

## Sildenafil does not affect the proliferation of human lymphocytes in the *in vitro* transplant model

Beata Kaleta<sup>✉</sup> and Agnieszka Boguska

Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland

**Sildenafil is used in the treatment of erectile dysfunction and pulmonary arterial hypertension. Numerous studies revealed beneficial effects of its use in renal, liver, heart and bone marrow transplant recipients. Some reports suggested that the drug modulates the function of the immune system, however, its influence on antigen-induced proliferation of lymphocytes remains unknown. Thus, the aim of the study was to investigate the effects of sildenafil on human peripheral blood mononuclear cells (PBMCs) proliferation in a mixed lymphocyte reaction. It was demonstrated that the drug did not affect auto- and alloantigen-induced proliferation of PBMCs and showed no cytotoxic effect.**

**Key words:** mixed lymphocyte reaction, PDE5 inhibitor; peripheral blood mononuclear cells; sildenafil

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✉ e-mail: [kaletabeata1@gmail.com](mailto:kaletabeata1@gmail.com)

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**Abbreviations:** ED, erectile dysfunction; GMP, guanosine 3,5-monophosphate; IL, interleukin; MLR, mixed lymphocyte reaction; NO, nitric oxide; PAH, pulmonary arterial hypertension; PBMCs, peripheral blood mononuclear cells; PDE5, type 5 phosphodiesterase; TNF- $\alpha$ , tumor necrosis factor alpha

### INTRODUCTION

Sildenafil (5-[2-ethoxy-5-(4-methylpiperazin-1-ylsulfanyl)phenyl]-1-methyl-3-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one; C<sub>22</sub>H<sub>30</sub>N<sub>6</sub>O<sub>4</sub>S) is a selective inhibitor of type 5 phosphodiesterase (PDE5), a major enzyme hydrolyzing cyclic guanosine 3,5-monophosphate (cGMP) to GMP. cGMP is expressed in cardiomyocytes, vessel and uterine smooth muscle cells, endothelial, epithelial, myometrial and immune cells (Nichols *et al.*, 2002; Kukreja *et al.*, 2005). As a second messenger, cGMP mediates the action of nitric oxide (NO), regulating a broad array of physiological processes (Dhayade *et al.*, 2016). By blocking the breakdown of cGMP, sildenafil acts to prolong the effects of this cyclic monophosphate.

Sildenafil is the first line therapy of erectile dysfunction (ED) and pulmonary arterial hypertension (PAH) (Essayan, 2001). ED is common in male patients with renal diseases and in patients after kidney transplantation, in which the drug is used effectively and safely (Zhang *et al.*, 2005; Sharma *et al.*, 2006). A growing body of evidence shows that sildenafil exerts immunomodulatory effects, however, most studies were performed in animal models (Serafini *et al.*, 2006; Szczycka & Obmnska-Mrukowicz, 2010; Pifarre *et al.*, 2011; 2014). Not much information is available about the impact of sildenafil on the

human immune system. Our recent studies demonstrated for the first time that sildenafil downregulates natural killer cell activity (Boguska *et al.*, 2018) and osteopontin (Kaleta & Boguska, 2017) production in human peripheral blood mononuclear cells (PBMCs). Moreover, we showed that sildenafil upregulates tumor necrosis factor (TNF- $\alpha$ ) production but has no effect on interleukin (IL)-6 and IL-1 $\beta$  synthesis (Kaleta *et al.*, 2019), as well as on phagocytic activity of granulocytes (Boguska *et al.*, 2018). There are reports suggesting that selective PDE5 inhibitors affect human endothelial, bone marrow and cancer cells proliferation (Erdogan *et al.*, 2007; Li *et al.*, 2007; Wang *et al.*, 2008; He *et al.*, 2017), however, the influence of sildenafil on antigen-induced proliferation of lymphocytes has not been studied up to date.

To address this subject, the aim of the study was to investigate the effects of sildenafil on healthy human PBMCs proliferation in a mixed lymphocyte reaction (MLR). MLR is an efficient *in vitro* model for studying T-cell activation and proliferation (Potter & Moore, 1977).

### MATERIALS AND METHODS

**Isolation of peripheral blood mononuclear cells and mixed lymphocyte reaction.** 30 healthy blood donors participated in the study. PBMCs were isolated from heparinized blood by centrifugation on Histopaque-1077 (Sigma, St. Louis, USA). PBMCs were resuspended in Parker's medium (Biomed, Lublin, Poland) supplemented with 2 mM L-glutamine, 0.23% Hepes (both from Sigma, St. Louis, USA), 0.1 mg/ml gentamicin (KRKA, Novo Mesto, Slovenia) and 10% fetal bovine serum (FBS, Gibco, Darmstadt, Germany). Half of the isolated PBMCs were inactivated by gamma-irradiation for 90 min. Sildenafil citrate (Sigma, St. Louis, USA) was dissolved in 0.9% sodium chloride (0.9% NaCl, Fresenius Kabi, Bad Homburg, Germany) to the initial concentration of 100  $\mu$ g/mL.

