Effects of sildenafil citrate and etanercept treatment on TNF-α levels in peripheral blood of women with recurrent miscarriage

Wpływ terapii cytrynianem sildenafilu lub etanerceptem na poziom TNF-α w krwi obwodowej kobiet z nawracającymi poronieniami

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Abstract

Objectives: *The aim of the study was to determine serum concentrations of a proinflammatory cytokine, tumor necrosis factor-alpha (TNF-α), in patients with recurrent abortions undergoing treatment with sildenafil or etanercept.*

Material and methods: *Serum TNF-α concentrations were determined for 24 patients with recurrent miscarriages (aged 32.7±4.64 years) deemed eligible for sildenafil therapy, and 7 patients treated with etanercept (aged 37.65±5.45 years). Measurements were performed before and after therapy. The control group included 10 healthy women (aged 33.3±5.49 years), who gave birth at least once without pregnancy-related complications. The levels of serum TNF-α were measured by Elisa.*

Results: *Patients treated with etanercept had significantly elevated levels of TNF-α before therapy as compared to the control group (41.4±28.4 vs. 16.6±7.2 pg/ml). Moreover, we found a tendency for the concentration of TNF-α to increase in sera of patients treated with sildenafil after therapy completion (19±29 vs. 15.4±26.7 pg/ml). Treatment with etanercept resulted in a significant reduction of serum TNF-α levels (41.4±28.4 vs. 25.4±3.2 pg/ml).*

Conclusions: *Therapy of recurrent abortions with anti-TNF-α drugs appears to be encouraging. Administration of blockers of phosphodiesterase type 5 or TNF-α blockers before conception seems to be a promising future therapy of immune-dependent recurrent miscarriages, limiting the teratogenic influence of the drugs on the fetus.*

Key words: **TNF-α -Tumor Necrosis Factor-α** / **sildenafil citrate** / **etanercept** / / **NK cells** / **phosphodiesterase type 5** / **recurrent miscarriage** /

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Otrzymano: **10.09.2014**
Zaakceptowano do druku: **08.02.2015**

Streszczenie

Cel pracy: *Celem pracy było oznaczenie stężenie cytokiny pozapalnej – czynnika martwicy nowotworów (TNF-α), w surowicy pacjentek z nawracającymi poronieniami poddanych terapii cytrynianem sildenafilu lub etanerceptem.*

Materiał i metody: *Do badań i terapii zakwalifikowano pacjentki, u których wcześniej wykluczono inne przyczyny poronień niż immunologiczne tj.: anatomiczne, genetyczne, hormonalne czy mikrobiologiczne. U 24 pacjentek w wieku 32,7 ±4,64 lat objętych terapią cytrynianem sildenafilu, jak również u 7 pacjentek w wieku 37,65 ±5,45 z podwyższoną aktywnością komórek NK poddanych terapii etanerceptem oznaczono stężenie TNF-α w surowicy krwi. Leki podawano na 3-5 dni przed planowanym poczęciem by wykluczyć ich możliwy teratogenny wpływ. W obu grupach badania przeprowadzono przed i po zastosowaniu leku. Grupę kontrolną stanowiło 10 zdrowych kobiet, które przynajmniej raz rodziły bez komplikacji w czasie ciąży. Średnia wieku kobiet grupy kontrolnej to 33,3 ±5,49 lat. Poziom TNF-α w surowicy krwi oznaczano testem Elisa.*

Wyniki: *Pacjentki z grupy leczonej etanerceptem miały istotnie podwyższony poziom TNF-α przed terapią w porównaniu do grupy kontrolnej (41,4 ±28,4 vs 16,6 ±7,2 pg/ml). W grupie pacjentek leczonych sildenafilem stwierdzono tendencję do wzrostu stężenia TNF-α w surowicy po terapii (19 ±15,4 vs 29 ±26,7 pg/ml). Leczenie etanerceptem skutkowało istotnym obniżeniem poziomu TNF-α w surowicy pacjentek (41,4 ±28,4 vs 25,4 ±13,2 pg/ml).*

