NOVEL BIOPHARMACEUTICAL USE OF SILDENAFIL CITRATE IN TREATMENT OF UNEXPLAINED RECURRENT MISCARRIAGE: FIRST LONGITUDINAL CLINICAL STUDY OF 50 CASES FROM EGYPT

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ABSTRACT

Present study was designed to fully investigate clinical as well as biopharmaceutical potential and mechanism of vaginally sildenafil citrate (SC) tablets on treatment of first trimester pregnant women with a history of unexplained recurrent spontaneous miscarriage (F-URSM), by altering blood antioxidants redox status as well as lipid peroxidation (MDA) and nitrosative stress (NO) biomarkers. Study was conducted on two groups of patients and a control group. The first patient group (F-URSM1) received SC (25 mg intravaginally, 4 times/day for 24 days, n= 20) and the second patient group (F-URSM2) received SC (25 mg intravaginally, 3 times/day for 13 days, n= 20). The control group comprised of 10 matched first trimester normal pregnant ladies (FTP). We evaluated effectiveness of this treatment on making changes on the balance between oxidant - antioxidant parameters as well as uterine indices to patient and control groups. SC treatment with different doses significantly increased antioxidants under investigation: [total antioxidant capacity (TAC), reduced glutathione (GSH), glutathione S- transferase (GST), catalase (CAT), and superoxide dismutase (SOD)] than in untreated F-URSM patients (0
day), while MDA and NO also uterine indices (PI, RI, S/D ratio) were significantly decreased after treatment. In conclusion, we report novel evidence that intravaginal SC can correct and reverse the imbalance between ROS and antioxidant defense by restoring and augmenting the capacity of antioxidant as well as modulating lipid peroxidation and nitrosative stress. It may act by inhibiting myometrial contractions and improvement of vasoconstriction through increasing blood flow causing relaxation of uterine arteries.

**Key words:** Sildenafil citrate; Antioxidants; Unexplained recurrent spontaneous miscarriage (URSM); Oxidative stress; Abortion.

**INTRODUCTION**

Recurrent spontaneous miscarriage (RSM) is a condition in which three or more consecutive, spontaneous abortions occur before 20 weeks gestation.\[1,2\] It affects 0.5% to 3% of women of reproductive age. The etiology of RSM may be linked to chromosomal abnormalities, uterine anatomic anomalies, endocrinological factors, environmental toxins and immunologic disorders such as antiphospholipid antibody syndrome, clotting disorders, and sperm DNA fragmentation.\[3\] However, 50%-60% of these patients following investigation are lacking a clear reason and are classified as unexplained (URSM).\[4\]

Recent reports implicate increase in oxidative stress which originates from developing embryo in early pregnancy.\[4\] It was proposed that antioxidants may aid in the treatment of such condition. 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, 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.\[5\]

In the past few years, much interest has been focused on the role of nitric oxide (NO) as a modulator of uterine blood flow.\[6\] Previous animal research has demonstrated that NO release lead to the relaxation of vascular smooth muscle through a cGMP-mediated pathway.\[7\] Endothelial and inducible NO synthase isoforms have also been identified in the vascular endothelium of human endometrium and myometrium.\[8\] Based on the obstetric uses of nitroglycerine (NTG); NO donor, for uterine relaxation and tocolysis, who speculated that NTG could be used to improve endometrial development.\[9\] Treatment with NTG patches did
produce a trilaminar sonographic pattern and increased endometrial thickness, but was impractical due to an unacceptably high rate of headaches, nausea and hypotension.

El-Far et al., [2] showed for the first time, in preliminary report, that SC might be a novel, interesting, safe antiabortive option in the treatment of threatened miscarriage in four patients with a history of URSM. Several new vasodilator drugs have been used to improve blood flow. SC induces vasodilation through inhibition of type 5 phosphodiesterase (PDE5). [10] PDE5 is responsible for the degradation of cGMP to guanosine monophosphate. Therefore, inhibiting PDE5 delays the breakdown of cGMP, increases vasodilatation [7] and potentiates the effects of NO on the vascular smooth muscle. Although sildenafil citrate has been developed for erectile dysfunction, it has other medical indications now. [11-13]

The protective role for SC on PDE-5 is achieved possibly by inhibiting free radical formation and supporting antioxidants redox system in men. [14] A recent report suggested that SC stimulates vasodilation in myometrial biopsies collected from intrauterine growth restriction pregnancies at the time of Cesarean section. [10] These findings were preceded by observations that sildenafil citrate rapidly reduces mean arterial pressure while simultaneously increasing heart rate and blood flow to the uterus in an ovine model of surgically induced menopause. [15] Collectively, out of our experience, available data indicate that SC may be used as a valuable therapeutic aid to reduce the incidence and/or severity of RSM.

