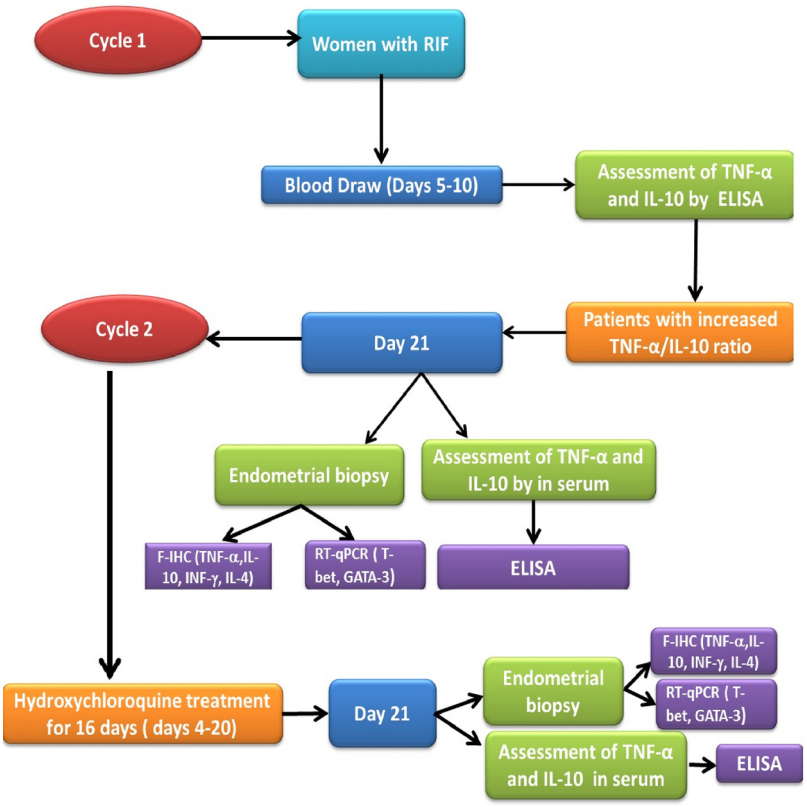

Fig. 1. The flowchart of the experimental study design.

Table 2
Primer sequences.

	Sequence (5'- > 3')	TM(°C)	Primer Size(bp)	Product size(bp)
T-bet				
Forward	CCTATGCGGACTCTGCCCATG	62.90	21	104
Reverse	CGGATGGGGGCAATCTCAGTC	62.85	21	
GATA-3				
Forward	CTTCGGATGCAAGTCCAGGCC	63.63	21	126
Reverse	CAGGCGTTGCACAGGTAGTGT	63.16	21	
Beta-actin				
Forward	GCA TGG GTC AGA AGG ATTCCT	61.88	21	106
Reverse	TCG TCC CAG TTG GTG ACG	61.83	21	

primary 10 min at 95 °C as activation step, 40 cycles comprising of denaturation at 95 °C, 10 s, annealing at 60◦ C, 15 s, and extension at 72 °C, 15 s were performed by Mic Real-Time PCR (Bio. Molecular Systems, Australia) detection system. Gene expression was analyzed by comparing with the reference gene in each PCR run. Sequences of the primers are summarized in Table 2. Amplification was confirmed by using electrophoresis analysis on 2% agarose gel followed by DNA sequencing performed by Bioneer (Bioneer Corporation, Daejeon, South Korea). Relative gene expression levels were obtained using the ΔΔCt standard 2-ΔΔct calculations and expressed as a fold change of a control sample. Amplification specific transcripts were further confirmed by obtaining melting curve profiles. All reactions were performed in triplicate.

