Access this article online

Quick Response Code:

Website: www.ijp-online.com

DOI: 10.4103/ijp.ijp_312_23

Off-target effect of high-dose sildenafil on adenosine 5'-diphosphate and collagen-induced platelet activation through mitogen-activated protein kinase pathway in treated BALB/C mice and *in vitro* experiments: A preliminary study

Sudharshanan Balaji[1,2], Yash Raj Patnaik[2], William Rasican Surin[2]

Abstract:

Sildenafil, a common over-the-counter pill often self-administered at high doses for erectile dysfunction, has been reported to rarely cause prothrombotic events and sudden cardiac death in a few case reports. Therefore, we investigated the *in vitro* and *in vivo* effect of sildenafil treatment and dosage on platelet activation and mitogen-activated protein kinase (MAPK) phosphorylation. BALB/C mice were segregated into four groups, each having four mice each (control, low [3.25 mg/kg], medium [6.5 mg/kg], and high [13 mg/kg] sildenafil), and after the treatment, blood was drawn from each mouse and washed platelets prepared. Washed platelets were incubated with CD41 PE-Cy7 and Phospho-p38 MAPK PE antibodies and analyzed using a flow cytometer for platelet activation and adenosine 5'-diphosphate (ADP)/collagen-induced MAPK phosphorylation. Washed platelets obtained from the venous blood of 18 human volunteers, were incubated with PAC-1 FITC and Phospho-p38 MAPK PE antibodies, and platelet activation (ADP and collagen), followed by flow cytometry analysis. There was a significant increase in both platelet activation as well as MAPK phosphorylation in the presence of collagen in the high-dose (13 mg/kg) sildenafil group ($P = 0.000774$). Further, increased platelet activation was observed in samples that were treated with high-dose sildenafil as compared to the untreated samples ($P < 0.00001$). These studies show the risk of prothrombotic episodes in patients on high-dose sildenafil (100 mg), in those with even mild endothelial dysfunction due to ADP, and collagen-induced platelet activation through MAPK phosphorylation, which was not seen in the low-and intermediate-dose cohorts.

Keywords:

Flow cytometry, mitogen-activated protein kinase, platelet, sildenafil citrate

[1]Department of Urology, Government Kilpauk Medical College, Chennai, Tamil Nadu, [2]Department of Microbiology and Cell Biology, Indian Institute of Science, Bengaluru, Karnataka, India

Address for correspondence:
Dr. William Rasican Surin, Department of Microbiology and Cell Biology, Indian Institute of Science, Bengaluru, Karnataka, India.
E-mail: wrsurin@iisc.ac.in

Submitted: 22-May-2023
Revised: 15-Nov-2023
Accepted: 18-Mar-2024
Published: 30-Apr-2024

Introduction

Sildenafil is commonly prescribed to treat erectile dysfunction. Chronic usage and self-administration may cause adverse effects ranging from priapism to hypotension to sudden cardiac death.[1]

Previous research has indicated that human platelet and corpus cavernosum PDE5s have similar responses to several drugs, implying

How to cite this article: Balaji S, Patnaik YR, Surin WR. Off-target effect of high-dose sildenafil on adenosine 5'-diphosphate and collagen-induced platelet activation through mitogen-activated protein kinase pathway in treated BALB/C mice and *in vitro* experiments: A preliminary study. Indian J Pharmacol 2024;56:136-40.

a similar mechanism of action.[2] Whether sildenafil causes aggregation or has an independent antithrombotic effect is still unclear.[3] About 30% of patients do not respond to initial doses of the drug, often leading to the patient being prescribed a higher dose.[4] α and β isoforms of mitogen-activated protein kinase (MAPK) are commonly found on human platelets. Platelets induced with thrombin and/or collagen have been known to activate p38 MAPK-dependent phosphorylation.[5] We aimed to understand the effect of sildenafil treatment and dosage on platelet activation and MAPK phosphorylation, both *in vitro* and *in vivo*.