The aim of this study is to fully investigate biopharmaceutical potential of intravaginal SC on treatment of F-URSM in longitudinal clinical study of 50 cases, through its effect on amelioration of oxidant stress and improve uterine blood flow. Intravaginal tablets were used; the rationale is to decrease the incidence of systemic side-effects by delivering the medication in close proximity to the target organ.

MATERIALS AND METHODS
Patients and samples
This study is a longitudinal prospective study where women were admitted to the Department of antenatal care at Mansoura University Hospital (MUH) during the period from (August 2008-September 2012). Inclusion criteria were: first trimester pregnant women with a history of unexplained recurrent spontaneous miscarriage (URSM; patients were routinely referred to the antenatal clinic where they were investigated for any possible causes; transabdominal
ultrasound confirmed- gestational weeks 6-8 weeks, singleton pregnancies), exclusion criteria were patients having any possible causes of abortion: anatomical, genetic (karyotypes were determined for all couples), endocrinological, infectious, or immunological (anticardiolipin, antinuclear and antiphospholipids antibodies) or other possible causes of RSM. Patients who were enrolled in the study were informed about the nature and the aim of the study and signed a written consent. The study was approved by the Medical Research Ethics Committee of Mansoura University.

Safety was considered a further end point of this study, data being collected throughout the study period, and included adverse event, vital signs and changes in laboratory test were valued for standard hematology and biochemistry variables. The dosage was extrapolated from the maximum recommended daily dose for men and from our previous preliminary reported data, \(^2\) given in three or four divided doses to minimize peak and trough effects.

RSM was diagnosed by uterine bleeding (light defined as spotting only, or heavy) +/- painful uterine contractions, closed and viable fetus with positive heart activity detected by ultrasonographic examination. Both groups were admitted to MUH for medical care until the end of the first trimester and received the usual treatment used in their previous pregnancies that ended with miscarriages including: rest, gestagens (200 mg/day), low dose aspirin (75mg/day), antispasmodics (40 mg/day) and folic acid (500 microgram/day) from the onset of threatened miscarriage until vaginal bleeding stopped. Subjects didn't take any medication including nitrite before pregnancy. Diets were comparable in all groups.

The first group of patients (F-URSM1) consisted of 20 first trimester pregnant women with a history of URSM, self-administered intravaginal SC tablets were given at a dosage of 25 mg, 4 times a day for 24 days (total dose 100mg/day). The second patient group (F-URSM2) consisted of 20 first trimester pregnant women with a history URSM, self-administered intravaginal SC tablets were given at a dosage of 25 mg, 3 times a day for 13 days (total dose 75mg/day). The FTP control group comprised 10 first trimester pregnant women with a single fetus, having at least one previous normal pregnancy, with no history of abortion, ectopic pregnancy, pre-term delivery or stillbirth. Gestational ages were calculated from the date of the last menstrual period and confirmed by crown-rump length measurement.

After an overnight fast, a blood sample (8mL) was obtained by sterile venipuncture. Two mL were transferred to heparinized vacutainer tube; plasma was separated for NO
determination. The remaining 6 mL was transferred to EDTA or heparin tubes (according to the desired protocol) for preparation of erythrocytes. Blood samples in anticoagulant-containing tubes were used for hematological assays and preparation of hemolysate. Samples were obtained at zero day for all groups, 13 days and 24 days for URSM1 group and 13 days for URSM2 group. Hemoglobin and PCV were performed for all groups.

Transabdominal pulsed color Doppler imaging was performed for each woman immediately before treatment at zero day for all groups, 13 days and 24 days for URSM1 group and 13 days for URSM2 group.

**Biochemical assays**

**Chemicals:** All chemicals used in this study were of analytical grade and were purchased from Sigma chemical company, (St. Louis, MO, USA), otherwise is mentioned.

**Determinations of oxidative-nitrosative stress parameters**

Malondialdehyde (MDA) was determined by the method described by Stocks.\(^{[16]}\) Plasma nitrite levels were estimated by colorimetric assay according to the method of Green.\(^{[17]}\)

**Determinations of antioxidant parameters**

Plasma (TAC, GSH, CAT) and erythrocyte (SOD, GST), were all determined spectrophotometrically (Uvikon 930 spectrophotometer, Kontron Instruments, Milan, Italy) using a commercially available kit according to the manufacturer’s protocols (Bio-diagnostic, Cairo, Egypt). Serum SOD was determined by the method of Dechatelet.\(^{[18]}\) Catalase activity in erythrocyte was determined according to Chance.\(^{[19]}\)

**Determination of pulsatility index (PI), Resistance index (RI) and systolic/diastolic ratio (S/D ratio) using Doppler**

