BIOPHARMACEUTICAL IMPACT OF VAGINAL SILDENAFIL CITRATE THERAPY ON SOME INFLAMMATORY CYTOKINES AND T/NK CELLS SUBSETS IN UNEXPLAINED RECURRENT SPONTANEOUS MISCARRIAGE: FIRST LONGITUDINAL CLINICAL STUDY OF 50 CASES FROM EGYPT

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ABSTRACT

Various immunological abnormalities have been reported in women with unexplained recurrent spontaneous miscarriage (URSM). The definition of pregnancy as a "T\(_H\)2" or anti-inflammatory state was proved by numerous studies and a shift in T\(_H\)2 cytokines (e.g. IL-4, IL-6, IL-10) towards T\(_H\)1 cytokines (TNF-\(\alpha\), IFN-\(\gamma\)) may be a potential relevant factor in RSM. In a previous study, we were the first to show the novel biopharmaceutical use of vaginal sildenafil citrate (SC) in treatment of URSM. The aim of this study is to evaluate the effect of vaginal SC tablet administration at different doses on the NK and T-cell subsets (as evaluated by CD3, CD4, CD8, CD16, and CD56 expression) and on some pro- and anti-inflammatory cytokines (TNF-\(\alpha\), IL-6 and IL-10) in patients with first trimester URSM in a longitudinal clinical study and to correlate this with successful pregnancy outcome. We investigated the effects of vaginal SC on some immunological abnormalities found in F-URSM, the study was conducted on two groups of patients in addition to a control group a control group. The first patient group (F-URSM1) received SC at a dose of 25 mg intravaginally, 4 times/day for 24 days (n = 20), while the
second patient group (F-URSM2) received SC at a dose of 25 mg intravaginally, 3 times/day for 13 days, (n= 20). The control group comprised of 10 matched first trimester normal pregnant (FTP). Patients also received their usual treatment used in their previous pregnancies ended with miscarriages. The percentage of T-cells subsets, CD16+CD56+ NK cells, TNF-α producing cells, as well as serum IL-6 and IL-10 levels were determined in patient and control groups. Intravaginal SC treatment increased the pregnancy success rate that was more observed in F-URSM1 (65%) than in the F-URSM2 (55%) patients groups. Moreover, different doses significantly increased IL-10 level in the patients under investigation, while the percentage of TNF-α producing cells and CD56+CD16+ NK cells in peripheral blood were significantly decreased after SC treatment. In conclusion, the use of intra-vaginal SC as anti-abortive agent in URSM patients is partly due to its ability to correct the percentage of TNF-α producing cells, NK cells), and IL-10. Accordingly, SC may offer a potential pharmaceutical therapeutic strategy through ameliorating various immunological abnormalities that might contribute to URSM.

Key words: Sildenafil citrate; TNF-α; Cluster of differentiation (CD); Interleukins; NK-cells; Unexplained recurrent spontaneous abortion.

INTRODUCTION

Recurrent spontaneous miscarriage (RSM) is defined as three consecutive pregnancy loss prior to 20 weeks from the last menstrual period. 1% to 2% of women experience RSM [1]. Multiple etiologies for RSM have been reported including autoimmune (20%), endocrine (17% - 20%), anatomic (10% - 15%), genetic (2% - 5%) factors and infection (0.5% - 5%). However, about 40% - 50% of RSM are of unknown etiology and are classified as URSM [2].

As the fetus is secured from the humoral immunity during normal pregnancy, cell-mediated immunity (cells and cytokines) was considered an important etiologic factor in URSM [3]. A disrupted normal inflammatory events necessary for natural parturition may initiate and intensify the cascade of inflammatory cytokine production involved in adverse pregnancy outcomes, such as implantation failure, pregnancy loss, preeclampsia, preterm labor, intrauterine growth restriction, and fetal inflammatory syndrome [4, 5].

In normal pregnancy, Burns et al., [6] found that CD4+ T-helper are low in the first part of gestation, are increasing progressively from the third trimester, while CD8+ cells (suppressor/cytolytic lymphocytes T) are increasing in the moment of birth. Natural Killer
(NK) cells are CD3-/TCR- large granular lymphocytes that express CD56 and/or CD16 and mediate non-MHC restricted cytotoxic functions. They were found to play a crucial role in the altered immune repertoire found in RSM. Previously, it was reported that 37.1% of women with URSM have elevated peripheral blood NK cells.