2.7. Tissue collection and immunohistochemistry

The endometrial biopsies were immediately placed into 10% neutral-buffered formalin for overnight fixation and then embedded into paraffin wax. TNF-α, IFN-γ, IL-4 and IL-10 expression were determined with the use of fluorescent immunohistochemistry. The endometrium sections with 5 μm thickness were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 min and permeabilized by 0.3% Triton X100 for 30 min and blocked in 10% normal goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature. The samples were incubated at 4 °C overnight with primary antibodies for TNF-α (Mouse monoclonal [2C8] to TNF-α; abcam), IFN-γ(Rabbit polyclonal to IFN-γ[ab25101]; abcam), IL-4(Mouse monoclonal to IL-4 [ab2503]; abcam) and IL-10(Rabbit polyclonal to IL10 [ab34843]; abcam) diluted in PBS. The FITC-conjugated secondary antibodies were diluted with PBS and the samples were incubated with the secondary antibody at room temperature and kept in the dark for 1 h. The sections were subsequently mounted with 4′6-diamidine-2′-phenylin-dole dihydrochloride (DAPI) from Roche to stain nuclei and preserve fluorescence signals. The fluorescent signals were analyzed by means of fluorescence microscopy (Olympus BX51). For each patient endometrial tissue samples were analyzed in triplicate. The triplicate tissue were serial section from the same paraffin tissue block. All samples were imaged with the use of a ×400 objective lens. For further quantitative analysis of the immunostaining results, an Image J (NIH) picture analysis software was used to calculate mean pixel fluorescence intensity.

2.8. Statistical analysis

Statistical analysis was performed using SPSS PC Statistics (version 22; SPSS Inc., Chicago, IL, USA). Paired *t*-test was applied to compare the results of immunologic studies before and after hydroxychloroquine treatment. P-values < 0.05 were reported to be statistically significant.

3. Results

3.1. TNF-α and IL-10 cytokine secretions in RIF patients before and after treatment with hydroxychloroquine

Hydroxychloroquine was given in an attempt to reduce the TNF-a /

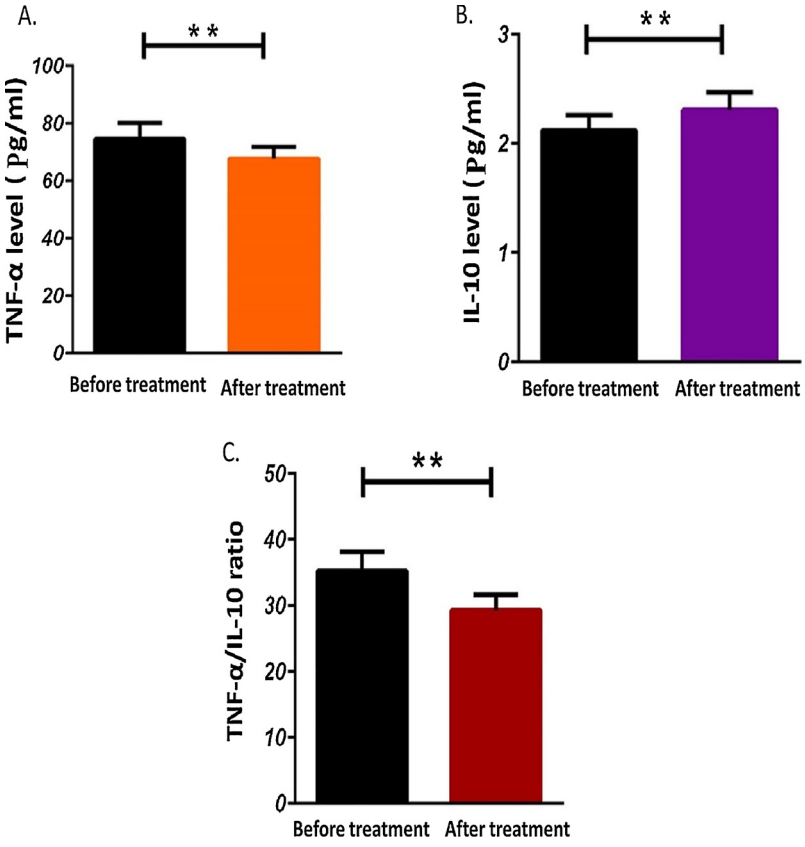

Fig. 2. Cytokine concentrations were measured in serum of women with recurrent implantation failure, before and after treatment with hydroxychloroquine. The concentration of cytokines was measured using specific cytokine ELISA detection kits (R &D Systems). A) TNF-α level before and after treatment l. B) IL-10 level before and after treatment. C) TNF-α/ IL-10 ratio before and after treatment (**P-value < 0.001). (P ≤ 0.05: statistically significant).