Uterine arteries Doppler examinations were performed using a (Philips ALTU1 trasound HDI 4000, Bothell, WA, USA 98041) and it was performed transabdominally with the women lying on a standard hospital bed, the probe was placed on the lower quadrant of the abdomen, moved laterally until the paracervical vascular plexus was observed and imaging was used to identify the UtA at the apparent crossover with the external iliac artery. Measurements were taken approximately 1 cm distal to the crossover point. Once it had been ensured that the angle was $< 30^\circ$, the pulsed Doppler gate was placed over the whole width of vessels. All scans were performed by the same observer who immediately entered the data into a
computed database. Uterine arteries were identified with color Doppler imaging close to the uterine arteries. A stable recording of at least 10 s was required before acceptance of the signals for storage and analysis of the flow pattern. At least three uniform consecutive waveforms were analyzed for calculation of the PI, RI, and S/D ratio for both uterine arteries. Mean (right + left/2) indices were calculated for each patient.

Statistics
The findings 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 ver. 17 (IBM, US).

RESULTS
Results are summarized in different tables and figures. Age and obstetric history of three studied groups are shown in table 1.

Table 1: Age and obstetric histories of studied groups

<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>
<td>(3 – 12)</td>
<td>(4 – 11)</td>
<td>(2 – 4)</td>
</tr>
<tr>
<td>Number of 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>
Altered antioxidants as well as lipid peroxidation and nitrosative biomarkers status following SC treatment

MDA and NO levels were significantly decreased after 13 days of treatment with different doses of SC in both F-URSM treated groups, the highest significant decrease was observed in F-URSM1 group who received 100 mg/day SC for 24 days as compared with the day before treatment (zero day) in the two groups and also to FTP control group ($p < 0.05$; in all comparison; table 2, figure 1). On the other hand, no significant difference found in the levels of MDA and NO in both F-URSM treated groups using different doses of SC after 13 days of treatment, a significant effect of treatment on MDA and NO were observed at 24 days.

Activities of SOD (Serum and erythrocyte) and CAT (plasma and erythrocyte) were significantly increased in both treated groups of F-URSM with different doses of SC at 13 and 24 days compared with zero day (before treatment). It is worth mention that the highest significant increase seen in plasma CAT activity was observed at 24 days following SC treatment and showed to be with normal range of FTP control group ($p < 0.05$; in all comparison; table 3, figure 2).

Plasma TAC levels were significantly increased after 13 days and 24 days of treatment with SC at different doses in both F-URSM groups as compared with the day before treatment ($p < 0.05$; in all comparison; table 2, figure 3).

Table (2) shows that plasma GSH and erythrocyte GST levels were found to be significantly increased after 13 days and 24 days following treatment with SC at different doses in both F-URSM treated groups as compared with zero day (before treatment) ($p < 0.05$; in all comparison, figure 4).

Uterine arteries blood flow indices

No significant differences were observed between right and left uterine arteries indices under investigation, and so the mean values were used for analysis. No significant changes in the levels of RI and S/D were found in F-URSM groups at zero day (before treatment) as compared with FTP control group. On the other hand, PI found to be significantly higher at 0 day as compared to FTP control group. Through the observational period in F-URSM treated groups, a significant decrease observed in PI, RI and S/D at 13 days as well as 24 days following SC treatment at different doses as compared with untreated zero day ($p < 0.05$ in each comparison; table 3, figure 5).
Table 2: Means and standard deviation (SD) of measured parameters (MDA, NO, TAC, GSH and GST) in both F-URSM groups as compared with FTP control group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>MDA, n moles/ml packed cells</th>
<th>NO, µmol/L</th>
<th>TAC, mM/L</th>
<th>GSH, Mg/dl</th>
<th>GST, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-URSM1</td>
<td>0 Day Before treatment (n=20)</td>
<td>4.415 ± 0.843#</td>
<td>43.25 ± 10.18#</td>
<td>0.20 ± 0.067#</td>
<td>2.36 ± 0.71#</td>
<td>517.69 ± 100.6#</td>
</tr>
<tr>
<td></td>
<td>13 Days After treatment (n=13)</td>
<td>1.76 ± 0.69 *#</td>
<td>24.57 ± 5.33 *#</td>
<td>0.33 ± 0.077*#</td>
<td>3.02 ± 0.93*#</td>
<td>611.27 ± 83.67*#</td>
</tr>
<tr>
<td></td>
<td>24 Days After treatment (n=13)</td>
<td>1.11 ± 0.48 *#$</td>
<td>15.31 ± 3.25 *#$</td>
<td>0.59 ± 0.13*#$</td>
<td>3.25 ± 0.89*#$</td>
<td>684.01 ± 99.8*#$</td>
</tr>
<tr>
<td>FTP control group (n=10)</td>
<td></td>
<td>0.93 ± 0.46</td>
<td>7.2 ± 1.99</td>
<td>0.75 ± 0.14</td>
<td>5.04 ± 1.31</td>
<td>776.51 ± 55.55</td>
</tr>
</tbody>
</table>