Wnioski: *Terapia anty-TNF-α wydaje się być obiecująca w leczeniu nawracających poronień o podłożu immunologicznym. Podawanie przed poczęciem blokerów fosfodiesterazy typu 5 lub blokerów TNF-α wydaje się być ciekawą i przyszłościową metodą terapii nawracających poronień, prawdopodobnie ograniczającą teratogenny wpływ leków na płód.*

Słowa kluczowe: **TNF-α -czynnik martwicy nowowtworów / cytrynian sildenafilu / / etanercept / komórki NK / fosfodiesteraza typu 5 / nawracające poronienia /**

Introduction

Sildenafil citrate inhibits the activity of phosphodiesterase type 5 (PDE5), thus preventing degradation of cyclic guanosine monophosphate (cGMP). PDE5 is found in particularly high concentrations in the corpus cavernosum, the erectile tissue of the penis. It is also found in the retina and vascular endothelium. The drug is a specific inhibitor of PDE5. Its molecule contains a structural motif of piperazine and its nucleoside analogue of the heterocyclic base-guanine-1H-pyrazolo [4,3-d] pyrimidine [1]. Sildenafil has a similar geometric shape as cGMP, which is a common regulator of ion channel conductance, glycogenolysis, and cellular apoptosis. It also relaxes smooth muscle tissues. In blood vessels, relaxation of vascular smooth muscles leads to vasodilation and increased blood flow [2, 3]. Vaginal administration of sildenafil was successfully used to improve uterine blood flow [4].

Pulmonary arterial hypertension (PAH) is the most common disease where sildenafil therapy is continued during pregnancy. The physiological changes which occur during pregnancy and delivery can cause immediate death in patients with PAH. Maternal mortality is estimated at 30-56%. Patients who decided to continue their pregnancy, subjected to oral therapy with sildenafil, gave birth normally and their blood pressure stabilized [3]. The results obtained by Sher et al., in 2000 and 2002, showed that sildenafil improved blood flow in the uterine arteries and increased endometrial thickness in patients with spontaneous recurrent miscarriage and after *in vitro* fertilization (IVF) [4,5,6].

Zinger observed sufficient growth of the endometrium for embryo implantation and development after intravaginal administration of sildenafil to 2 patients with Asherman's syndrome (secondary infertility after previous surgical removal of the endometrium) [7].

Previous studies on the influence of sildenafil on the fetus were performed on mice and rats [8,9,10], and showed no teratogenic effects of the drug on the fetus. Rat fetuses had increased weight after administration of sildenafil [9,10]. Sildenafil administration during pregnancy in women with PAH did not adversely affect the survival of the fetus [11, 12]. Some studies showed that sildenafil significantly improves blood flow in the uterus, resulting in increased endometrial thickness [13]. The quality of the endometrium is an important factor in achieving successful implantation [15].The first study on the influence of sildenafil on the immune system of women with recurrent miscarriage was performed by El-Far et al. These authors described the effect of sildenafil on oxidative stress and a decrease in the level of TNF-α and the number of NKT cells ($CD3^+$ $CD56^+$ $CD161^+$) in women with recurrent pregnancy loss (RPL) [16]. Our previous studies have shown that sildenafil also reduces the activity of NK cells in peripheral blood in patients with RPL after natural conception or after *in vitro* fertilization [17]. On the other hand, sildenafil is known to enhance the activity of nitric oxide, which induces proinflammatory cytokines, including TNF-α. TNF-α is a potent activator of NK cells [18]. Therefore, we decided to examine the before and after therapy serum levels of TNF-α in patients treated with sildenafil.

We also investigated the effect of another drug, etanercept, on the level of TNF-α in sera of women with RPL. Etanercept (Enbrel) is a TNF-α antagonist used in treating autoimmune diseases such as rheumatoid arthritis, juvenile rheumatoid and psoriatic arthritis, plaque psoriasis, and ankylosing spondylitis. Etanercept is a fusion protein composed of TNF receptor 2 and the constant end of the IgG1 antibody [19]. Etanercept and sildenafil should have opposite effects on the production of TNF-α.

The aim of the study was to investigate the influence of sildenafil and etanercept on the level of TNF-α in women with recurrent miscarriage.