IL-10 ratio to a normal range. Measurement of TNF-α and IL-10 cytokines secretion in the serum isolated from women with RIF indicated a significant lower concentration of TNF-α ($p < 0.001$) as Th1-associated inflammatory cytokine and a significant higher concentration of IL-10 ($p < 0.001$) as Th2-associated anti-inflammatory cytokine ($p < 0.001$) after treatment, in comparison with the menstrual cycle before treatment. Moreover, TNF-α/IL-10 ratio was significantly decreased in women with RIF after treatment. (Fig. 2).

3.2. mRNA expression levels of transcription factors related to Th1 and Th2 cells in women with recurrent implantation failure, before and after treatment

T-bet is selectively expressed in Th1 clones whereas GATA-3 is selectively expressed in Th2 clones. Expression of these genes is rapidly induced in primary T-cells developing along the Th1 or Th2 pathway. T-bet and GATA-3 mRNA levels in two consecutive cycles without treatment and with treatment were evaluated and then compared with each other. As shown in Fig. 3 the mRNA level of GATA-3 significantly increased ($p < 0.0001$) and the mRNA level of T-bet and T-bet/GATA3 ratio significantly decreased ($p < 0.0001$) after treatment with hydroxychloroquine in comparison with the menstrual cycle before treatment. The change in T-bet expression was proportionally greater than for GATA-3, and this was reflected in the T-bet/GATA-3 ratio. To confirm the differentiation status of these Th1 and Th2 cells, the expression of Th1-specific IFN-γ, TNF-α and Th2-associated IL-4 and IL-10 mRNA was analyzed by RT-PCR. The variation of T-bet and GATA-3 expression and its correlation with Th1/Th2 status was reflected more closely by the ratio of T-bet to GATA-3. Indeed, the T-bet/GATA-3 ratio significantly increased under Th1 conditions and decreased under Th2 conditions. (Fig. 3B)

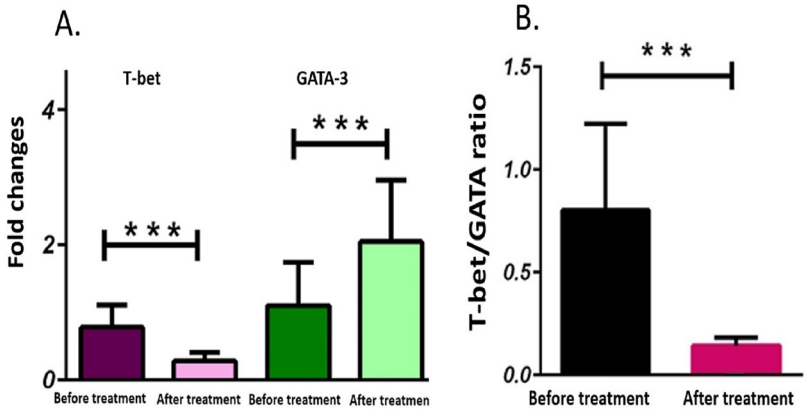

Fig. 3. mRNA expression of transcription factors in endometrial tissue, pretreatment and after -treatment, in the time of implantation window was measured by qRT-PCR. Gene expression data were normalized to β-actin expression. A) The mRNA expression level of T-bet before treatment was significantly higher in comparison with after treatment ($P < 0.0001$). Also, the mRNA expression of GATA-3 significantly increased after treatment (***P-value < 0.0001). B) T-bet/ GATA3 ratios were significantly decreased after treatment (***P-value < 0.0001).