Table 3: Means and standard deviation (SD) of measured parameters (SOD and CAT activities) and uterine indices in both F-URSM groups as compared with FTP control group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>SOD of inhibition</th>
<th>U/gm Hb</th>
<th>CAT U/L</th>
<th>IU/gm Hb</th>
<th>PI</th>
<th>RI</th>
<th>S/D ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Day Before treatment (n=20)</td>
<td>41.99 ± 7.08#</td>
<td>12.91±3.92#</td>
<td>158.5 ± 71.5#</td>
<td>24.6 ± 5.02#</td>
<td>2.47 ± 0.66#</td>
<td>0.93 ± 0.16</td>
<td>5.47 ± 1.65</td>
</tr>
<tr>
<td></td>
<td>13 Days After treatment (n=13)</td>
<td>51.74 ± 5.65*#</td>
<td>22.43 ± 3.6*#</td>
<td>340.4 ± 71.7*#</td>
<td>35.9 ± 4.25*#</td>
<td>1.32 ± 0.61*#</td>
<td>0.69 ± 0.1*#</td>
<td>2.76 ± 0.61*#</td>
</tr>
<tr>
<td></td>
<td>24 Days After treatment (n=13)</td>
<td>62.52 ± 6.35*#$</td>
<td>30.07 ± 3.14*#$</td>
<td>528.9 ± 118.8*#$</td>
<td>47.8 ± 5.9*#$</td>
<td>0.98 ± 0.38*#$</td>
<td>0.58 ± 0.09*#$</td>
<td>1.97 ± 0.49*#$</td>
</tr>
<tr>
<td></td>
<td>F-URSM1</td>
<td>39.5 ± 4.66#</td>
<td>12.38 ± 4.1#</td>
<td>178.9 ± 66.7#</td>
<td>23.7 ± 4.26#</td>
<td>2.38 ± 0.7#</td>
<td>0.9 ± 0.14</td>
<td>5.21 ± 0.97</td>
</tr>
</tbody>
</table>
13 Days After treatment (n=11)

<table>
<thead>
<tr>
<th></th>
<th>FTP control group (n=10)</th>
<th>13 Days After treatment (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>52.7 ± 5.26*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.6 ± 4.64*#</td>
</tr>
<tr>
<td></td>
<td></td>
<td>372.9 ± 74.1*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.95 ± 5.1*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.24 ± 0.26*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.61 ± 0.06*#</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.33 ± 0.48*#</td>
</tr>
</tbody>
</table>

(*) Significant, *p*<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.

(#) Significant, *p*<0.05; before treatment (zero day), (13 days) and (24 days) in F-URSM groups as compared with FTP control group.

($) Significant, *p*<0.05; 13 days after treatment in F-URSM1 group as compared with 24 days after treatment in F-URSM1 group.

Figure 1 Showing mean values of malondialdehyde (MDA) and nitric oxide (NO) in (F-URSM) groups as compared with (FTP) control group.

Figure 2 Mean values of SOD and CAT activity in both F-URSM groups as compared with FTP control group.
Figure 3 showing mean values of TAC in plasma in F-URSM groups as compared with first trimester pregnancy control group (FTP).

Figure 4 Showing mean values of GSH and GST in (F-URSM) groups as compared with first trimester pregnancy (FTP) control group.
Figure 5 Uterine artery visualized by transabdominal Doppler Ultrasonography in predicting URSM by Doppler index before (A) and after 13 days of vaginal SC treatment (B). The PI, RI and S/D were decreased compared with the baseline, indicating increased uterine blood flow.

Adverse effects
All patients completed the whole study period of treatment using different doses and none presented with serious adverse effects. Only swelling of labia associated with a thick discharge was reported by some patients. The incidence of commonly recorded adverse effects reported by subjects during the use of intravaginal 100 mg/day SC were (69.2 %) in F-URSM1 group and (66 %) in F-URSM2 group. In addition, no teratogenic or fetotoxic effects were reported in any case under investigation.

Pregnancy outcome
In F-URSM1 group a success pregnancy rate of 65% (13 out of 20 patients) was observed. In F-URSM2 the success rate of 55% (11 out of 20 patients) was reported. Normal babies were delivered in all cases for both treated groups, following up made up to 2 years (figure 6).

Figure 6 Showing pregnancy success and failure rate in F-URSM1 group (A); F-URSM2 group (B).
Correlations

All correlations between different studied parameters are shown in F-URSM1 group 24 days after treatment (table 4). Positive correlations were observed between antioxidants and each other under investigation. Also, highly significant negative correlations were found between all antioxidants used in this study and oxidative stress biomarker namely MDA & NO. A highly significant positive correlations were found between (PI, RI, S/D) and oxidative stress. Highly significant negative correlations were found between (PI, RI, S/D) and antioxidants.