Methods

Tyrode buffer, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, adenosine-5′-diphosphate, Tris base, apyrase, and collagen Type I from rat tail were procured from Sigma Aldrich (MERCK Group, Bengaluru, India). PAC1-FITC antibody was from BD Biosciences Inc. (USA). Anti-mouse CD41 PE-Cy7 and anti-mouse Phospho38 MAPK PE antibody and permeabilization and fixation buffer were purchased from Invitrogen (Thermo Fisher Scientific, Massachusetts, USA). All other chemicals used were of analytical grade. Sildenafil citrate was purchased as soluble tablets from Mankind™, under the brand Manforce™ in different concentrations of 25 mg and 100 mg, dissolved uniformly in saline (0.9% NaCl), and used for the experiments.

Animals

Male BALB/C mice, aged 12 weeks, 25–30 g, were housed at 24°C ± 0.5°C temperature, with 12 h day/night cycle with humidity (55% +10%), supplied with chow pellets and water. The animal study was conducted according to the Institute Animal Ethics Committee approval (CAF/Ethics/892/2023). Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines were duly adhered to while performing all experiments.

Sample collection from human volunteers

The effect of sildenafil citrate on human platelets was studied *in vitro*. Informed consent from 18 self-declared healthy human male volunteers aged 18–24 years was obtained with prior approval from the Institutional Human Ethics Committee (601/2021). The study was conducted adhering to the principles in the Declaration of Helsinki and the ICMR National Ethical Guidelines for Biomedical and Health Research on Human Participants, 2017.

Effect of sildenafil citrate on collagen and adenosine 5′-diphosphate-induced platelet activation in mice platelets *in vivo*

Platelets extracted from mice as detailed by Lugun *et al*. with small modifications.[6] BALC/C mice were grouped into four groups of four mice each (control, low-dose sildenafil [3.25 mg/kg], medium-dose sildenafil [6.5 mg/kg], and high-dose sildenafil [13 mg/kg]). The sildenafil groups were treated orally with 3.25, 6.5, and 13 mg/kg of sildenafil citrate, corresponding to 25, 50, and 100 mg of human dosage, respectively. After 1 h of sildenafil citrate treatment, the mice were anesthetized with isoflurane, and 1 ml of blood was collected from each mouse by cardiac puncture in 1:10 acid citrate dextrose solution. Thereafter, the blood was processed to obtain washed platelets as detailed by Lugun *et al*.[6] The final count of washed platelets was set to 0.8–1.2 × 10^9/ml.

Platelet wash from each mouse was labeled with anti-mouse CD41 PE-Cy7 and anti-mouse Phospho38 MAPK PE antibodies by incubation at 37°C for 1 h in the dark. Platelet activation was induced by adding collagen (100 µg/ml) or adenosine 5′-diphosphate (ADP) (100 µM). It was analyzed by BD FACSCanto™ II flow cytometer (BD Biosciences, Inc.) at the excitation wavelength (488 nm) and emission wavelengths (564–606 nm and 750–810). The platelet activation was evaluated by measuring the mean fluorescence intensity (MFI) of the platelet marker (CD41 PE-Cy7) and phospho38 MAPK PE phosphorylation. Data acquisition and analysis were done using BD FACSDiva software (6.1.3).

Effect of sildenafil citrate on collagen and adenosine 5′-diphosphate-induced platelet activation in mice platelets *in vitro*

A volume of 1 ml blood was collected through a syringe by cardiac puncture in male BALB/C mice after isoflurane exposure and transferred into a 1.5 ml Eppendorf Tube containing 3.8% trisodium citrate in 1:9 proportion and processed as described above to prepare the washed platelets.