Dysregulation of NK cells has been associated with RSM, infertility and preeclampsia. It has been reported that women with RSM and unexplained infertility have increased peripheral blood NK cells when compared to normal controls. Furthermore, down-regulation of NK cells in women with RSM was associated with a favorable pregnancy outcome. Although Emmer et al. reported no difference in NK cell levels or cytotoxicity before pregnancy in women with RSM when compared to normal controls, a longitudinal study in URSM women revealed that higher percentages of CD56⁺, CD16⁺ NK cells were present during early pregnancy, paralleled by an increase in cytotoxic NK cell reactivity as compared with controls.

In addition, women with URSM or multiple implantation failures (MIF) have significantly elevated TH1/TH2 cytokine ratios in the peripheral blood when compared to normal controls. Furthermore, El-Far et al. have found a correlation between URSM and elevated TNF-α.

Treatment of URSM is a challenging issue. The currently available lines of treatment according to simplicity of use, reliability and degree of invasiveness include corticosteroids, aspirin, heparin and immunoglobulins (besides good antenatal care), but up to now there are no prospective randomized studies, powerful enough, to determine a significant difference between two therapeutic protocols, with any of the above mentioned pharmacological agents. Several therapies have been advocated in patients with a history of URSM and an elevated levels of peripheral blood NK cells. Intravenous gamma immunoglobulin (Ig) is one of these treatment lines. However, a recent large placebo-controlled study found limited efficacy of intravenous gamma Ig in treating URSM patients. Another proposed therapy which is reported to increase implantation rates in these patients is intravenously administered intralipid. Other treatment modalities are sildenafil citrate (SC) that increases blood flow to the uterus and increases lining thickness in non-pregnant women with the history of URSM, and prednisolone, was shown to effectively suppress NK cell cytotoxicity in vitro. El Far et al. have recently reported novel preliminary as well as first longitudinal clinical study of 50 cases from Egypt, they were the first to show
Mohamed El-Far et al. World Journal of Pharmacy and Pharmaceutical Sciences

The biopharmaceutical use of SC as novel antiabortive agent in cases of URSM by restoring and augmenting the capacity of antioxidants as well as modulating lipid peroxidation and nitrosative stress and improvement of vasoconstriction through increasing blood flow causing relaxation of uterine arteries.

SC induces vasodilation through inhibition of type 5 phosphodiesterase (PDE5)\(^{[25]}\). PDE5 is responsible for the degradation of cGMP to guanosine monophosphate. Therefore, inhibiting PDE5 delays the breakdown of cGMP and increases vasodilatation\(^{[26]}\) and potentiates the effects of nitric oxide (NO) on the vascular smooth muscle. Although SC has been developed for erectile dysfunction, it is now used for other medical indications such as cardiovascular conditions and diabetes mellitus, depression, pulmonary hypertension, pre-eclampsia, IUGR, infertility patients with Asherman’s syndrome, inflammation, chronic heart failure and renal insufficiency, hypertensive disorders, and even to cancer treatment\(^{[27-30]}\). Also, oral administration of SC from day 5 of menstrual cycle to ovulation found to cause significant increase in subendometrial vascularity in apparently fertile women without effecting an increase in endometrial thickness or volume\(^{[31]}\). El-Far et al., recently used intravaginal SC and showed significant increase in blood flow in uterine arteries\(^{[13, 24]}\) Intravaginal administration of SC was found to decrease the incidence of systemic side-effects by delivering the medication in close proximity to the target organ\(^{[32-34]}\).

The aim of this study is to evaluate the effect of intravaginal SC administration at different doses on the percentages of T-cell subset, NK cells, and TNF-α producing cells in addition to the levels of IL-6 and IL-10 in patients with URSM in a longitudinal clinical study and to correlate this with successful pregnancy outcome.

**MATERIAL AND METHODS**

**Patients and samples**

Forty first trimester pregnant women with a history of RSM admitted to the Department of antenatal care at Mansoura University Hospital (MUH) suffering from threatened abortion including uterine bleeding (light defined as spotting only, or heavy) +/- painful uterine contractions, closed and viable fetus with positive heart activity detected by ultrasonographic examination until the end of the first trimester were included in our study. They were investigated and anatomic, genetic, microbiologic, and hormonal causes of miscarriages were excluded. A written consent was taken from each patient to participate in this study. The study was approved by the Medical Research Ethics Committee at Mansoura University.
During this time the patients received the usual treatment used in their previous pregnancies that ended with miscarriages including: gestagens (200 mg/day), low dose aspirin (75mg/day), antispasmodics (40 mg/day) and folic acid (500 microgram/day) and rest. No other therapy including nitrite was introduced before pregnancy for all subjects.