Table 4: Correlations coefficient (r) values between oxidant and antioxidant measured parameters after 24 days of treatment in URSM1 group.

<table>
<thead>
<tr>
<th></th>
<th>MDA</th>
<th>NO</th>
<th>P-CAT</th>
<th>S-SOD</th>
<th>TAC</th>
<th>GSH</th>
<th>GST</th>
<th>PI</th>
<th>RI</th>
<th>S/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>-</td>
<td>0.764</td>
<td>-0.745*</td>
<td>-0.803*</td>
<td>-0.825*</td>
<td>-0.731*</td>
<td>-0.734*</td>
<td>0.710*</td>
<td>0.812*</td>
<td>0.849*</td>
</tr>
<tr>
<td>NO</td>
<td>0.764*</td>
<td>-</td>
<td>-0.973*</td>
<td>-0.966*</td>
<td>-0.978*</td>
<td>-0.927*</td>
<td>-0.982*</td>
<td>0.736*</td>
<td>0.814*</td>
<td>0.822*</td>
</tr>
<tr>
<td>P-CAT</td>
<td>-0.745*</td>
<td>-0.973*</td>
<td>-</td>
<td>0.958*</td>
<td>0.948*</td>
<td>0.913*</td>
<td>0.972*</td>
<td>-0.740*</td>
<td>0.778*</td>
<td>-0.804*</td>
</tr>
<tr>
<td>S-SOD</td>
<td>-0.803*</td>
<td>-0.966*</td>
<td>0.958*</td>
<td>-</td>
<td>0.965*</td>
<td>0.954*</td>
<td>0.933*</td>
<td>-0.700*</td>
<td>0.971*</td>
<td>0.802*</td>
</tr>
<tr>
<td>TAC</td>
<td>-0.825*</td>
<td>-0.978*</td>
<td>0.948*</td>
<td>0.965*</td>
<td>-</td>
<td>0.935*</td>
<td>0.965*</td>
<td>-0.741*</td>
<td>0.821*</td>
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<tr>
<td>GSH</td>
<td>-0.731*</td>
<td>-0.927*</td>
<td>0.913*</td>
<td>0.954*</td>
<td>0.935*</td>
<td>-</td>
<td>0.899*</td>
<td>0.641*</td>
<td>0.821*</td>
<td>0.764*</td>
</tr>
<tr>
<td>GST</td>
<td>-0.734*</td>
<td>-0.982*</td>
<td>0.972*</td>
<td>0.933*</td>
<td>0.965*</td>
<td>0.899*</td>
<td>-</td>
<td>0.749*</td>
<td>-0.808*</td>
<td>-0.804*</td>
</tr>
<tr>
<td>PI</td>
<td>0.710*</td>
<td>0.736*</td>
<td>-0.740*</td>
<td>-0.700*</td>
<td>-0.741*</td>
<td>-0.641*</td>
<td>-0.749*</td>
<td>-</td>
<td>0.741*</td>
<td>0.886*</td>
</tr>
<tr>
<td>RI</td>
<td>0.812*</td>
<td>0.814*</td>
<td>-0.778*</td>
<td>-0.971*</td>
<td>-0.821*</td>
<td>-0.821*</td>
<td>-0.808*</td>
<td>0.741*</td>
<td>-</td>
<td>-0.933*</td>
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<tr>
<td>S/D</td>
<td>0.849*</td>
<td>0.822*</td>
<td>0.804*</td>
<td>-0.802*</td>
<td>-0.821*</td>
<td>-0.764*</td>
<td>-0.804*</td>
<td>0.886*</td>
<td>0.933*</td>
<td>-</td>
</tr>
</tbody>
</table>

(*) Significant, p < 0.05 in each correlations.

DISCUSSION

It is well known that levels of free radicals are controlled by various cellular defense mechanisms including enzymatic (SOD, CAT, GST, etc) and non-enzymatic (as GSH, vitamins, etc) component. [20] Our present results showed significant increase in blood antioxidant (SOD, CAT, GST, GSH and TAC) with a concomitant decrease in MDA and NO following SC treatment. This indicates that SC can augment various cellular defense mechanisms by increasing antioxidants levels and can also modulate lipid peroxidation and nitrosative stress in treated patients with other disease. So, SC can serve as powerful antioxidant and improve significantly the redox imbalance which El-Far et al., previously showed to play a role in URSM. [12, 21, 22] It is well known that the disturbance of the pro-oxidant/antioxidant balance due to increased free radical production, antioxidant enzyme
inactivation and excessive antioxidant consumption may be the main reasons in oxidative damage.\textsuperscript{[23]} We here provided novel biochemical evidence that SC treatment can significantly plays a major role in maintenance of proper oxidant-antioxidant status in F-URSM cases via its significant antioxidant properties, this is in accordance with recent studies in many experimental models, which revealed beneficial effect of SC that could constitute a key mechanism for the decrease of oxidative stress.\textsuperscript{[24-27]}

