



Effect of Sildenafil Citrate on Peripheral Natural Killer Cells in women with Recurrent Pregnancy Loss

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Abstract: Back ground Researchers' defined recurrent miscarriage as three or more pregnancy losses, the available research data denotes that the risk of abortion is 30% and 33% after two miscarriages and three miscarriages, consecutively. Sildenafil citrate (a type 5-specific phosphodiesterase inhibitor, augments the vasodilatory effects of NO. Vagina sildenafil augments the vasodilatory effects of NO. NK cytotoxicity has been reported to be predictive of subsequent pregnancy loss in women who had recurrent spontaneous abortions (RSA). **Aim:** Is to investigate whether sildenafil citrate affect peripheral natural killer cells successful in women with history of recurrent pregnancy loss. **Methodology:** An interventional research study, with one arm clinical trial: one group of study subjects were administered the sildenafil citrate without comparative placebo effect, conducted at the Obstetrics and Gynecology Outpatient clinic at Ain Shams University hospital throughout 2017-2018. The sample size consists of 77 women within the reproductive age (from 18 to 35 years old) having a history of recurrent pregnancy loss. **Results:** There was a statistical significant reduction in peripheral blood natural killer cell activity after having vaginal sildenafil. (p value <0.001), there was no statistical significant correlation between peripheral blood natural killer cell activity (%) and other demographic characteristics (age, BMI, parity, miscarriages, time from last miscarriage, p values before = 0.204, 0.929, 0.416, 0.302, 0.627, consecutively, p values after intervention = 0.133, 0.709, 0.380, 0.410, 0.559, consecutively, p values of reduction = 0.794, 0.458, 0.687, 0.181, 0.944, consecutively). **Conclusions:** The current research reveals and displays that vaginal sildenafil citrate is a potentially promising agent in managing cases with recurrent miscarriage and it reduces the natural killer cell activity levels.

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1. Introduction

Researchers' defined recurrent miscarriage as three or more pregnancy losses, the available research data denotes that the risk of abortion is 30% and 33% after two miscarriages and three miscarriages, consecutively. Correspondingly the up-to-date guidelines by the American Society for Reproductive Medicine define recurrent pregnancy loss as two or more pregnancy losses, which have been confirmed by either ultrasound or histopathological study.**1,2,3.**

Research studies furthermore classify recurrent pregnancy loss as primary or secondary. Primary recurrent pregnancy loss cases have never reached viability, while in the secondary type, live birth have occurred at some time. On the other hand, there is no particular terminology given to multiple spontaneous miscarriage interposed with normal gestation. It is crucial to perform an early assessment in clinical scenarios where fetal cardiac activity have been documented before the loss, in women who are older

than 35 years and/or in couples with clinical infertility history. Incidence of Recurrent pregnancy loss affects around 0.4–1% of couples. The clinical risk of losing the gestation is more in early pregnancies, frequently in the first gestational trimester. There is about 22–57% miscarriage risk with pregnancy less than 6 weeks.**4,5,6.**

Etiologies involve genetic abnormalities, endocrinological disorders, uterine anomalies, antiphospholipid syndrome, thrombophilias (heritable or acquired), infections, immunological abnormalities, and environmental factors. Correspondingly elevated number of prior miscarriages, increased maternal age, lifestyle factors, and familial factors are different risk factors for recurrent pregnancy loss.**7,8,9.**

Natural killer cells and miscarriage: NK cells comprise about 10–15% of peripheral blood lymphocytes. Two distinct subsets of human NK cells are possible, depending on the cell surface density of the CD56 molecule. Approximately 90% of peripheral

blood human NK cells are CD56dim and express high levels of FcγIII (CD16) as well as perforin. In contrast, a minority (approximately 10%) of NK cells are CD56bright and CD16dim. These CD16dim cells are the primary source of NK cell derived cytokines and thought to be an important regulatory subset. An abnormal increase in peripheral blood NK cell parameters (either in NK cell absolute values or in proportion (%) prior to conception or during early pregnancy) is associated with recurrent miscarriage and infertility with multiple implantation failures. **10,11,12.**

Sildenafil citrate (a type 5-specific phosphodiesterase inhibitor, augments the vasodilatory effects of NO. Vagina sildenafil augments the vasodilatory effects of NO. NK cytotoxicity has been reported to be predictive of subsequent pregnancy loss in women who had recurrent spontaneous abortions (RSA). **14,15,16.**

Aim .