The patients were divided into two groups according to the dose of intravaginal SC. Group I (F-URSM1) consisted of 20 first trimester pregnant women with a history of URSM, to whom intravaginal SC was administered in a dose of 25 mg four times a day for 24 days (total dose 100 mg/day) and group II (F-URSM2) comprised of 20 first trimester pregnant women with a history of URSM, self-administered SC (25 mg intravaginally three times a day for 13 days; total dose 75 mg/day). In addition, a control group contained 10 first trimester normal pregnant women (FTP) with at least one previous successful normal pregnancy outcome and no history of miscarriages (group III).

The dosage was extrapolated from the maximum recommended daily dose for men and from our previous preliminary reported data study by El Far et al. [13, 24], given in three or four divided doses to minimize peak and trough effects. Age and obstetric history of the three studied groups are shown in table (1).

Table 1 Age and obstetric history of studied groups [13]

<table>
<thead>
<tr>
<th>Groups</th>
<th>F-URSM1 (n = 20)</th>
<th>F-URSM2 (n = 20)</th>
<th>FTP (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>27</td>
<td>26.5</td>
<td>25.5</td>
</tr>
<tr>
<td>Range</td>
<td>(19 – 35)</td>
<td>(20 – 33)</td>
<td>(22 – 33)</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Range</td>
<td>(0 – 2)</td>
<td>(0 – 3)</td>
<td>(1 – 3)</td>
</tr>
<tr>
<td>Gravidity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>5.5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Range</td>
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<td>(4 – 11)</td>
<td>(2 – 4)</td>
</tr>
<tr>
<td>Previous URSM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Range</td>
<td>(2 – 11)</td>
<td>(3 – 8)</td>
<td>0</td>
</tr>
</tbody>
</table>

Blood sample (6ml) was obtained by veinepuncture under sterile conditions. 3 ml of blood was drawn without anticoagulant; serum was used to measure levels of IL-6 and IL-10. In addition, 3 ml were collected on EDTA tubes and immediately transported to the
flowcytometry laboratory for determination of T-cells subsets, NK and TNF-α producing cells. Samples were obtained once from the control group, at zero day and 13 days from F-URSM1 and F-URSM2 groups, besides a third sample from F-URSM1 patients after 24 day of treatment.

**Determination of serum IL-6 and IL-10**

Enzyme-linked immunosorbent assays (ELISA) kits were used for determination of concentrations of IL-6 (IMMUNOTECH, a Beckman Coulter Company, Marseille Cedex 9, France) and IL-10 (ANOGEN, CANADA). The manufacturer’s protocols were followed, and recombinant reference cytokine samples served as positive controls for calibration.

**Detection of T-cell subsets, NK and TNF-α producing cells by Flow Cytometry**

**Cell separation and staining procedure**

Red blood cells were lysed by incubating with hypertonic ammonium chloride lysing solution (0.8 gm ammonium chloride, 0.1 gm EDTA, and 0.01 gm Dihydrogen potassium phosphate in 1 liter), for 10 minutes at room temperature (RT). Specimen were then washed with phosphate buffered saline (PBS) to remove cytophilic antibodies and re-suspended in appropriate amount of PBS. For evaluation of T- and NK-cells, detection of CD3, CD4, CD8, CD16, and CD56 with different fluorescently labeled monoclonal antibodies (mAbs) was done using three color flowcytometry according to manufacturer recommendations (Dakocytomation, Denmark, and Beckman Coulter, France). The mAbs were used in different combinations of fluorochromes; namely fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin-cyanine5 (PECy5). One hundred microliter of cell suspension (containing about 5×10^5 cells) was mixed with 10 µl of the fluorescently labeled mAb and incubated in the dark at RT for 30 min. Washing with PBS containing 2% bovine serum albumin was done twice and the pellet was resuspended in PBS and analyzed immediately on flowcytometer (Coulter Epics XL, Coulter, Miami, FL, USA). The percentage of antibody positive cells was calculated by comparing with the appropriate control.

For assessment of intracellular TNF-α, the cells stained with antibodies against surface antigens and then permealized using IntraPrep Permealization Kit (Beckman Coulter, France). Fifty microliter of EDTA PB sample were mixed with 100 µl of IntraPrep reagent 1 (fixative), incubated for 15 min at RT protected from light, and washed with PBS. 100 µl of IntraPrep reagent 2 (permealization) were mixed with the cells and incubated for 5 min at RT
without vortexing or shaking. The tube was shook carefully and manually for 2-3 seconds and then 10-20 µl of the mAb was added, vortexed, and incubated for 20 min at RT protected from light. Then, the mixture was washed and resuspended in PBS and analyzed on the flow cytometer immediately (Coulter Epics XL, Coulter, Miami, FL, USA).