Present study showed that NO and MDA levels in patients of both untreated F-URSM groups (zero day) were found to be significantly higher than in FTP control group which confirms our prior observations,\textsuperscript{[2, 21, 22]} that shows the connection between URSM and oxidative stress, which is not only corroborated by the increase in oxidative-nitrosative stress generation seen in early pregnancy but also can be related to decreased levels of antioxidants needed to neutralize and scavenge excessive ROS present in women affected by recurrent miscarriage. When lipid peroxide level is elevated as MDA, they induce various pathogenic intracellular signals involving of inflammatory genes, calcium, G-proteins, cAMP, cGMP, phospholipase C and D, protein kinase C, IL-1, IL-6, TNF-α, ceramide, and MAP kinase cascade leading to cellular dysfunction,\textsuperscript{[28-30]} and increased vascular smooth muscle cell growth\textsuperscript{[31, 32]} and inhibit the synthesis of endothelial cell-derived vasodilator prostacyclin. Disruption of platelet cell membrane is another action of peroxides, which results in thromboxane elevation.\textsuperscript{[33]} This abnormal prostaglandin action is supposed to be a triggering factor of endothelial cell damage of vasculopathic diseases of pregnancy as in miscarriage, disorganized placental blood flow may lead to hypoxia and reperfusion injury with a resultant increase in the oxygen tension within the early placenta.\textsuperscript{[34, 35]} This, in turn, causes a disruption in the balance between NO and reactive oxygen species (ROS), finally resulting in decreased NO bioavailability\textsuperscript{[31, 32]} therefore, this mechanisms may explain one of the major causes in both untreated F-URSM groups in the present study that ended with miscarriage as compared to FTP control group.

We observed a significant decrease in MDA and NO levels in response to intravaginal SC administration in different doses up to 13 and 24 days in both F-URSM groups that went successfully to term as compared to the day before treatment (zero day). This present findings are in tune with earlier report demonstrating decreased NO and MDA after SC treatment in induced experimental Huntington's disease in rats.\textsuperscript{[36]} Because of the antiatherogenic and antithrombotic properties of NO and the proatherogenic and prothrombotic actions of
endogenous oxidants, a decreased NO bioavailability with increased oxidative and nitrosative stresses will result not only in impaired, endothelium-dependent vasorelaxation but also in the acceleration of thermogenesis and onset of acute atherothrombotic events. Also, other studies showed the effect of SC in enhancing cGMP levels which may also inhibit the production of MDA via the inhibition of NADPH oxidase-mediated generation of ROS on liver injury. Thus, we conclude for the first time that SC beneficial effects at intravaginal local level would be a regulator of free radical generation and NO bioavailability in URSM cases. This hypothesis may also well explain why no increase in NO and MDA levels could be detected following intravaginal SC therapy with different doses up to 13 and 24 days in both F-URSM groups in the current study as compared to the day before the treatment (zero day).

NO seems to play a biphasic role in URSM pathogenesis depending on the nitric oxide synthase (NOS) isoenzyme, a protective effect of constitutive NOS (cNOS)/NO and pro-inflammatory effect of inducible NOS (iNOS)/NO. Low concentration of NO can be protective, by promoting vasodilatation and limiting neutrophil adhesion to vascular endothelium. Our results agree with the studies on other different diseases which conclude that SC may act functionally with amplifying the action of NO without increase its production. This effect was rapid and reversible and suggests that it may be regulated by NO release at a local level, indicating that treatment with SC is a useful salvage treatment approach for URSM patients.

On the other hand, SOD and CAT are the first line of defense against oxygen toxicity and their functions are to provide a defense against the potentially damaging reactivity of superoxide anion. The present study, showed that the levels of SOD and CAT in both F-URSM groups before treatment (zero day) revealed a highly significant decreased activity as compared with that of FTP control group may be due to its consumption in protecting the cell from a harmful effect of ROS, this indeed would support our present and previous finding of decreased SOD and CAT activities in untreated URSM patients (Table 3). CAT and SOD activities in both treated F-URSM groups that went successfully to term at 13 day and 24 day after SC treatment with different doses were found to be significantly increased as compared to the day before treatment (zero day), this statistically highly increase may be attributed to the inhibition of free radicals and lipid peroxidation due to the effect of SC.