Is to investigate whether sildenafil citrate affect peripheral natural killer cells successful in women with history of recurrent pregnancy loss.

2. Methodology

An interventional research study, with one arm clinical trial: one group of study subjects were administered the sildenafil citrate without comparative placebo effect, conducted at the Obstetrics and Gynecology Outpatient clinic at Ain Shams University hospital throughout 2017-2018. The sample size consists of 77 women within the reproductive age (from 18 to 35 years old) having a history of recurrent pregnancy loss **Inclusive research criteria were as follows** Age range was 18 - 35 years old., History of unexplained recurrent miscarriages with loss of three or more pregnancy at gestational age more than 8-10 weeks in order to exclude genetic and chromosomal causes of RPL., six or more months have elapsed since the last abortion., no other therapy was allowed during the course of treatment. **Exclusive research criteria were as follows** Detailed history exclude any other causes of RPL (anatomical, endocrinal, infections), consanguineous marriage, Concomitant use of organic nitrites, or nitrates, Severe hepatic, renal or cardiovascular impairment.

Every research study subject recruited have undergone the following:

Verbal consent about the experimental nature of management protocol before initiation of therapy. Detailed past, family and obstetric histories. **Explanation and counselling about possible side effects including:**

I-Common effects: headache, flushing, dyspepsia, nasal congestion, blurring of vision,

photophobia, acyanopsia (blue vision), dizziness, postural hypotension.

II-Other minor effects: palpitation diarrhea, vomiting, sweating, backache, and arthralgia.

III- Serious effects: loss of peripheral vision, allergic reaction, sudden hearing loss and chest pain.

Management protocol implemented

Cases recruited have self -administrated sildenafil citrate (25mg vaginal, four times a day) during the proliferative phase of menstrual cycle for 3-6 days in most cases for 3 days from 5th to 7th day of the cycle. **2-Withdrawal** of 10 ml of venous blood sample twice to be collected in citrated lab. Tube and peripheral NK cells will be measured by flow cytometry, first time before start of treatment and second time after 3-6 days.

Flow Cytometry

Ten ml of venous blood sample was withdrawn from cases in heparinized tubes. Specimens were incubated in the dark at room temperature for 15 min, then 1 ml of lysing solution was added and mixed well, to be incubated again in the dark at room temperature for 10 min, centrifuged for 5 min at 2000 rpm (42.48 xg) and the supernatant was discarded. Cells were then washed with phosphate buffer saline (PBS) and the cell pellet was re-suspended in 200- 500 ml PBS. Flow cytometric analysis was performed on the Becton Deckinson FACS caliber flow cytometer using Cell Quest Software (Becton Deckinson). Cells negatively stained for CD3, positively for CD56 were selected and CD16 percentage was analyzed. The results were expressed as the percentage of the positive cells relative to the isotype control (%) and expression or mean fluorescence intensity (MFI) which is defined as the ratio between the MFI of the cells incubated with the tested monoclonal antibodies and the MFI of the cells incubated with isotype matched control.

Research study outcome:

Primary research outcome:

Assessment of decrease in peripheral natural killer cells after 3-6 days of administration of sildenafil citrate.

Secondary research outcome:

Continuation of a healthy pregnancy in patients included in the study till 20th gestational weeks to ensure they did not undergo recurrent miscarriage.