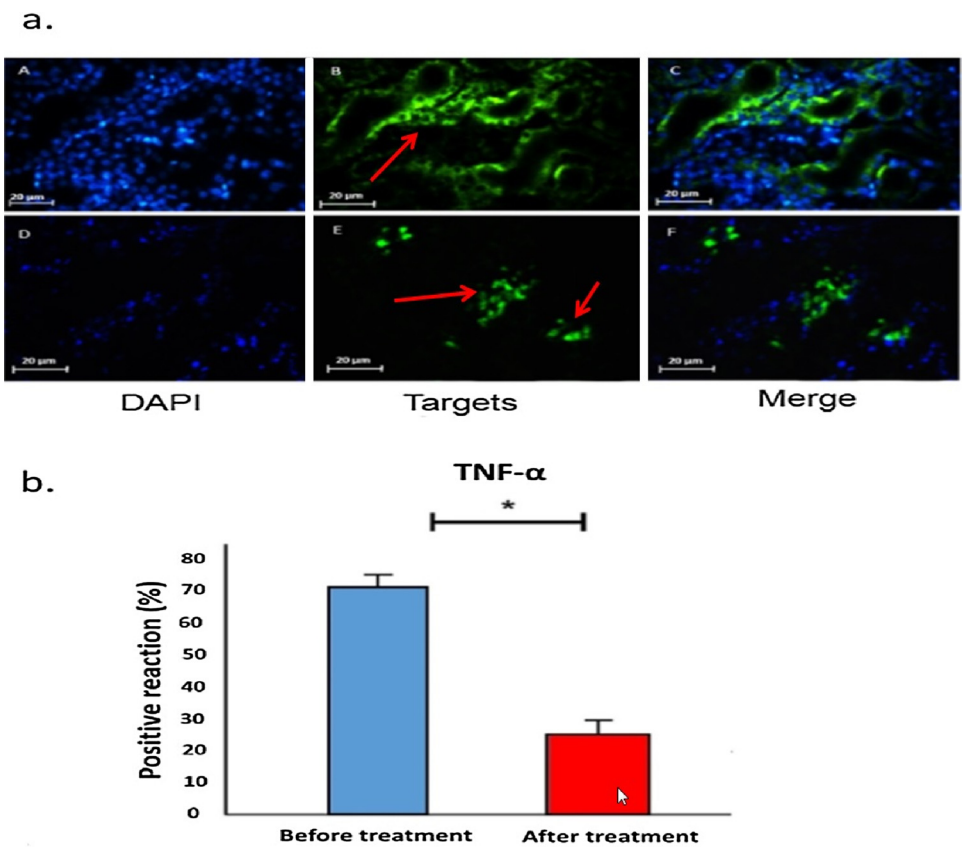

Fig. 4. a) Cellular localization of TNF-α in endometrial cells. Fluorescent immunohistochemistry staining of TNF-α before treatment (A–C) and after treatment (D–F) were performed on the endometrium (n = 17). DAPI was used to stain the nuclei (A, D). Then TNF-α - specific signal and DAPI could be merged together, indicating that TNF-α protein is localized in the Endometrial cells(C). Localization of TNF-α change after hydroxychloroquine treatment was observed. (F) Magnification = × 400. Scale bar = 20 µm. b) Corresponding bar graphs represent the quantitative analysis of TNF-α expression in endometrial tissue. For each patient endometrial tissue samples were analyzed in triplicate. TNF-α expression in endometrial tissue significantly decreased after treatment (*P-value < 0.05).