Other studies in another diseases showed that SC depresses hydrogen peroxide generation by
mimicking SOD and CAT and thus preventing generation of reactive oxygen species.\textsuperscript{14, 28, 42, 43} High levels of superoxide anion and hydrogen peroxide increase oxidative stress,\textsuperscript{44} and it is possible that increased SOD and CAT activities are a response to the increased superoxide anion and probably represent a defense mechanism which activated to protect the cell against an oxidative stress insult. Quenching of oxidative stress by SC as we reported here, could sustain the bioavailability of nitric oxide for vasodilatation, which may be another mechanism of action of this drug, as most cases of URSM are associated with oxidative stress.\textsuperscript{2, 21}

Our results are in accordance with others which stated that PDE-5 inhibitor as SC, increases activities of the antioxidant enzymes, such as SOD and CAT level after spinal cord injury in rat\textsuperscript{45} and ovaries I/R injury.\textsuperscript{46} So, This increased antioxidant enzymes activities may be involved as a protective effect of SC against the increased oxidative stress, SC thus exhibits an antioxidant effects which block the expression of inflammatory cytokines by inhibiting generation of ROS, inhibition of free radical and lipid peroxidation and through enhancing cGMP level and inhibiting the expression of NADPH oxidase,\textsuperscript{28, 47-49} present data support antioxidant redox systems observed in F-URSM that went successfully to term after treatment with different doses with intravaginal SC up to 13 and 24 days as compared to normal FTP control group and the day before treatment, this is in agreement with others who reported that this reduction in oxidative stress results in improvement of endothelial function and increase in red blood cell CAT, SOD, and GSH values after SC treatments\textsuperscript{14, 30, 45, 50} and recently in other diseases.\textsuperscript{24-27}

Also, F-URSM patients that ended with miscarriage were associated with significantly lower levels of plasma (TAC, GSH) & erythrocyte GST as compared to FTP control group. Our novel reported observation are the first of its kind, which indicate that intravaginal SC with different doses at 13 & 24 days after treatment in F-URSM groups that went to term as compared to FTP control group reactivate and restore the antioxidant defense mechanism mainly plasma (TAC & GSH) and erythrocyte GST also create an evidence to reverse serious side effect of excessive ROS in F-URSM patients. This increase in TAC, GSH and GST activity, may be due to the marked elevation in the levels of antioxidants which we reported in SOD and CAT in the same treated groups, which agrees with the former results and findings by previous studies, they have found a protective effect of increased cGMP levels due to SC in conditions associated with increased oxidative stress by increasing antioxidant
activity. Previous work of Perk et al., Ebrahimi et al., and Zhao et al. may explain our present results that a critical and catalytical subunit of NADPH oxidase, and the levels of intracellular reactive oxygen species (iROS) induced by lipid peroxidation were markedly inhibited by SC leading to increase antioxidants.

Our results showed the presence of positive correlations found between estimated antioxidants (SOD, CAT, TAC, GST and GSH) and each other's and also negative correlations between oxidative stress biomarkers (MDA & NO) and some estimated antioxidant (SOD, CAT, TAC, GST and GSH) in the F-URSM1 group (Table 4). This support other authors regarding the correlation between oxidative-antioxidative status, showing decreasing oxidative stress and increasing antioxidant values after treatment using SC in erectile dysfunction cases. Concerning association between SOD activity and NO levels, SOD found to be intimately involved in the regulation of superoxide anion and the metabolites of nitrogen, as \( \text{O}_2^- \) when combined and reacts rapidly with NO forms peroxynitrite (ONOO-). Peroxynitrite is cytotoxic and much more reactive than NO and \( \text{O}_2^- \) and causing different chemical reactions in biological system including nitration of tyrosine residues of proteins, triggering of lipid peroxidation, inhibits the mitochondrial electron transport, and oxidation of biological thiol compounds. This tends to indicate the absence of any harmful lipid peroxidation or nitrosative stress upon using intravaginal SC. The present results showed a modulating effect of SC by increasing antioxidants namely SOD, CAT, TAC, GST and GSH through preventing \( \text{O}_2^- \) production and reverted back the altered levels of oxidative stress (MDA & NO). In general we conclude that SC treatment can augment the antioxidant defense system together with a suppressive action of it on MDA level and lipid peroxidation as well as NO level and its nitrosative stress.