Washed platelets from each mouse were incubated with anti-mouse CD41 PE-Cy7 and anti-mouse Phospho38 MAPK PE antibodies for 1 h at 37°C, followed by 30 min of incubation with sildenafil citrate (10, 20 or 40 mg/ml). Platelet activation was induced by adding collagen (100 µg/ml) or ADP (100 µM). It was analyzed by BD FACSCanto™ II flow cytometer (BD Biosciences, Inc.) at the blue excitation wavelength (488 nm) and emission wavelengths (564–606 nm and 750–810).

Effect of sildenafil citrate on collagen and adenosine 5′-diphosphate-induced platelet activation in human platelets *in vitro*

A volume of 5 ml of venous blood was collected from the 18 volunteers by sterile venepuncture technique in 1:9 ratio acid citrate solutions in a 15 ml falcon tube. Following centrifugation at 275 g for 20 min at 20°C, the

platelet-rich plasma (PRP) obtained was subject to 1500 g for 15 min at 20°C. The supernatant was decanted, and the pellet was suspended in modified Tyrode's HEPES Buffer (134 mM NaCl, 0.34 mM $NaHPO_4$, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, and pH 7.3) to which 0.15/mL ADPase of apyrase was added with half an hour incubation at 37°C.

Then, PRP was centrifuged at 1500 g for 15 min at 20°C. The platelet pellet was suspended in 300 µL of modified Tyrode's HEPES Buffer and labeled with PAC1 FITC antibody for 1 h at 37°C, followed by 30 min of incubation with sildenafil citrate (10, 20, or 40 mg/ml).

Thereafter, platelet activation was induced by collagen (100 µg/ml) or ADP (100 µM) and was analyzed by BD FACSCanto™ II flow cytometer (BD Biosciences, Inc.) at the blue excitation wavelength (488 nm) and emission wavelengths (515–530 nm and 750–810 nm).

Statistical analysis

Statistical analysis was done between different groups using IBM SPSS Statistics (Version 27, Armonk, NY: IBM Corp) and $P < 0.05$ was considered to be statistically significant.

Results

Effect of sildenafil citrate on collagen and adenosine 5′-diphosphate-induced platelet activation in mice platelets *in vivo*

Sildenafil citrate was studied for its effect on collagen and ADP-induced platelet activation. The CD41 PE-Cy7 antibody binds the fibrinogen receptor GPIIb present in the platelets. Upon activation by platelet agonists, collagen, and ADP, there is increased externalization of the fibrinogen receptor GPIIb, leading to increased binding of CD41 PE-Cy7. Thus, CD41 PE-Cy7 binding correlates with the increased activation of platelets. Further, Phospho38 MAPK labeling correlates with the phosphorylation level of Phospho38 MAPK in the MAPK signaling pathway.

The upper right quadrant of the dot plot depicts the collagen and ADP-induced platelet activation and phosphorylation [Figure 1]. We observed a significant increase in platelet activation and MAPK phosphorylation in the presence of collagen in the high-dose sildenafil group (13 mg/kg) compared to the control group [Figure 2] ($P = 0.000774$), while there was no significant difference in collagen-induced