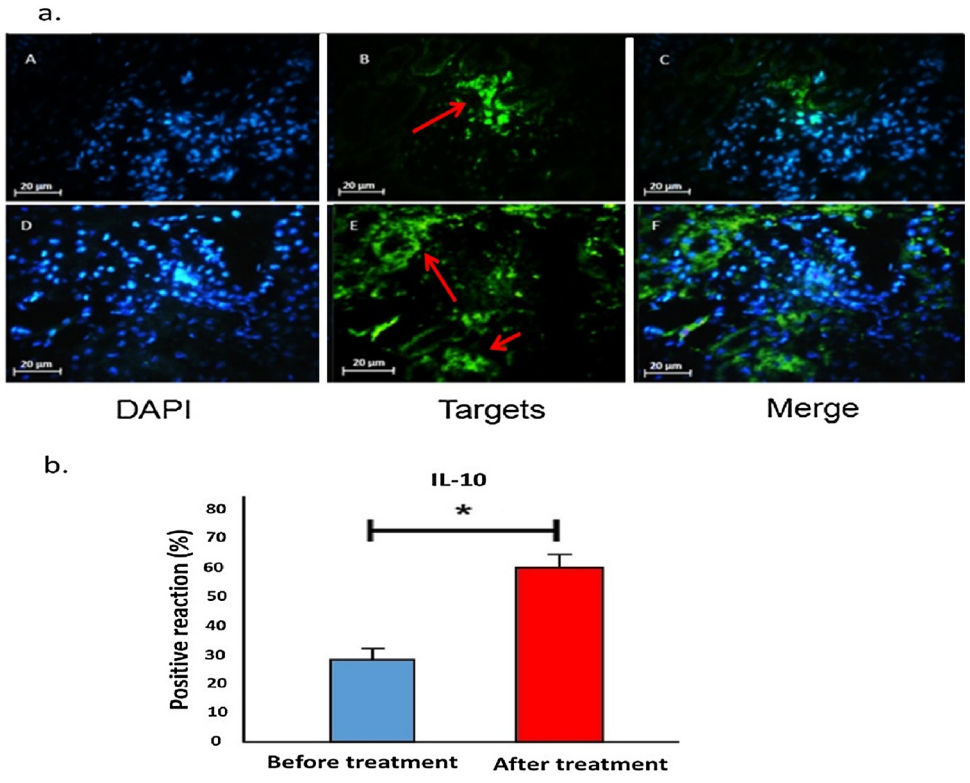

Fig. 5. a) Cellular localization of IL-10 in endometrial cells. Fluorescent immunohistochemistry staining of IL-10 before treatment (A–C) and after treatment (D–F) were performed on endometrium in women with RIF (n = 17). DAPI was used to stain the nuclei (A, D). Then the pictures were merged together (C, F). For each patient endometrial tissue samples were analyzed in triplicate. Magnification = × 400. Scale bar = 20 µm. b) Corresponding bar graphs represent quantitative analysis of IL-10 expression in endometrial tissue. IL-10 expression in endometrial tissue significantly increased after treatment (*p value < 0.05).

3.3. Cellular localization of TNF-α, IFN-γ, IL-10, IL-4

Cellular Localization of Th1 (TNF-α, IFN-γ) and Th2 (IL-10, IL-4) related cytokines in endometrial tissues was determined before and after treatment in women with RIF. Seventeen endometrial sections taken in day 20 of the cycle were analyzed by immunohistochemistry. We observed only very weak reactivity for IL-10 and IL-4 in endometrial cells before treatment (Figs. 5 and 7). The positive reactions