Studies suggest that uterine artery perfusion may regulate endometrial receptivity, and that poor uterine perfusion could be one of the causes associated with pregnancy complications such as spontaneous abortion, intrauterine growth restriction and pre-eclampsia. The color Doppler ultrasound has become a first level diagnostic procedure to measure the penile blood flow of men with erectile dysfunction and might be a new useful method to study blood flow in uterine arteries in URSM patients. In the present study, PI and RI values for uterine arteries at the first trimester of gestation in normal pregnant women are in agreement with those reported by Antonio Gadelha, Tamura and Liao. Ours was the first longitudinal study on the effects of intravaginal SC with different doses on uterine blood flow.
in first trimester pregnant women with a history of URSM using color Doppler ultrasound before and after treatment with SC. Sildenafil citrate rapidly reduces mean arterial pressure and simultaneously increases blood flow to uterus by a significant decrease in PI, RI and S/D ratio in both F-URSM groups that went successfully to term compared to baseline (zero day) following the treatment with intravaginal SC in different dose up to 13 and 24 days in both F-URSM groups. This finding could indicate that SC exhibits a positive relaxant effect by acting directly on the dysfunctional uterine endothelium and improving its function, thereby improving relaxation of uterine artery and increasing blood flow via increasing cGMP levels. It has been showed that treatment with SC may exert an indirect NO-cGMP dependent effect and a direct effect by lowering the intracellular free Ca2+ concentration that mediated via the activating of calcium pumps, the inhibition of voltage-gated Ca2+ channels and the inhibition of Ca2+-induced G protein–coupled receptor activation.\textsuperscript{[64]}

Positive correlations found between uterine indices (PI, RI, S/D) ratio and oxidative stress (MDA & NO) and negative correlation between PI, RI, S/D ratio and antioxidants measured in F-URSM1 group. Our data suggest that SC used as a therapeutic agent may improve uterine arteries vasodilatation, decreasing peripheral resistance, and increasing flow within the uteroplacental bed in both treated F-URSM groups and prolongation of pregnancy by nitric oxide release at local level, these results also explain the positive correlation between PI and NO found in the studied group under investigation.

Progesterone is vital in the maintenance of pregnancy and there are reports that insufficient production of progesterone could result in poor pregnancy outcome.\textsuperscript{[65]} Other studies have shown that about 17% of women with recurrent abortion may have a potential endocrine abnormality, with the majority having insufficient progesterone production.\textsuperscript{[66]} Although treatment with progesterone has been advocated for the prevention of threatened miscarriage in the participating patient in this study but it did not reduce the frequency of URSM as was used in the all previous pregnancies that ended with miscarriages. In our present study SC therapy was introduced in combination with progesterone as this support our present finding in pregnant women with F-URSM, so the improvement in the continuation of pregnancy in the present study is attributed to the additive effect of the local vasodilator used as all other measures including progesterone were used in the previous unsuccessful pregnancies. This study demonstrated enhanced SC exposure in the uterus following its SC administration could be used to target uterus for therapeutic benefits.
Previous work shows the benefit of using intravaginal SC in non-pregnant women with a history of URSM during proliferative phase of the menstrual cycle and showed improving endometrial thickness. [67] Our data show the potential use of SC as oxygen free radical scavenger in first trimester pregnant women with a history of URSM. From our present first longitudinal clinical experience since (2008 to 2012), the administration of vaginal sildenafil has shown to be free of major clinical side effects.

We did not consider repeated dosages of SC in patients with F-URSM in this study for safety concerns because, unfortunately, there is a paucity of data on repeated dosage and pharmacokinetics of oral 100 mg of SC, and none for the vaginal administration. However, it has been shown that the plasma concentration of SC is dose proportional [68], and therefore, if we would administer another 100 mg dose of SC 4 h after the initial dose, it is reasonable to assume that the mean plasma concentration after several doses would rise above the one found after a single dose, and possibly increase the chance of unwanted side effects. We used 100 mg vaginal dose of SC divided into three or four doses, to avoid a rise in plasma concentrations above the side effects threshold. The dosage of 100 mg also has been used in previous trials with vaginal SC, divided into four daily doses of 25 mg through several days in assisted reproductive therapy in non-pregnant women [69]. In the present study the high dose of intravaginal SC (100 mg/day) and the long duration of administration (24 days) found to be more effective in enhancing the biological parameters that help in maintaining the pregnancy by increasing antioxidant and decreasing oxidative stress (MDA and NO) more than the low dose (75mg/day) and short duration of administration of intravaginal SC (13 days). Also, based on these findings, it is also possible to propose the use of transvaginal ultrasonography with Doppler flowmetry in the URSM to assess uterine artery flow and local perfusion in women at risk for spontaneous abortion and obtain a prognostic cut-off value.

The overall ongoing successful pregnancy rate in F-URSM groups was 60 % (24 out of 40 patients) and the overall ongoing failure pregnancy rate in F-URSM groups 40 % (16 out of 40 patients), further work may be needed to increase the successful pregnancy rate if possible.

CONCLUSION
The mechanism of SC action as novel pharmaceutical antiabortive agent in treatment of URSM patients depends on two main topics: firstly, the increases of blood flow in uterine
arteries. Secondly, augmentation of antioxidants levels as well as suppression of lipid peroxidation and nitrosative stress.

REFERENCES