3. Results

The demographic characteristics of the recruited research study cases in which Mean±SD of age, BMI, parity, miscarriages, and time from last miscarriage =26.8±3.6 years, 24.9±2.1 kg/m², 1.1±1.2, 3.5±1.0, 8.1±1.5 months, consecutively as regards the Peripheral blood natural killer cell activity (%) there was a statistical significant reduction in peripheral

blood natural killer cell activity after having vaginal sildenafil. (p value <0.001) table 1,2,3 figure 1,2.

Table (1): Demographic characteristics of the studied cases

Variables	Mean±SD	Range
Age (years)	26.8±3.6	19.0–35.0
BMI (kg/m ²)	24.9±2.1	19.0–30.1
Parity	1.1±1.2	0.0–3.0
Miscarriages	3.5±1.0	3.0–7.0
Time from last miscarriage (months)	8.1±1.5	6.0–11.0

Total=77

Table (2): Peripheral blood natural killer cell activity (%) of the studied cases

Time	Mean±SD	Range	^P
Before	16.2±5.4	2.7–31.6	<0.001*
After	11.1±4.3	1.0–19.6	
Reduction	5.1±1.5	1.7–13.5	

Total=77, ^Paired t-test, *Significant

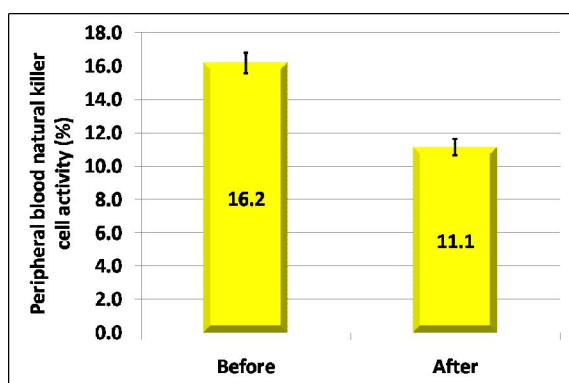


Figure (1): Peripheral blood natural killer cell activity (%) of the studied cases

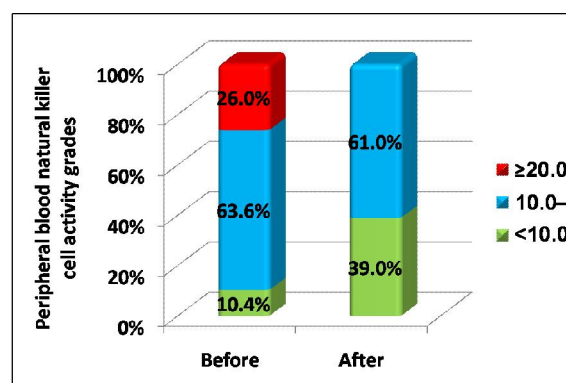


Figure (2): Peripheral blood natural killer cell activity grades of the studied cases

Table (3): Peripheral blood natural killer cell activity grades of the studied cases

Grades	Mean±SD	Range
Before		
<10.0	8	10.4
10.0–	49	63.6
≥20.0	20	26.0
After		
<10.0	30	39.0
10.0–	47	61.0
Reduction		
<10.0	56	72.7
10.0–	21	27.3

Total=77

4. Discussion

Recurrent spontaneous miscarriage is the loss of three or more consecutive pregnancies before 20 gestational weeks. The etiology of most recurrent miscarriages cases remains unclear. The majority of recurrent spontaneous miscarriage cases after full investigations are categorized as idiopathic. **6.**

Natural killer (NK) cells have a corner stone role in maintenance of gestation, assay has been invented to assess natural killer cells in peripheral blood and menstrual. **9.**

Recent research studies reveal and display the critical role of NK cells in embryonic implantation. **10.**

Recurrent spontaneous abortion (RSA) is a frequent clinical scenario that complicates pregnancy causing couples to represent by unexplained infertility issues. Peripheral and uterine NK cells (pNK, uNK) could be discriminated in accordance to the receptor expression patterns investigators have shown a correlation between elevated peripheral nk and uterine NK to recurrent miscarriage clinical scenarios. **11.**