**Statistics**
The data were expressed as the mean ± SD. Statistical and correlation analyses were undertaken using independent One-way ANOVA with post-hoc tukey test and Pearson's rank correlation coefficient test, respectively. A P-value <0.05 was accepted statistically significant. All the previous statistical analyses of data were carried out by SPSS software version 17 (IBM, US).

**RESULTS**
Forty patients diagnosed with URSM were included in our study in addition to 10 normal pregnant women as a control group. The patients were classified according to intravaginal SC dose into URSM1 and URSM2 (100 mg/day for 24 days and 75 mg/day for 13 days respectively). Age and obstetric history of three studied groups are shown in table 1.

**Levels of IL-6, IL-10, TNF-α producing cells**
No significant change was shown in the level of IL-6 before and after (13 or 24 days) treatment with different doses within each F-URSM group. However, the level of IL-6 was significantly lower in both patients’ groups at all time points when compared with the FTP control group. On the other hand, the level of serum IL-10 showed a highly significant increase after intravaginal treatment with SC in both F-URSM groups (p< 0.05, table 2, figures 1 and 2). The most significant increase in IL-10 was observed at day 24 in F-URSM1 group (received 100 mg/day SC) as compared with zero day in the same group, with 13 days in F-URSM2 group, and also with FTP control group (p < 0.05).

As regards the percentages of TNF-α producing cells, it showed a highly significant decrease after 13 days and 24 days of intravaginal SC in both F-URSM patients’ groups treated with different doses of intravaginal SC when compared with zero day (p< 0.05, table 2, figures 1 and 2). Of note, the most observed decrease in TNF-α producing cells was observed after 24 days of intravaginal SC in F-URSM1 group (received 100 mg/day) when compared either with day zero in the same group or with the 13 days in F-URSM2 group and also when compared to FTP control group (p < 0.05).
On the other hand, no significant difference found in the levels IL-10 levels or on percentages of TNF-α producing cells in both F-URSM treated groups using different doses of SC after 13 days of treatment.

**T-cell subsets and NK cells**

The percentages of CD3⁺ T-lymphocytes, CD3⁺/CD4⁺ (T- helper) and CD3⁺/CD8⁺ (T-cytotoxic) cells showed no significant change between patient and control groups. These percentages also did not change significantly before and after treatment with intravaginal SC treatment with different doses (table 2, figure 3 A).

It was of interest, to investigate the effect of intravaginal SC treatment on NK-cells as appreciated from the percentages of CD16⁺ and CD56⁺ (table 2). The percentage of CD16⁺ and CD56⁺ NK cells were significantly higher in all patients before receiving intravaginal SC and start to decrease 13 days after treatment in both patients groups under investigation as compared to zero day ($p < 0.05$; in all comparison). Furthermore, the percentage of CD16⁺ and CD56⁺ NK cells in F-URSM1 were decreased significantly after 24 days of treatment as compared to zero day. At this time point, there was no remarkable difference noticed in the NK cell percentage between F-URSM1 and FTP control groups ($p < 0.05$, table 2, figure 3 B).

**Figure 1** Percentage of TNF-α positive cell in F-URSM groups as compared with FTP control group.
Table 2 Means and standard deviation (SD) of IL-6 and IL-10 levels and percentages of TNF-α producing cells, T-cell subsets and NK cells in F-URSM groups as compared with FTP control group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>IL-6, (Pg/ml)</th>
<th>IL-10, (Pg/ml)</th>
<th>TNF-α&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% CD3+ve&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% CD3+ve/CD4+ve&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% CD3+ve/C D8+ve&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% CD16+ve&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% CD56+ve&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>Zero Day</td>
<td>Before</td>
<td>Treatment</td>
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<td>13 Days</td>
<td>After</td>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 Days</td>
<td>After</td>
<td>treatment</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>FTP</td>
<td>control</td>
<td>group</td>
<td></td>
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</tbody>
</table>

URSM; unexplained recurrent spontaneous miscarriage. FTP; first trimester pregnancy.

<sup>a</sup>; % out of lymphocytes.

<sup>b</sup>; % out of CD3<sup>+</sup>cells.

(*) Significant, <i>p</i> < 0.05; before treatment (zero day) as compared with (13 days, 24 days) after treatment in F-URSM1 group and also before treatment (zero day) as compared with (13 days) after treatment in F-URSM2 group.

(#{sup}<sup>2</sup>; Significant, <i>p</i> < 0.05; before treatment (zero day), (13 days) and (24 days) in F-URSM groups as compared with FTP control group.