DOI: 10.17772/gp/57823

Material

Sildenafil citrate group

The study included 24 patients (aged 32.7±4.64 years) with RM treated with sildenafil citrate. Serum concentrations of TNF-α were determined before and after drug administration. All assays were performed at least 6 months after the last miscarriage and administration of drugs other than sildenafil. According to the WHO, recurrent miscarriage is defined as three or more consecutive pregnancy losses before 20 weeks of pregnancy with the same partner. Factors other than immunological, including genetic, anatomical, hormonal, and microbiological were excluded before qualifying for the study group. In all cases, complete medical history, surgical and social data were collected. All couples had peripheral blood chromosome assessment.

Intravaginal pessaries were prepared by the local hospital pharmacy at WIM from the oral tablets (Viagra – Pfizer). Patients were administered sildenafil intravaginally at the dose of 25 mg, 4x/day for 3-6 days, in most cases from day 5 to 7 of the menstrual cycle (the proliferative phase). Patients with a small increase in endometrial thickness applied sildenafil for 6 days. The level of TNF-α was measured after at least 14 days (luteal phase of the menstrual cycle).

Etanercept group

Serum concentrations of TNF-α before and after therapy were determined in 7 patients (aged 37.65±5.45 years) with increased NK cell activity enrolled for etanercept therapy. Assays were carried out at least 6 months after the last miscarriage and administration of drugs other than etanercept. Women received 4 doses (25 mg) of etanercept, 2x/week prior to conception in the proliferative phase. The study was conducted in the luteal phase of the menstrual cycle.

Control group

The control group consisted of 10 healthy women who gave birth at least once, without any complications during pregnancy. Average patient age in the study and the control groups was very similar (control group 33.3±5.49 years). We did not determine the level of TNF-α after administration of sildenafil or etanercept in multiparous women, because there was no approval of the Bioethics Committee for performing such assays. Each subject knowingly signed the consent to participate in the study according to the protocol approved by the Bioethics Committee of the Military Medical Institute in Warsaw. The study was conducted with the approval of the Bioethics Committee No: 67/2003 Military Medical Chamber and No. 70/2006 Military Medical Institute.

Methods

In order to determine the concentration of TNF-α in the study and the control groups, blood samples were collected to collection tubes. Then, blood was centrifuged for 20 min. at 550g and sera were collected and stored at -20°C or -75°C.

The concentration of TNF-α was measured by Elisa ("OptEIA" kit, Becton Dickinson), according to the manufacturer's instructions using Elisa microplate reader (Ledetect 96) at a wavelength of 450 nm.

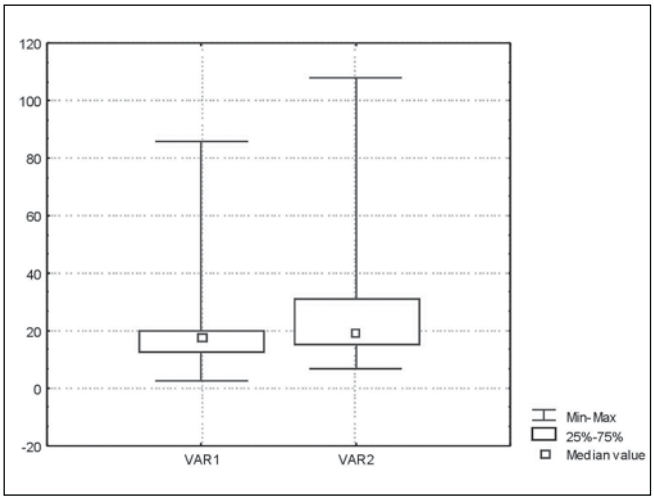

Figure 1. Serum concentration of TNF-α before and after sildenafil therapy (VAR1 vs.VAR2).