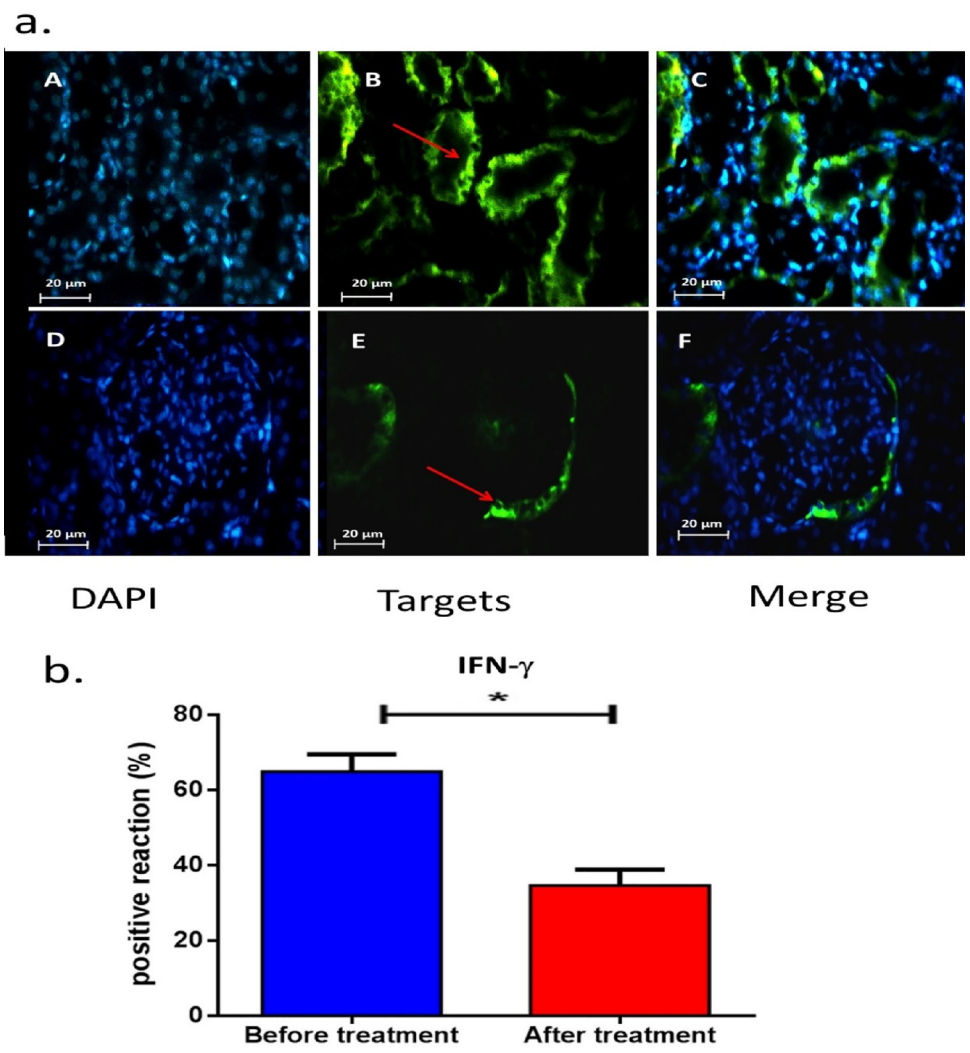

Fig. 6. a) Cellular localization of IFN-γ in endometrial cells. Fluorescent immunohistochemistry staining of IFN-γ before treatment (A–C) and after treatment (D–F) were performed on the endometrium in women with RIF (n = 17). DAPI was used to stain the nuclei (A, D). Then IFN-γ- specific signal and DAPI could be merged together, indicating that IFN-γ protein is localized in the Endometrial cells(C). Localization of IFN-γ changes after hydroxychloroquine treatment was observed (F). For each patient endometrial tissue samples were analyzed in triplicate. Magnification = × 400. Scale bar = 20 µm. b) Corresponding bar graphs represent the quantitative analysis of IFN-γ expression in endometrial tissue. IFN-γ expression in endometrial tissue significantly decreased after treatment (*P-value < 0.05).

(%) of IL-10 and IL-4 were significantly increased after treatment in comparison with the menstrual cycle before treatment (P-value < 0.05 and P-value < 0.001 respectively). As shown in Figs. 4 and 6 TNF-α and IFN-γ proteins are localized in the endometrial tissue. The positive reactions (%) of TNFα and IFN-γ were significantly decreased after treatment with hydroxychloroquine (P-value < 0.05).

4. Discussion

Repeated implantation failure (RIF) is often determined when embryos of good-quality fail to implant following at least three consecutive IVF attempts [10]. The correct balance between Th1 cytokines and Th2 cytokines is necessary to achieve good reproductive outcome [5,10,12]. Therefore immunotherapy has been empirically used in couples with recurrent implantation failure, based on the evidence that immunological factors may be responsible for lack of embryo implantation [13]. In the present study, the immunomodulatory effects of hydroxychloroquine in RIF patients with immunological abnormalities were examined. The importance of the balance of T helper cells in pregnancy has accumulated significant support over recent years and numerous studies have demonstrated an association between significant increase of TNF-α/IL-10 ratio and RIF [9,14].

one of the methods that is provided to diagnose infertility in people with implantation failures includes determining the ratio of TNF-α/IL-10 and the comparison of this ratio with those normal pregnancies to determine whether a patient with RIF with immunological reasons responds to treatment with immunosuppressive drugs or not [6,15]. Immunological causes may be among the most important factors that adversely affect endometrial receptivity and embryo implantation. The endometrium allows implantation mediated by several factors such as immune cells growth factors, chemokins, cytokines and adhesion molecules at implantation window.

Hydroxychloroquine, originally antimalarial drugs, now have been widely used in the treatment of autoimmune diseases because of its anti-inflammatory and immunomodulatory effects [16–18]. The overall safety of hydroxychloroquine use is well established with long-term administration showing a favorable safety profile, thereby enhancing the potential for clinical studies furthermore hydroxychloroquine does not appear to be associated with any increased risk of congenital defects, spontaneous abortions, fetal death, prematurity or decreased numbers of live births in patients with autoimmune diseases and is safe in pregnancy [17,19–21].