The mixed lymphocyte reaction (MLR) was performed by seeding  $1 \times 10^5$  responder PBMCs into the wells of a 96-well plate, and  $1 \times 10^5$  irradiated (stimulatory) PBMCs in the following combinations:

AA<sub>ir</sub>BB<sub>ir</sub>AB<sub>ir</sub>BA<sub>ir</sub> donor 1 and 2

AA<sub>ir</sub>BB<sub>ir</sub>AB<sub>ir</sub>BA<sub>ir</sub> donor 3 and 4, etc.

A, B – PBMCs (responding cells), A<sub>ir</sub>, B<sub>ir</sub> – irradiated PBMCs (stimulating cells)

Cells were co-cultured with sildenafil at concentrations of 0.06  $\mu$ M, 0.6  $\mu$ M and 6  $\mu$ M. Drug concentrations were selected on the basis of the near therapy doses, according to their pharmacokinetics (C<sub>max</sub> and area un-

der the time-concentration curve, AUC). 0.6  $\mu\text{M}$  is an average serum level of sildenafil after a single 100 mg oral administration (Nichols *et al.*, 2002). 0.06  $\mu\text{M}$  and 6  $\mu\text{M}$  are 10-fold lower and 10-fold higher, respectively. The concentration of 6  $\mu\text{M}$  was used to test the potential toxic effects. Control cultures contained equivalent volumes of the medium.

PBMCs were cultured for 5 days at 37°C in a humidified atmosphere with 5%  $\text{CO}_2$ . After 5 days, PBMCs were pulsed with 1  $\mu\text{Ci}/\text{well}$  of  $^3\text{H}$ -thymidine (113 Ci/nmol, NEN, Boston, USA) for the last 18 h of the incubation and harvested. The amount of  $^3\text{H}$ -thymidine incorporated into the cells was measured using the scintillation counter (Wallac, PerkinElmer, Boston, USA), giving the level of radioactivity as 'Corrected Counts per Minute' (CCPM). Experiments were performed in triplicates.

**Cell viability assay.** PBMCs viability was examined using trypan blue staining. After 5 days of culture, PBMCs were collected and stained with 0.4% trypan blue. The number of total and dead cells was counted using a hemocytometer. Values were expressed as a percentage of control culture viability (100%).

**Statistical analysis.** All analyses were performed with Statistica version 12.5. The results were expressed as mean  $\pm$  standard deviation (SD). After testing all the data for normality (Shapiro-Wilk test), one-way ANOVA was used to compare values obtained with or without sildenafil treatment. A probability value of  $p < 0.05$  with a 95% confidence interval was considered to indicate a statistically significant difference.

The study was approved by the Ethics Committee of the Medical University of Warsaw (No. KB/164/2017) and all subjects provided their written informed consent. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000.

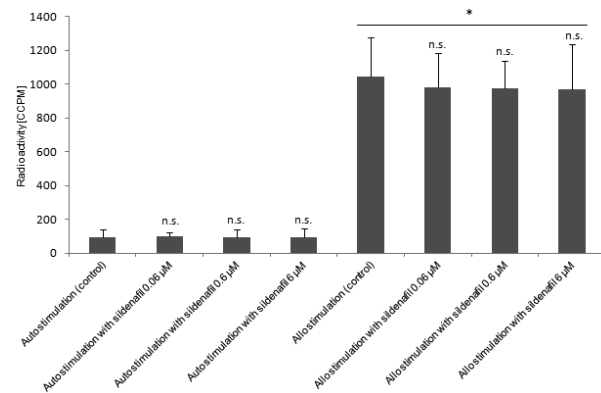
## RESULTS

As expected, the addition of allogeneic irradiated PBMCs from one donor to responding PBMCs from unrelated second donor resulted in an increase in lymphocytes proliferation ( $p < 0.01$  in comparison to the autostimulated variants). Sildenafil at all tested concentrations (0.06, 0.6 and 6  $\mu\text{M}$ ) did not affect auto- and alloantigen-induced proliferation of PBMCs ( $p > 0.05$ ) (Fig. 1) as well as PBMCs viability (percentage of viable cells was  $96.5\% \pm 1.9\%$  [range: 96–97%]) ( $p > 0.05$ ) (Fig. 2).

## DISCUSSION

Despite the fact that PDE5 inhibitors showed efficacy and safety, it is important to know whether they can affect the human immune system as they are used in patients after organ transplantation. Thus, studies in transplant settings, including *in vitro* transplant models, as presented in the current work, are required. The results of our analysis demonstrated for the first time that sildenafil has no significant effects on the auto- and alloantigen-induced proliferation of human PBMCs and does not affect the cells viability.