The current research study findings have revealed and displayed as regards the demographic characteristics of the recruited research study cases in which Mean±SD of age, BMI, parity, miscarriages, and time from last miscarriage =26.8±3.6 years, 24.9±2.1 kg/m², 1.1±1.2, 3.5±1.0, 8.1±1.5 months, consecutively as regards the Peripheral blood natural killer cell activity (%) there was a statistical significant reduction in peripheral blood natural killer cell activity after having vaginal sildenafil. (p value <0.001) Furthermore there was no statistical significant correlation between peripheral blood natural killer cell activity (%) and other demographic characteristics (age, BMI, parity, miscarriages, time from last miscarriage values before =0.204, 0.929, 0.416, 0.302, 0.627, consecutively, p values after intervention =0.133, 0.709, 0.380, 0.410, 0.559, consecutively, p values of reduction =0.794, 0.458, 0.687, 0.181, 0.944, consecutively)

A previously conducted research study similar to the current research as regards the aim and research methodology in which the research team of investigators aimed to assess the impact of sildenafil on peripheral natural killer cell activity levels in cases with a past history of recurrent miscarriage. The research team recruited Thirty-eight non pregnant cases having a past history of recurrent miscarriage and 37 healthy cases with past successful pregnancy clinical outcomes. Cases have self-administered sildenafil suppositories (25 mg intravaginally, four times a day) for 36 days similar to the current research study The following results were revealed and displayed the NK-cell activity levels were statistically significantly fallen after vaginal sildenafil administration course of treatment. The research team came to the conclusion that Vaginal sildenafil could be a promising effective management option before conception in cases with history of reproductive failure. **14.**

Another group of researchers conducted a study supporting ours aiming to investigate the ability of sildenafil to downregulate regulate osteopontin (OPN) and protein in peripheral blood mononuclear cells (PBMCs) from healthy blood donors. OPN is expressed by a wide variety of cell types, including immune cells. Osteopontin (OPN) is a member of the small integrin binding ligand N-linked glycoprotein family proteins. OPN is expressed by a wide variety of

cell types such as immune system cells [T and B cells, natural killer (NK) cells, NK T cells, dendritic cells, macrophages, neutrophils]. bone cells, neurons, epithelial cells, pericytes, fibro blasts, hepatocytes, tubular cells, vascular smooth muscle cells. OPN functions are linked to various physiological and pathological conditions. Sildenafil is a selective inhibitor of type 5 phosphodiesterase. Sildenafil has recently been found to have immunomodulatory effects in animal models and in studies performed in humans. PMA-stimulated and unstimulated PBMCs from 16 healthy blood donors (men) were cultured with sildenafil (at concentrations of 400ng/ml and 4µg/ml). OPN level in culture supernatants was measured by enzyme-linked immunosorbent assay. The analysis of OPN gene expression was performed by real-time PCR. Cell viability was assessed by trypan blue staining. PMA plus ionomycin stimulation of PBMCs resulted in a significant increase of OPN production and gene expression (p < 0.001). Sildenafil significantly decreased OPN secretion (p < 0.05) and gene expression (p < 0.05) in stimulated PBMCs; however, had no effect on OPN in unstimulated PBMCs. Sildenafil did not affect PBMCs viability. Sildenafil downregulates OPN in natural killer cells. Despite accumulating evidence for the immunomodulatory effects of sildenafil on human immune system cells. Accordingly to this study sildenafil citrate immunomodulatory effect could be effective in down regulating natural killer cells in women with recurrent pregnancy 15,16.