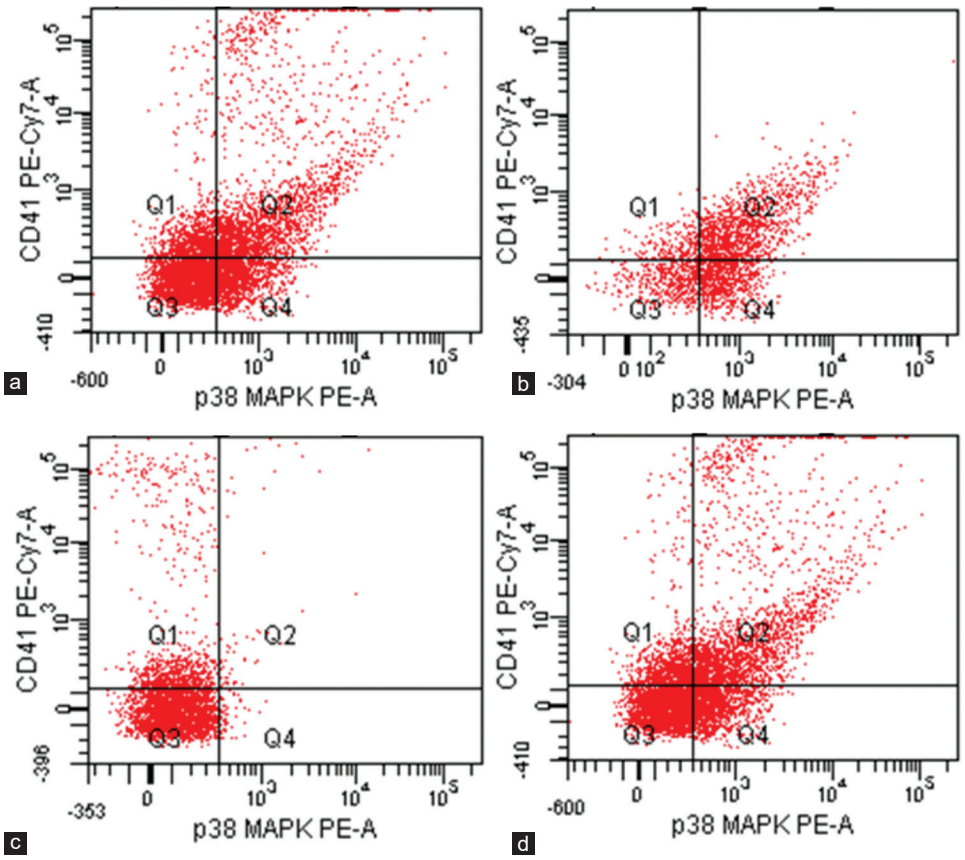

Figure 1: Collagen and adenosine 5'-diphosphate (ADP)-induced platelet activation and mitogen-activated protein kinase phosphorylation in BALB/C mice treated with high-dose (13 mg/kg) sildenafil versus untreated mice. The top two graphs represent high-dose sildenafil treated mice with (a) collagen and (b) ADP-induced platelet activation, respectively. The bottom two graphs indicate untreated mice group with (c) Collagen and (d) ADP-induced platelet activation, respectively

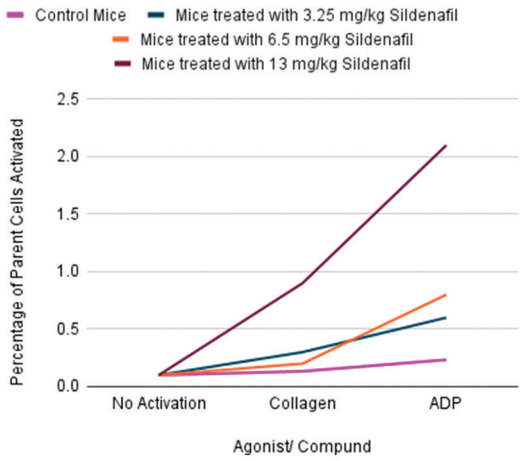

Figure 2: Effect of high-dose sildenafil on platelet activation and mitogen-activated protein kinase phosphorylation

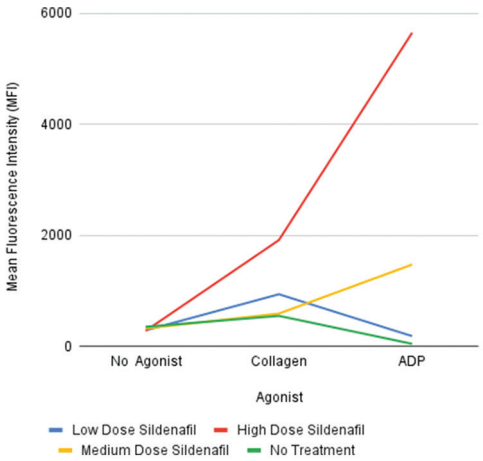

Figure 3: Effect of *in vitro* sildenafil on human platelet activation induced by adenosine 5'-diphosphate (ADP) and collagen

platelet activation between the low (3.25 mg/kg) and intermediate (6.5 mg/kg) groups for the control untreated group.