($^<sup>2</sup>$) Significant, <i>p</i> < 0.05; 13 days after treatment in F-URSM1 group as compared with 24 days after treatment in F-URSM1 group.
Figure 2 Mean values ± SD of IL-6 (A) and IL-10 (B) levels in F-URSM groups as compared with FTP control group.
Figure 3 (A) Percentage of T-lymphocytes and subsets (B) Percentage of NK cells in F-URSM groups as compared with FTP control group.

Table 3 Correlations Coefficient (r) Value Between Different Studied Parameters After 24 Days Of Treatment In Urm1 Group. (*) Significant, P<0.05 In Each Correlations

<table>
<thead>
<tr>
<th></th>
<th>CD3⁺</th>
<th>CD3⁺/CD4⁺</th>
<th>CD3⁺/CD8⁺</th>
<th>CD16⁺</th>
<th>CD56⁺</th>
<th>TNF-α</th>
<th>IL-6</th>
<th>IL-10</th>
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<tr>
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<td>-</td>
<td>.051</td>
<td>.071</td>
<td>.016</td>
<td>-.037</td>
<td>.119</td>
<td>-.125</td>
</tr>
<tr>
<td>CD3⁺/CD8⁺</td>
<td>.511</td>
<td>.051</td>
<td>-</td>
<td>-.403</td>
<td>-.413</td>
<td>-.456</td>
<td>.477</td>
<td>.459</td>
</tr>
<tr>
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<td>.009</td>
<td>.071</td>
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<td>.848*</td>
<td>-.85*</td>
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<tr>
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<td>.016</td>
<td>-.413</td>
<td>.950*</td>
<td>-</td>
<td>.720*</td>
<td>-.88*</td>
<td>-.91*</td>
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<tr>
<td>TNF-α</td>
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<td>-.037</td>
<td>-.456</td>
<td>.84</td>
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<td>-</td>
<td>-.791</td>
<td>-.691</td>
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<td>-.884*</td>
<td>-.791*</td>
<td>-</td>
<td>.886*</td>
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<tr>
<td>IL-10</td>
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<td>-.125</td>
<td>.459</td>
<td>-.865*</td>
<td>-.910*</td>
<td>-.691*</td>
<td>.886*</td>
<td>-</td>
</tr>
</tbody>
</table>

CORRELATIONS

We studied the correlations between different studied parameters are shown in F-URSM1 group 24 days after treatment (table 3). According to the results obtained in this study highly significant negative correlations were found between CD16⁺ and CD56⁺ NK cells percentage and the levels of IL-10 and IL-6.

DISCUSSION

URSM represent about 40% - 50% of RSM which may be largely due to overactive circulating NK cells with elevated cytotoxicity may lead to RSM. There is a continuous quest to develop targeted and safe treatments for such patients[35].
$T_{H1}/T_{H2}$ cytokines are regulatory elements in several immunological processes and the balance in their production is a major factor affecting the immune response. When the mechanisms allowing feto-maternal tolerance are not completely understood it is difficult to establish how their dysfunction can induce spontaneous abortion $^{[36]}$. Recent attention has focused on elucidating the immunobiological roles of cytokines in normal human pregnancy following the accumulated reports of complex cytokines activity within uteroplacental tissues $^{[37]}$. T helper cells can be differentiated into subsets with distinctive patterns of cytokine release. It has been proposed that $T_{H1}$-type responses (e.g. the production of IL-2, TNF-$\alpha$ and IFN-$\gamma$) are systemically suppressed in pregnancy and that local expression of $T_{H2}$ cytokines (e.g. IL-4, IL-6, and IL-10) in placental tissue might be beneficial for fetal survival $^{[38, 39]}$. TNF-$\alpha$, causes embryo toxicity $^{[38]}$ and causes an increase in the synthesis of prostaglandins, which cause uterine contractions. EL-Far et al., $^{[14, 15, 24]}$ confirmed that there is elevated serum levels of TNF-$\alpha$ in women with a history of URSM and whose pregnancies ended in miscarriage when compared to values obtained from normal control group. Moreover, this highly significant increase in TNF-$\alpha$ content affects the implantation of the embryo adversely and decreases the blood flow to the embryo by causing thrombosis in the blood vessels $^{[40]}$. On the other hand, $T_{H2}$ cytokines have important roles in placental development and in the prevention of placental rejection $^{[38]}$.