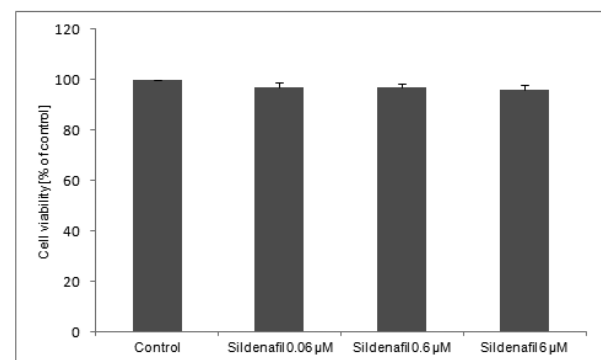
As mentioned above, sildenafil is used in patients with ED and PAH (Essayan, 2001; Boswell-Smith *et al.*, 2006). ED is common in male patients with renal diseases and in patients after kidney transplantation, in which the drug is effective and safe (Sharma *et al.*, 2006). In addition, multiple studies revealed beneficial effects of sildenafil use in renal, liver, heart and bone marrow



**Figure 1.** Effect of sildenafil (0.06, 0.6 and 6  $\mu\text{M}$ ) on peripheral blood mononuclear cells (PBMCs) proliferation in a mixed lymphocyte reaction (MLR).

The proliferation of lymphocytes cultured with irradiated PBMCs from the same donor (autostimulation) and from the second unrelated donor (allostimulation) was examined on the DNA synthesis level by the measurements of  $^3\text{H}$ -thymidine incorporation. The results are presented as a level of radioactivity measured as 'Corrected Counts per Minute' (CCPM). Data are presented as mean  $\pm$  standard deviation of 30 experiments performed in triplicates, \* $p < 0.01$  in comparison to autostimulated variants, n.s.=not statistically significant.

transplant recipients (Russo *et al.*, 2004; Zhang *et al.*, 2005; Lledo-Garcia *et al.*, 2007; De Santo *et al.*, 2008; Boffini *et al.*, 2009; Lansaponara *et al.*, 2013; Singh *et al.*, 2014). Moreover, accumulating data indicate that sildenafil can play an important role in the regulation of the immune system in animals (Serafini *et al.*, 2006; Szczypka & Obmnska-Mrukowicz, 2010; Pifarre *et al.*, 2011; Pifarre *et al.*, 2014). However, the data about its influence on human immune cells are limited. It was demonstrated that incubation of PBMCs isolated from myeloma and head and neck cancer patients with sildenafil restored  $\text{CD4}^+$  T cells proliferation (Serafini *et al.*, 2006). Another study (Pifarre *et al.*, 2014) provided evidence that sildenafil enhances the ability of healthy human regulatory T cells (Tregs) to down-regulate the proliferation of the effector T cells (Teffs). He and colleagues (2017) revealed that sildenafil suppressed the proliferation of hemangioma endothelial cells (HemECs) *in vitro*. Further analyses confirmed that sildenafil inhibited the prolifera-



**Figure 2.** Effect of sildenafil (0.06, 0.6 and 6  $\mu\text{M}$ ) on peripheral blood mononuclear cells (PBMCs) viability.

PBMCs were incubated with sildenafil for 5 days. Cell viability was measured using trypan blue staining. Results are expressed as the percent of control cells viability. Data are presented as mean  $\pm$  standard deviation for each sildenafil dose, n=30.

tion of pulmonary artery smooth muscle cells (PASMCs) (Li *et al.*, 2007; Wang *et al.*, 2008). Erdogan and colleagues (2007) likewise demonstrated the antiproliferative effects of sildenafil on human endothelial cells. In contrast, Doganci *et al.* (2015) showed that the drug-induced proliferation of human umbilical vein endothelial cells (HUVECs). In another study, Santos and others (Santos *et al.*, 2014) revealed that the treatment with sildenafil increased the proliferation of the subventricular zone (SVZ)-derived neural stem cells (NSCs).

Since PDE5 is expressed in human immune cells and sildenafil increases cGMP levels, we speculated that the drug, which is used in transplant recipients, could affect the proliferation of PBMCs. The present study, although on a limited number of participants, demonstrated that sildenafil had no significant effects on the auto- and alloantigen-induced proliferation of human lymphocytes in the *in vitro* transplant model. Based on the contradictory observations of previous authors and the results of the current study, we conclude that the effects of sildenafil on cellular proliferation are variable and dependent on the type of cells studied. Therefore, further functional studies are required to elucidate the potential mechanisms of immunomodulatory actions of this PDE5 inhibitor.

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#### Conflicts of Interests

None declared.

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