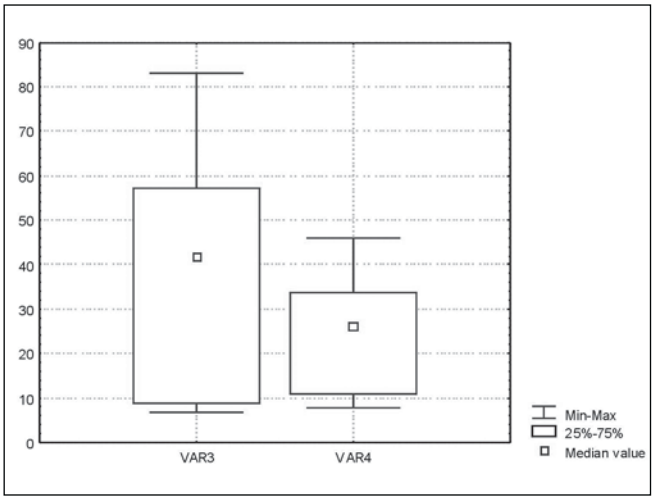

Figure 2. Serum concentration of TNF-α before and after etanercept therapy (VAR3 vs. VAR4).

Statistical analysis

Statistical analysis of the results obtained within individual groups was performed by non-parametric test of Wilcoxon matched pairs. Differences between the groups were determined by the Mann -Whitney test. The p value of <0.05 was considered statistically significant. Statistica 9 (StatSoft) software was applied for statistical analysis.

Results

The level of TNF-α in sera of women with RM was higher than in the control group. We found particularly high concentrations of TNF-α in sera of women qualified for treatment with etanercept as compared to controls (Table I). Patients treated with sildenafil had higher level of TNF-α after therapy than before administration of the drug. Often, the level of the cytokine was 3-4-fold higher after therapy (Figure 1). Treatment with etanercept, a TNF-α antagonist, resulted in a statistically significant decrease in the level of this cytokine (Table I, Figure 2). However, mean level of TNF-α after treatment was still higher as compared to controls.

Table I. Concentration of TNF-α in sera of the control group vs. concentration of TNF-α in sera of patient from the group qualified for sildenafil therapy-2 or etanercept therapy-3, 2'- concentration of TNF-α after sildenafil therapy, 3'-concentration of TNF-α after etanercept therapy (*p<0.05, min-minimum value, max.-maximum value, SD-standard deviation, 1-3 quartile).

Serum TNF-α concentration (pg/ml)								
group	Number of patients	Min.	Max.	1 quartile	3 quartile	median	mean	SD
1	n=10	6.2	31.2	9.85	19.8	17.5	16.6	7.2
2	n=24	2.5	85.6	13	19.83	17.7	19	15.4
2'	n=24	6.8	108	15.5	31.5	19.3	29	26.7
3	n= 7	6.75	83	9.22	57	42	41.4*	28.4
3'	n= 7	7.7	45.8	11.07	33.85	26.4	25.4	13.2

Discussion

Sildenafil citrate is a phosphodiesterase type 5 inhibitor which augments the vasodilatory effects of nitric oxide (NO) by preventing the degradation of cGMP.

Constant and smooth blood flow in the uterine arteries can be achieved by administration of drugs which increase the level of NO [20]. Endometrium growth depends on the uterine artery blood flow [6, 10, 15]. The growth of the endometrium in the proliferative phase is important for the implantation of the embryo [4, 5]. Drugs which block the activity of TNF-α are considered as an alternative treatment for recurrent miscarriage [21]. So far, there has been no evidence of embryotoxic effects of TNF-α antagonists. Data on the safety of using these drugs in pregnant women is limited. Some reports suggest that TNF-α blockers may increase the risk of VACTERL syndrome (V – vertebral anomalies, A – anal atresia, C – congenital heart defects, T – tracheostomy esophageal fistula, E – esophageal atresia, R – renal abnormalities or radial dysplasia, L – limb abnormalities) [21, 22, 23].

The largest database containing data on the use of etanercept during pregnancy was created by the Organization of Teratology Information Specialists (OTIS). In recent reports by OTIS, no statistically significant effects of etanercept therapy on the development of malformations and deformations of the fetus were mentioned. Studies were performed on female patients with rheumatoid diseases during the first trimester of pregnancy. Recent FDA guidelines suggest that etanercept therapy should be avoided at the time of conception in women with rheumatoid arthritis [25]. So far, individual applications of etanercept have been reported during pregnancy in patients suffering from ankylosing spondylitis or rheumatoid arthritis [23]. Premature births and reduced birth weight were observed after administration of etanercept (25 mg every 14 days or 25 mg/week). In breastfeeding mothers taking etanercept, the drug level is negligible (below ≤ 4ng/ml) in breast milk [13].