The results obtained in earlier studies showed a modulating effect of SC on cytokine production by $T_{H1}$ and $T_{H2}$ lymphocytes $^{[41, 42]}$. Previous study showed that SC significantly suppressed NO, IL-1$\beta$ and TNF-$\alpha$ production $^{[41]}$. Recently, we had addressed the beneficial effects of SC in URSM patients $^{[24]}$.

The aim of this study was to determine the effect of intravaginal SC in different doses on IL-6, IL-10, T-lymphocytes, NK and TNF$\alpha$ producing cells. 40 women with URSM were included in the study and classified into 2 groups (URSM1 and URSM2) according to the intravaginal SC dose administered URSM2 (100 mg/day for 24 days and 75 mg/day for 13 days respectively).

Our present results indicate that IL-6 levels are significantly diminished in both URSM groups before treatment as compared to FTP control group which may suggest a role in URSM. This finding of is consistent with previous reports $^{[38, 43-45]}$. Treatment with SC had no significant effect on the level of IL-6 in our F-URSM patients (either post day 13 or day 24) supporting other studies showing that SC treatment has no effect on IL-6 in an animal model.
of insulin resistance [46, 47]. As regards IL-10, our study showed a highly significant decrease in IL-10 levels in both F-URSM groups at zero day as compared to FTP control group. This result is in agreement with other authors who also confirmed that pregnancy appears to be a \( \text{T}_{\text{H}}-2 \) dominate state with low levels of IL-10 [12, 48]. Our novel reported observation was the highly significant increase of IL-10 level in both treated F-URSM groups (at either 13 or 24 days) as compared to zero day and to the control FTP group. Moreover, those women completed their pregnancy successfully to term. This statistically significant increase in IL-10 has been attributed to the modulating effect of SC on cytokines production and is in accordance with those of Essayan et al [49]. The effect of SC treatment on IL-10 and the absence of such effect on IL-1 confirm the previous study stated that \( \text{T}_{\text{H}}-1 \) lymphocytes are more sensitive to the inhibitory action of SC than \( \text{T}_{\text{H}}-2 \) lymphocytes [42]. It is worth mentioning that longer continuous SC therapy for 24 days found to be better than 13 days protocol towards increasing not only IL-10 concentrations, but also to give better success rate of pregnancy as we very recently showed by El-Far et al.,[13] this supports our novel findings of direct proportional between higher SC dose and higher IL-10 levels productions.

In the present study, percentage of TNF-\( \alpha \) positive cells of untreated F-URSM groups (zero day) were found to be statistically significantly increased as compared to the FTP normal control group. This percentage is decreased after treatment with low and high dose of vaginal SC in both F-URSM groups that went successfully to term as compared to the day before treatment (zero day). Of note, these women went successfully through their pregnancy into full term. Present study represents first longitudinal clinical study which indicate clearly that continuous SC therapy possesses immunomudlatory properties of this drug, which are believed to play a significant role in the pathogenesis of URSM and in consistence with our recent studies [13, 24] and other studies showing that SC down regulates TNF-\( \alpha \) in diabetic rats [50], and recently has a role in nephrotoxicity in rats [51].

In URSM-1 group, a positive correlation was found between IL-6 and IL-10 that were negatively correlated with TNF-\( \alpha \). This could be explained by the hypothesis that IL-10 counteracts the harmful effects of the inflammatory response which is based on the increased production of TNF-\( \alpha \), IFN-\( \gamma \) and NO [52]. This finding support those of Red-Horse, [53] who identified a link between the immune system and the reproductive endocrine system. The authors showed that the mechanisms by which the mother accepts the implanting fetus as an allograft remain unexplained [53]. Mononuclear cytotrophoblasts, the specialized cells of the
placenta that invade the uterus secrete IL-10, the immunosuppressive cytokine that modulates immune responses, helping to protect the fetal hemiallograft from rejection. IL-10 is involved in the maintenance of normal pregnancy, perhaps by suppressing IFN-γ and TNF-α production by TH1 cells [54], which may be down regulated after SC treatment. This is partly contradictory to another published study [55].

Flow cytometric immunophenotyping of circulating lymphocytes has been used to assess immune status in normal pregnancy and pregnancy complicated by infection or with placental abnormalities or with preeclampsia [6, 56, 57]. Because the literature reveals controversial results in the immune etiology of URSM and in the levels of circulating lymphocyte subsets in normal pregnancy, our study aims to extend current knowledge by quantifying circulating T-lymphocytes subsets and and NK cells using flowcytometry in FTP normal control and F-URSM groups.