Effect of sildenafil citrate on collagen and adenosine 5′-diphosphate-induced platelet activation in mice platelets *in vitro*

In the presence of sildenafil, there was significant platelet activation in ADP-induced platelet activation (P = 0.028871). However, no considerable platelet activation was observed in collagen-induced platelet activation.

Effect of sildenafil citrate on collagen and adenosine 5′-diphosphate-induced platelet activation in human platelets *in vitro*

There was a significant increase in the expression of MFI in samples treated with high-dose sildenafil when compared to the untreated samples when activated by both ADP and collagen (P < 0.00001). However, no significant difference was observed between the high-dose versus low-dose versus medium-dose samples [Figure 3].

Discussion

Sildenafil is an over-the-counter drug in India that has been prone to overuse and abuse over the years.[7] While Kontaras *et al*. have conclusively proven that there is no additional risk of sudden cardiac death in sildenafil users, the case report of Huber *et al*. shows otherwise.[8,9]

Clopidogrel, commonly used in myocardial infarction and prothrombotic states, is an effective and specific inhibitor of prostaglandin E1-induced adenylyl cyclase and, thereby, an inhibitor of ADP-induced activation of platelets, which has proven to be an effective mechanism to treat atherogenic and prothrombotic states.[10] Endothelial dysfunction, following vascular injury or exposure of subendothelial structures or stemming from metabolic disorders such as diabetes mellitus, is a common entity in India, which has many diabetic individuals.[11] In such conditions, when blood flows directly over exposed subendothelial structures containing collagen leading to platelet activation and aggregation.[12]

Based on this hypothesis, we carried out our experiments, which indicated that a high dose of sildenafil citrate (100 mg – adult human dose) in the presence of endothelial dysfunction (collagen/ADP exposure and increase) leads to increased platelet activation as compared to moderate (50 mg) and low dose (25 mg). This mechanism and pathophysiology can provide a possible explanation for the various case reports and articles demonstrating prothrombotic events following high-dose sildenafil usage, such as stroke, myocardial infarction, and deep vein thrombosis.[13-16]

Our results show increased activation of platelets induced by ADP and collagen following a high dose of sildenafil, in contrast with previous studies. Halcox *et al*. demonstrated that sildenafil inhibits ADP-induced platelet GPIIb/IIIa receptor activation.[17] Li *et al*. demonstrated in vitro Cyclic guanosine monophosphate (cGMP) induced platelet activation following sildenafil treatment.[3] Wallis *et al*. demonstrated that sildenafil does not significantly inhibit platelet activation,[18] whereas Yang *et al*. demonstrated that sildenafil reduced neointimal hyperplasia and inhibited platelet aggregation and activation through cGMP dependent protein kinase upregulation.[19]

Akand *et al*. showed an increase in ADP-induced platelet aggregation at high-dose sildenafil (100 mg) treated patients with erectile dysfunction compared with controls, which seems to align with our findings.[2]

However, this study needs to be validated in larger cohorts to get a better idea of the external validity of the hypothesis.

Conclusions

Our studies suggest mild endothelial dysfunctions result in increased exposure of platelets to subendothelial collagen and elevated levels of ADP in circulation, leading to increased platelet activation through the phosphorylation of MAPK. Moreover, high-dose sildenafil treatment in mild endothelial dysfunctions occurring in an array of diseases can predispose individuals to a higher incidence of platelet-mediated thrombotic events, which can be avoided in lower doses.

Financial support and sponsorship

Financial support in terms of REDA grant to W. R. S. is greatly acknowledged.

Conflicts of interest

There are no conflicts of interest.