The mode of administration of etanercept or sildenafil in our study excluded direct effects of these drugs on the fetus as drugs were taken prior to conception by each patient. In both treatment groups we observed a decrease in NK cell activity [16, 17].

In our study, the use of etanercept before conception proved to be safe for both, mothers and their offspring. Etanercept effectively reduced the level of TNF-α in sera of RPL patients. In contrast, sildenafil increased the concentration of TNF-α, however, the level achieved after therapy was similar to the level of this cytokine after etanercept therapy. El-Far et al., in their study performed on 4 RM patients, obtained opposite results. These authors found that sildenafil reduced the level of TNF-α in sera and the percentage of NKT cells [16]. This discrepancy could be explained by differences in treatment protocol. Patients in the study group of El-Far and colleagues applied 25mg of sildenafil 4x/day but for 24 days. It is possible that a sildenafil therapy longer than 6 days results in a decrease of TNF-α in peripheral blood of patients due to sildenafil regulatory effects. Margonis et al., showed in their study performed on animal model of colitis that sildenafil decreased the level of TNF-α in homogenized tissue but the level did not decrease to the level of the control group [32].

In our study, etanercept abrogated the level of TNF-α but still it was higher than in the control group and similar to the mean level obtained after sildenafil treatment. Moreover, all patients within six months after sildenafil administration conceived and deliver healthy children (data not shown).

A comprehensive paper on the impact of sildenafil on the immune system was published in 2013 [27]. That study was performed on a mouse model. It has been shown that sildenafil acts differently on immune cells of females and males [27]. It is believed that sildenafil has different mode of action in experimental animals and humans. Single use of sildenafil during pregnancy was reported in cases of pulmonary hypertension, which is a fatal disease. Administration of sildenafil in these cases was beneficial to both, the mother and the child. It did not cause damage to the fetus. In fact, the weight gain was higher, sildenafil prevented fetal resorption, and it normalized blood pressure in the uterine arteries [12, 28].

TNF-α during pregnancy can be secreted by decidualized NK cells and also by trophoblast cells. During normal pregnancy, NK cells ($CD56^{+/-}$) after contact with trophoblast cells do not produce TNF-α [29, 30]. In our studies, sildenafil decreased NK cell activity in women with RPL (the results were reported in [15]), but it has the tendency to increase serum level of TNF-α in patients.

The presence of TNF-α in the endometrium in early pregnancy has a positive impact on its outcome. TNF-α stimulates angiogenesis, is involved in the elimination of activated lymphocytes expressing the Fas receptor, and enhances the expression of Fas [28]. Toder et al., reported anti-apoptotic activity and regulation of cell proliferation by TNF-α during pregnancy [30]. During the first trimester of pregnancy, mRNA for TNF-α is detected in cytotrophoblast, as well as in syncytiotrophoblast villi [30].

DOI: 10.17772/gp/57823

The majority of authors described negative effects of TNF-α on the pregnancy outcome, i.e. hindering implantation, inhibition of remodeling of spiral arteries, and induction of Th1 cytokines which causes fetal resorption [18, 31].

In our study, therapy of recurrent miscarriage with sildenafil or etanercept had opposite effects on serum level of TNF-α in patients. Interestingly, both drugs were effective in the treatment of RM (data shown in [15, 17, 26]).

Maintenance of pregnancy requires complex interactions between different populations of immune cells. Further studies are needed to explain mechanisms of action of these drugs on immune cells and cytokines involved in pregnancy maintenance.

Acknowledgments

This study was supported by grants no.: N407 246634 from Ministry of Science and Higher Education and P05E 07926 from National Research Committee of Poland.

Oświadczenie autorów:

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2. Małgorzata Jerzak - zebranie materiału, analiza statystyczna wyników, przygotowanie manuskryptu.
3. Andrzej Górski – korekta pracy, kierownik jednostki, w której zostały wykonane badania.

Źródło finansowania:

projekt był finansowany z grantów: Ministerstwa nauki i Szkolnictwa Wyższego nr N407 246624 i KBN P05E 07926.

Konflikt interesów:

Autorzy nie zgłaszają konfliktu interesów oraz nie otrzymali żadnego wynagrodzenia związanego z powstawaniem pracy.

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