Our results show that there is no notable difference in the percentage of peripheral T-cells or T-cells subsets (CD3+, CD3+/CD4+ and CD3+/CD8+) between FTP control group and both F-URSM groups. Intravaginal SC treatment with different doses did not cause any significant effect on the percentages of CD3+, CD3+/CD4+, or CD3+/CD8+ cells. This result is in agreement with the study done by Darmochwal – Kolarz [58], who observed no change in the percentage of CD3+ lymphocytes and CD3+/CD4+ lymphocytes in the study group when compared to controls. Yamada et al., [59] observed that the difference of CD3+, CD3+/CD4+, and CD3+/CD8+ cells does not have important meaning in the immune system of pregnancy. However, this result is in conflict with other studies which observed a significantly higher CD4+ and CD3+/CD8+ T - cell percentage in pregnant recurrent miscarriage compared to healthy fertile women [58, 60]. Also, ex-vivo cultivation of isolated splenocytes with SC results in an increase in CD3+/CD4+ T - cells and a concomitant decrease in B - cells and also in central memory CD3+/CD8+ T-cells [61].

Our present study showed a significant increase in peripheral blood NK-cells as determined by anti-CD16 and anti-CD56 in both F-URSM patient groups before treatment (zero day) as compared to FTP control group. This is in agreement with other results that showed increased cytotoxicity of decidual or peripheral blood NK-cells against fetal antigens, which plays a role in URSM. Also, it is consistent with our preliminary results as well as and other studies which showed that NK-cell and either implantation failure or URSM patients [8, 24, 62-66]. Moreover, previous studies found that NK-cell activity may also predict URSM [67], which
denotes that the systemic regulation of NK-cells is essential for achievement of a successful reproductive outcome. In addition, a strong evidence was found that elevated numbers of peripheral blood NK cells and increased endometrial infiltration by NK-cells are related to pregnancy complications such as miscarriages. Although peripheral blood NK-cells are different from those of infiltrating the endometrium, peripheral blood NK-cells seem to be closely related with decidual NK-cells and may reflect decidual NK-cell functional status. Relying on this hypothesis, peripheral blood NK-cell enumeration by their surface antigens (CD16\(^+\), CD56\(^+\)) might predict the cause for URSM patients.

To the best of our knowledge, our present study is the first to explore immune responses of intravaginal SC in women with a history of URSM. Previous studies provide a potential immunomodulatory effect of sildenafil in mice. In this study NK-cells significantly decreased at 13 days after intravaginal administration of different doses of SC therapy in the both F-URSM patient groups, when compared to the base line (zero day). This decrease was more marked with higher SC dose after 24 days of treatment in F-URSM2 patient group, where the percentages of CD16\(^+\) and/or CD56\(^+\) NK cells were comparable to the FTP control group. This finding is in consistent with other studies which stated that down regulation of NK-cells with high-dose of intravenous of immunoglobulins in women with RSM is associated with a favorable pregnancy outcome. Our results are also in tune with others reported that the improvement in uterine artery flow has efficient influence on the local endometrial NK-cell population, and diminished NK-cell activity and this may promote successful pregnancy outcome in non-pregnant women with a history of RM. Thus, the percentages of NK-cells have clinical value for monitoring first trimester pregnant women with a history of URSM, and changes in NK-cells levels may predict pathological obstetrical outcome. On the other hand, this finding is in contrast with that of Michimata and Yamamoto, who suggested that the peripheral blood NK-cells levels has no association with miscarriage and no predictive value for pregnancy outcome. This may be due to technical and different conditions, we here report a novel finding of direct proportional and association between higher SC dose and higher decrease in CD56\(^+\), CD16\(^+\) NK cells levels which produce more success rate of pregnancy outcome.

NK cytotoxicity has been reported to be predictive of subsequent pregnancy loss in women who had URSM by releasing their granular components leading to apoptosis to target cells, or secretion of a wide range of cytokines. These results were confirmed by ours that
demonstrated a positive correlation between NK cells and TNF-α positive cells measured in the F-URSM1 group at day 24 post SC treatment (both were lowered significantly as compared to the baseline). Other studies showed that NO mediates the release of cytokines such as TNF-α from activated NK-cells and this has been implicated as a cause of implantation failure. This tend to support the positive correlation found between TNF-α positive cells and NO (which reflect the oxidative stress) measured in our previous studies [24, 13-15, 24] and other studies [51, 73] and confirm that SC may exerts its anti-inflammatory effects mainly through inhibition of NO generation, by cGMP-iNOS feedback and induces down regulation of pro-inflammatory mediators as TNF-α [74, 75].