In this study, for the first time, we reported the successful intervention with hydroxychloroquine in patients with RIF characterized by high TNF-α/IL-10 ratio. These patients were treated with hydroxychloroquine as an immunomodulatory drug, and then the immunologic parameters which involved in the implantation were evaluated. Interestingly, we observed that hydroxychloroquine decreased T-bet/GATA3 ratio by changing the T-bet and GATA-3 mRNA expression as the transcription factors respectively related to Th1 and Th2 cells, furthermore hydroxychloroquine significantly decreased serum level of TNF-α and increased serum level of IL-10 and reduced the TNF-α/IL-10 ratio in these patients in compared with their previous menstrual cycle before treatment. Our results are inconsistent with previous studies

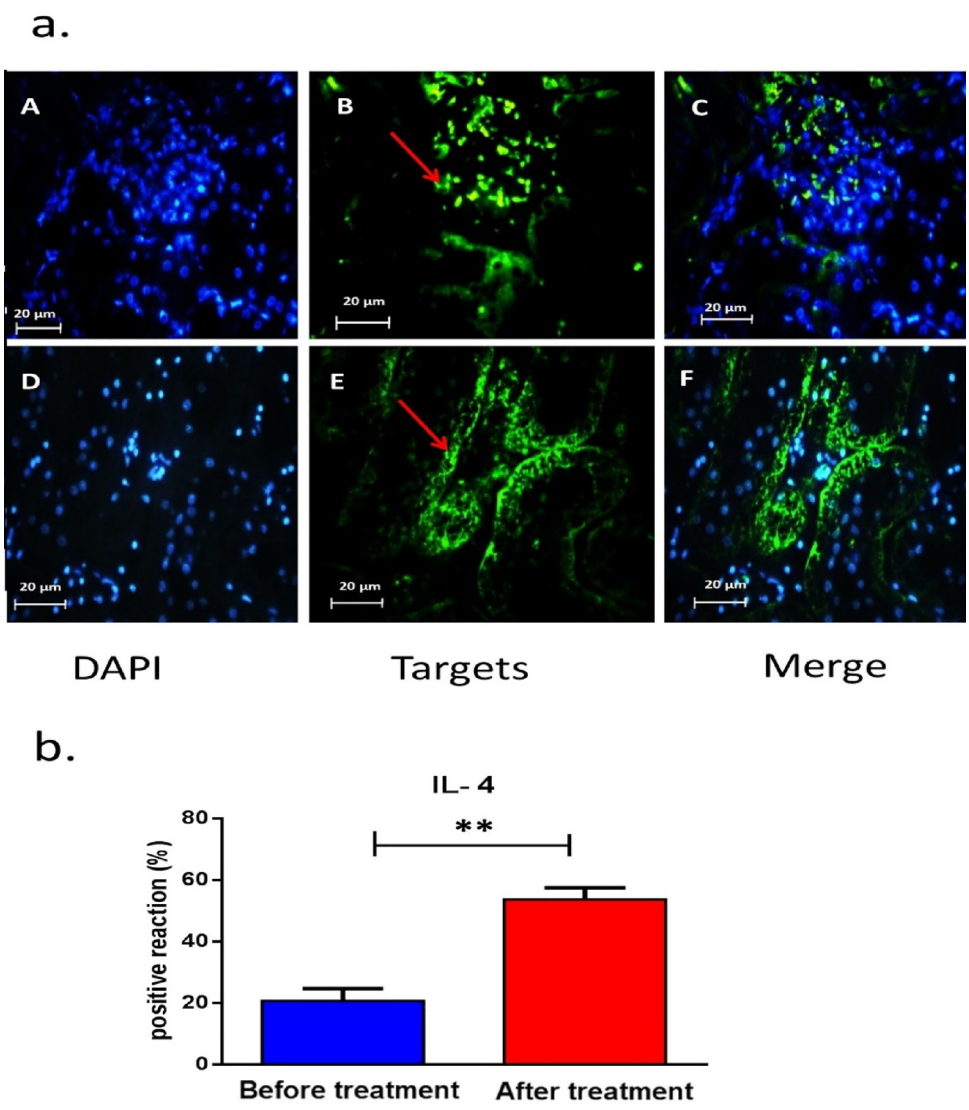

Fig. 7. a) Cellular localization of IL-4 in endometrial cells. Fluorescent immunohistochemistry staining of IL-4 before treatment (A–C) and after treatment (D–F) were performed on the endometrium in women with RIF (n = 17). DAPI was used to stain the nuclei (A, D). Then the pictures were merged together (C, F). For each patient endometrial tissue samples were analyzed in triplicate. Magnification = ×400. Scale bar = 20 µm. b) Corresponding bar graphs represent the quantitative analysis of IL-4 expression in endometrial tissue. IL-4 expression in endometrial tissue significantly increased after treatment (**P-value < 0. 001).