References

1. Tracqui A, Miras A, Tabib A, Raul JS, Ludes B, Malicier D. Fatal overdosage with sildenafil citrate (Viagra): First report and review of the literature. Hum Exp Toxicol 2002;21:623-9.
2. Akand M, Gencer E, Yaman Ö, Erişgen G, Tekin D, Özdiler E. Effect of sildenafil on platelet function and platelet cGMP of patients with erectile dysfunction. Andrologia 2015;47:1098-102.
3. Li Z, Xi X, Gu M, Feil R, Ye RD, Eigenthaler M, *et al*. A stimulatory role for cGMP-dependent protein kinase in platelet activation. Cell 2003;112:77-86.
4. McMahon CG. High dose sildenafil citrate as a salvage therapy for severe erectile dysfunction. Int J Impot Res 2002;14:533-8.
5. Börsch-Haubold AG, Ghomashchi F, Pasquet S, Goedert M, Cohen P, Gelb MH, *et al*. Phosphorylation of cytosolic phospholipase A2 in platelets is mediated by multiple stress-activated protein kinase pathways. Eur J Biochem 1999;265:195-203.
6. Lugun O, Bhoi S, Kujur P, Kumar DV, Surin WR. Evaluation of antithrombotic activities of *Solanum xanthocarpum* and *Tinospora cordifolia*. Pharmacognosy Res 2018;10:98-103.
7. Sathyanarayana Rao TS, Kumar VA, Raman R, Andrade C. Prolonged, longstanding, ultra-high-dose abuse of sildenafil. Indian J Psychiatry 2015;57:311-2.
8. Kontaras K, Varnavas V, Kyriakides ZS. Does sildenafil cause myocardial infarction or sudden cardiac death? Am J Cardiovasc Drugs 2008;8:1-7.
9. Huber BC, von Ziegler F, Bamberg F, Franz WM, Becker A. Cardiac arrest associated with sildenafil ingestion in a patient with an abnormal origin of the left coronary artery: Case report. BMC Cardiovasc Disord 2011;11:49.
10. Schrör K. The basic pharmacology of ticlopidine and clopidogrel. Platelets 1993;4:252-61.
11. Dhananjayan R, Koundinya KS, Malati T, Kutala VK. Endothelial dysfunction in type 2 diabetes mellitus. Indian J Clin Biochem 2016;31:372-9.
12. Farndale RW, Sixma JJ, Barnes MJ, de Groot PG. The role of collagen in thrombosis and hemostasis. J Thromb Haemost 2004;2:561-73.
13. Robles-Marhuenda Á, Romero Gallego-Acho P, Arnalich Fernández F. Deep vein thrombosis associated with sildenafil. Emergencias 2019;31:72.
14. Lee J, Kwon JH, Lee CH, Lee SM, Yim JJ, Yoo CG, *et al*. A case of acute pulmonary thromboembolism after taking tadalafil. Tuberc Respir Dis (Seoul) 2012;73:231-3.
15. Habek M, Petravić D. Stroke – An adverse reaction to sildenafil. Clin Neuropharmacol 2006;29:165-7.
16. Hayat S, Al-Mutairy M, Zubaid M, Suresh C. Acute myocardial infarction following sildenafil intake in a nitrate-free patient without previous history of coronary artery disease. Med Princ Pract 2007;16:234-6.
17. Halcox JP, Nour KR, Zalos G, Mincemoyer RA, Waclawiw M, Rivera CE, *et al*. The effect of sildenafil on human vascular function, platelet activation, and myocardial ischemia. J Am Coll Cardiol 2002;40:1232-40.
18. Wallis RM, Corbin JD, Francis SH, Ellis P. Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. Am J Cardiol 1999;83:3C-12C.
19. Yang HM, Jin S, Jang H, Kim JY, Lee JE, Kim J, *et al*. Sildenafil reduces neointimal hyperplasia after angioplasty and inhibits platelet aggregation via activation of cGMP-dependent protein kinase. Sci Rep 2019;9:7769.