Our present data are consistent with our preliminary and other previous studies [13-15, 24, 51, 73] and other studies [51, 73] that show positive correlation between TNF-α positive cells and oxidative stress. TNF-α induce $\text{O}_2^-$formation from NADPH oxidase leading to hydrolysis of cGMP, both of which would impair normal relaxation in uterine arteries. So, treatment with SC with different doses significantly reduce $\text{O}_2^-$ formation which induced by cytokines (TNF-α positive cells), and CD16+, CD56+ NK-cells, by blocking all these events.

Our demonstration then parallels finding by our preliminary and previous findings [13-15, 24] and others who described functional improvement of antioxidant defense system after SC treatment, a possible explanation for this benefit was recently described that SC reverted back the altered level of MDA which reflect lipid peroxidation and suppress pro-inflammatory cytokine production as TNF-α positive cells through improvement of antioxidant values. Therefore, this finding could support and indicate a positive correlation found between oxidative stress and TNF-α positive cells and also negative correlation between TNF-α positive cells and antioxidant in our preliminary studies [13, 24] and previous studies [50, 51].

Our present study provides certain insight with regards to the potential effects of intravaginal SC administration on F-URSM patients by increasing uterine arteries flow and significantly decreases TNF-α positive cells, CD16+, CD56+ NK cells by decrease vascular resistance (RI) and pulsatility index (PI) measured in our preliminary studies [13, 24]. These in turn provide a positive correlation between TNF-α positive cells, CD16+, CD56+ NK-cells and previous measured uterine indices. The effect of this treatment was a reduction in resistance to uterine artery blood flow by reducing NK cells [22]. Another important finding of the present study, is the negative correlations found between IL-10 and CD56+, CD16+ NK-cells in F-URSM1 group which agrees with the former results and findings by previous studies that T_\text{H}2
cytokines are believed to have various effects on NK cells, including inhibition of NK cell binding and cytotoxicity to vascular endothelium,\textsuperscript{76} inhibition of NK cell proliferation\textsuperscript{77}, and skewing of NK cell cytokine production toward a T\textsubscript{H}2 phenotype\textsuperscript{78, 79}. Enhancement of IL-10 production by progesterone-induced blocking factor (PIBF) leads to suppression of IL-12 production by human peripheral lymphocytes as well as inhibition of NK cytotoxic activity \textit{in vitro} which have additional antiabortive effects\textsuperscript{80}.

There were no complaints of side effects reported from most patients due to the use of intravaginal SC suppositories in IVF cases made it possible to decrease the incidence of side effects by delivering medication in close proximity to the target organ\textsuperscript{81}. The administration of vaginal sildenafil has shown to be free of major clinical side effects. This effect was rapid and reversible and suggests that it may be regulated by nitric oxide release at local level.

In our ongoing nearly 6-years experience, the administration of vaginal SC tablets has been found to be free of major clinical side effects. Moreover, because sildenafil is rapidly and completely cleared from the body within 24–48 hours of discontinuing its administration. In the present, treatment with SC leads to more balanced T\textsubscript{H}1/T\textsubscript{H}2 cytokine production during the course of treatment. The high dose of intravaginal SC for long duration (100 mg/day for 24 days; F-URSM1) found to be more effective in enhancing the biological parameters that help in maintaining the pregnancy by increasing T\textsubscript{H}2 (IL-10) and decreasing T\textsubscript{H}1 (TNF-\alpha) and NK-cells more than the low dose for short duration (75mg/day for 13 days). Our results show that the influence of SC on T\textsubscript{H}1/T\textsubscript{H}2 cytokine production was balanced during the course of treatment. The overall ongoing successful pregnancy rate in F-URSM patient groups was 60 % (24 out of 40 patients) and the overall ongoing failure pregnancy rate in F-URSM patient groups was 40 % (16 out of 40 patients), more work may be needed to increase the successful pregnancy rate if possible.

Further work defining the dose, timing, and optimal parameter of these agents may enhance the successful response rate of URSM patients for this challenging-to-treat population that may represent a valuable addition to the assisted reproductive technology therapeutic. Also, there is a need for investigating possible use of oral SC alone, we believe that it may not give same results, as it has been showed that oral SC give different results on endometrium thickness.

**CONFLICT OF INTEREST**

All authors had no conflict of interest.
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REFERENCES


20. Perricone, R., G. Di Muzio, C. Perricone, R. Giacomelli, D. De Nardo, et al. High levels of peripheral blood NK cells in women suffering from recurrent spontaneous abortion are
31. Raine-Fenning, N.J., B.K. Campbell, J.S. Clewes, N.R. Kendall, and I.R. Johnson. The reliability of virtual organ computer-aided analysis (VOCAL) for the semiquantification