A similar prior research study objective was to verify whether intravaginal sildenafil citrate tablets (25 mg intravaginal, 4 times/day for 24 days) are efficient as a novel agent in the management of threatened miscarriage in cases with a past history of unexplained recurrent miscarriage evaluating the concentrations of various antioxidants, the percentage of expression levels of tumor necrosis factor-α, which express one of the NK cell markers. The research group of investigators compared these results with healthy women in their first gestational trimester. **the investigators revealed and displayed that Sildenafil enhanced the antioxidants concentrations levels besides The percentage of natural killer cells and TNF- α in peripheral blood was higher in unexplained recurrent miscarriage cases in comparison to cases in their full term pregnancies. On the other hand, concentration levels were greatly reduced and improved after sildenafil administration. the research team came to a conclusion that sildenafil citrate is a potential antiabortive agent in the management of threatened miscarriage in cases with a history of unexplained recurrent miscarriage. 1,4,9.**

Another group of investigators interestingly conducted a previous research study in which they

assessed and measured peripheral NK cell activity before pregnancy after administration of sildenafil suppositories (25 mg intravaginally four times a day) during the proliferative phase of the cycle in cases with history of recurrent miscarriage. The cases self-administered sildenafil suppositories (25 mg intravaginally, four times a day for 3–6 days, in most cases for 3 days from the 5th to 7th day of the cycle). Peripheral blood NK-cell activity levels were assessed and determined by usage of flow cytometry. The peripheral blood NK-cell surface antigens CD16- and CD56 were also investigated by usage of flow cytometry the research team of investigators revealed and displayed the following results in which Peripheral blood NK-cell activity level was statistically significantly increased in the recurrent miscarriage cases compared with the control research group (15.67 +/-11.86% vs. 8.29 +/- 5.00%, consecutively; P value <0.05), those research findings are similar to the current research study findings additionally the research team revealed and displayed that there was no statistical significant differences in CD16 and CD56 expression levels between recurrent miscarriage research cases and the control research group furthermore finally the research investigators have shown that the NK-cell activity levels was statistically significantly reduced after vaginal sildenafil therapy in comparison to NK-cell activity levels before therapy in the recurrent miscarriage research group (P value <0.05) additionally those finding show great harmony and similarity to the current research study findings. The research team of investigators came to the conclusion that sildenafil could be a useful therapy in recurrent miscarriage cases. **5, 16.**

A prior research study similar to the current one showed that in vitro culture of endometrial cells with sildenafil citrate significantly reduces NK-cell activity levels, and this impact was independent of the sildenafil dosage added to the culture., since the low dosage was as effective as the high dosage of sildenafil. Consequently, vaginal sildenafil could be an effective management agent with justified usage for selected cases scenarios with reproductive failure. Since recurrent miscarriage and infertility embrace a dissimilar category of cases. It is crucial to fully investigate such categories of cases on Immunological basis before proceeding with further research studies.**6,8.**

Another researcher stated that Sildenafil Can Affect Innate and Adaptive Immune System in Both Experimental Animals and Patients The Effects of Sildenafil on the Lymphocytes Subpopulations of Healthy Human Blood Donors. The only data of the effects of sildenafil on healthy human lymphocytes in vitro comes from studies on human T regulatory

cells. Cocultures of T effector cells and T reg cells (0.25: 1 and 0.5: 1 ratio) isolated from healthy donor peripheral blood showed that sildenafil at the 10 μ M concentration influenced the ability of Tregs to downregulate T effector cell proliferation. Moreover, expression of Treg transcription factor Foxp3 was increased, suggesting that upregulation of Tregs is involved in T effector cell deactivation. Thus sildenafil immunoregulatory function could be useful in women with recurrent pregnancy loss.**13, 14.**

Limitation of the study is short term follow-up longer period are needed to assess going home babies.

Conclusions and recommendations

The current research reveals and displays that vaginal sildenafil citrate is a potentially promising agent in managing cases with recurrent miscarriage and it reduces the natural killer cell activity levels. However the molecular and cellular complexity of the process of placentation and implantation require the interpretation of the results with great care, as other variables could influence this clinical situation such as racial and ethnic differences that mirror different immunological expression due to genetic differences. Sildenafil citrate administered vaginally should be considered in metacentric fashioned future research studies to clearly consider its benefit and implement future clinical guidelines implementation aiding in improvement of management and care levels of cases having recurrent miscarriage clinical scenarios.

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