which reported that hydroxychloroquine can be used in treatment of reproductive failure in antiphospholipid syndrome by immunomodulatory functions, with complement pathway inactivation, TNF-alpha blocking, Th1/Th2 shifting effect and the inhibition of TLR [22]. Literature data suggests that hydroxychloroquine interfere with TNF-α release from human and murine cells, although it's exact mode of action is not fully understood [23,24]. Previous studies suggested that the immunoregulatory effects of hydroxychloroquine on the cytokine network might be important in the therapeutic effects of hydroxychloroquine in immune-mediated disorders [25–30]. study of Gilman et al demonstrated that hydroxychloroquine causes a dose-dependent reduction of the cytotoxicity, proliferation, and TNF alpha production resulting from allorecognition in mixed lymphocyte culture they stated that it may be useful in the prevention and treatment of graft-versus-host disease (GVHD) [31,32]. In another study by Koch et al pretreatment with hydroxychloroquine reduced the production of TNF-α in experimental autoimmune encephalitis. They suggested that the reduction of pro-inflammatory cytokines through hydroxychloroquine treatment could be a therapeutic avenue in MS [16]. Several characteristics make hydroxychloroquine an attractive agent to bring to clinical trial. hydroxychloroquine is usually well tolerated and widely used as a chronic treatment in SLE and RA [33,34].

According to our results T-bet/ GATA3 mRNA ratio, TNF-α/IL-10 ratio and Cellular localization of TNF-α, IFN-γ significantly decreased after treatment by hydroxychloroquine in women with RIF. These results showed that administration of hydroxychloroquine significantly increased the reactivity of IL-10 and IL-4 as anti-inflammatory cytokines in endometrial tissue and shifted the immune response toward the Th2 pattern and the shift to a Th2 pattern plays an important role in the good pregnancy outcomes. Several immunomodulatory drugs such as intravenous immunoglobulin G (IVIG), Etanercept (Enbrel), adalimumab (HumiraTM) and Tacrolimus have been previously reported to be effective in women with RIF by reducing Th1/Th2 lymphocyte ratio in peripheral blood. Increased successive rate of IVF by these medications in RIF patients who had an elevated peripheral blood Th1/Th2 ratio indicates a significance of Th1/Th2 immune regulation in implantation and maintenance of pregnancy [5,10,35,36]. In accordance with these studies, our results revealed that hydroxychloroquine treatment can be an appropriate candidate for reducing endometrial immune reaction occurring in implantation window, which is the time when the endometrium must receive the embryo [37].

According to our analyses performed after treatment,

hydroxychloroquine significantly reduced Th1-associated cytokine secretion and the mRNA level of T-bet after treatment, furthermore, the Th2 associated cytokine secretion and the mRNA level of GATA-3 increased in compared with the previous cycle without treatment. As a result, Th1/Th2 balance is affected by hydroxychloroquine, and a decrease in the ratio was observed through a shift toward Th2 responses. An increase of IL-10 could be also due to an expansion of T regulatory cells However, after treatment with hydroxychloroquine the increased localization of IL- 4 in addition to IL-10 in the endometrial tissue, as well as the increased GATA-3 mRNA expression as the transcription factor related to Th2 cells, suggest that immune responses are likely to shift to Th2 responses. We note the limitations of our study. The main limitation was that in this study we did not evaluate success rate of IVF after women's received hydroxychloroquine treatment. Another limitation was the reduced number of patients. In addition, the measurement of additional cells and cytokines related with the Th1 and Th2 profile, such as IL-6, IL1B and IL-13, in addition to TNFa and IL-10, would be of interest to have a more robust panorama of the inflammatory profile of these patients.

5. Conclusion

To our knowledge, this is the first study to show the ability of hydroxychloroquine to modulate the immune system in women with RIF with a high Th1/Th2 ratio during the implantation window. Our results demonstrated that hydroxychloroquine can shift toward Th2 type responses and decrease the Th1/Th2 ratio. these findings suggest that there is merit in further assessing hydroxychloroquine, a drug that has a proven safety profile in pregnancy, as an adjuvant therapy for increasing IVF success rates in women with the initial elevation Th1/Th2 ratio. These issues are fundamental and must be considered in future investigations to produce more consistent results that can benefit the field of obstetrics.

Conflict of interest

The authors declare that there is no conflict of interest.

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