Effects of sildenafil citrate on survival in the posterior leg replantation model

Rat arka bacak replantasyon modelinde sildenafil Sitrat'ın sağkalım üzerine etkileri

Sildenafil citrate and leg replantation

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Öz

Amaç: Sildenafil sitrat bir fosfodiesteraz (PDE) enzim inhibitörü ve bir vazodilatör ajandır. Anjiyoprotektif ve anjiyogenetik etkileri önceden bildirilmiştir. Bu çalışmanın amacı, sildenafil sitratın iskemik sıçan arka bacak replantasyonundan sonra sağkalıma olan etkisini araştırmaktır. Gereç ve Yöntem: Bu çalışmada Sprague-Dawley dişi sıçanlar kullanılmıştır. Çalışmanın ilk evresinde sildenafil sitratın sağkalım etkileri araştırıldı. Bu amaçla, kontrol ve çalışma gruplarında iskemik sıçanlara arka bacak replantasyonu yapıldı ve postoperatif dönemde 7 gün boyunca çalışma gruplarına 10 mg/kg/gün sildenafil sitrat uygulandı. İkinci aşamada, sildenafil sitratın doku etkisi analiz edildi. Çalışma grubuna iskemik sıçan arka bacak modeli için sildenafil sitrat uygulandı ve gastrocnemius kası ve femoral ven alınan doku örnekleri histolojik olarak analiz edildi. Damar dokusuna Ang-1, VEGF immünohistokimyasal boyaları uygulanırken, kas dokusunda PCNA immünohistokimyasal boyaları uygulandı. Kan numunelerinin katalaz düzeyleri biyokimyasal olarak analiz edildi. Bulgular ve Tartışma: Sildenafil sitratın anlamlı şekilde sağkalımı artırdığı gözlemlendi. Kas dokusunda nekrozu azalttığı, ayrıca endotel hasarını azaltırken antioksidan aktiviteyi arttırdığı da gözlendi.

Anahtar Kelimeler

Sildenafil Sitrat; Replantasyon; Revaskülarizasyon; Angiopoetin; İskemi-Reperfüzyon Hasarı

Abstract

Aim: Sildenafil citrate is a phosphodiesterase (PDE) enzyme inhibitor and a vasodilator agent. Its angioprotective and angiogenetic effects are widely known. The aim of this study was to investigate the effects of sildenafil citrate on survival of ischemic rat hind leg amputation after replantation. Material and Method: Sprague-Dawley female rats were used in the study. The survival effects of sildenafil citrate were investigated in the first phase of the study. For this purpose, ischemic rat hind leg replantation was performed on control and study groups and 10 mg/kg/day sildenafil citrate was administered to study groups for 7 days in the postoperative period. In the second phase, the tissue-level effects of sildenafil citrate were investigated. Tissue samples were taken from gastrocnemius muscle and femoral vein and they histologically analyzed. Ang-1 and VEGF immunohistochemical stains were applied to vein tissue, whereas PCNA immunohistochemical stains were applied to muscle tissue. Blood samples were analyzed for catalase. Results and Discussion: It was observed that sildenafil citrate macroscopically and significantly increased survival after hind leg replantation. It reduced necrosis. It also reduced endothelium damage and increased antioxidant activity.

Keywords

Sildenafil Citrate; Replantation; Revascularization; Angiopoietin; Ischemia-Reperfusion Damage

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Introduction

Peripheral organ amputations are problematic injuries in Plastic and Reconstructive Surgery practice. Proper timing of the surgery, meticulous surgical technique, and appropriate surgical and anesthetical environments and techniques are indispensable for a successful outcome. However, in order to increase the chance for survival or to cope with the consequences of less than ideal conditions, researchers try to find remedies for reducing the expected ischemic-reperfusion damage, by increasing the regenerative capacity of vascular endothelium, protecting the anastomosis from thrombotic events, and promoting vasodilator activity.

A specific phosphodiesterase (PDE-5) enzyme inhibitor, sildenafil citrate, is currently used for erectile dysfunction treatment due to its peripheral vasodilator effect. Its known effect is to ensure the progression of vasodilatation by preventing the degradation of c-GMP (guanidine monophosphate), which is the secondary messenger of nitric oxide (NO), primarily in vascular smooth muscle cells (1). The vasodilator effect of PDE-5 inhibitors was presumed to release endogenous mediators that would result in cardioprotective effects against ischemia/reperfusion injury of cardiac muscle. Treatment with sildenafil was also established to mediate increased levels of VEGF in the ischemic brain and induce capillary-like tube formation in a culture of both brain-derived endothelial cells and coronary arteriolar endothelial cells, suggesting neoangiogenetic properties (2). On the other hand, inhibition of PDEs was shown to exert a platelet-inhibitory effect by increasing the intracellular cyclic nucleotide content of the platelet, thus blocking cytoskeletal rearrangement, fibrinogen receptor activation, degranulation, and expression of proinflammatory mediators (3).

The aim of the study was to investigate the versatile effects of sildenafil citrate on survival in the ischemic rat hind leg replantation model.

Material and Method

The study was approved by the Eskisehir Osmangazi University (ESOGU) Ethical Committee on Animal Experiments (decision number of 101 dated 18.02.2009). 35 female Sprague-Dawley rats between 250-300 g in weight were used for the study. The rats were kept in the experimental animal laboratory of Eskisehir Osmangazi University Faculty of Medicine at constant room temperature and were supplied with limitless water and dry pellets. A separate cage was provided for each subject. The surgical procedure was performed by the same researcher during the study and the subjects were randomly distributed into groups. Subjects were anesthetized intraperitoneally with 80 mg/kg ketamine (Ketalar 500 mg, solution for injection in vials, Pfizer) for all surgical procedures.

The sildenafil citrate sample to be administered to the subjects was obtained in compliance with Hart et al. using the commercially available product (Viagra tablet 100 mg, Pfizer Inc., NY) (4). The study was planned to be two steps.

In the first part of the study, the effects of sildenafil citrate on survival were determined in the ischemic rat hind leg replantation model. The right hind legs of the subjects were amputated at the inguinal level. Femoral and sciatic nerves were protected during amputation in order to prevent autocannibalization that could arise during the follow-up period. The control group (n=7) received 1 cc of saline and the study group (n=7) received 10mg/kg sildenafil citrate intraperitoneally immediately after amputation (Figure 1).

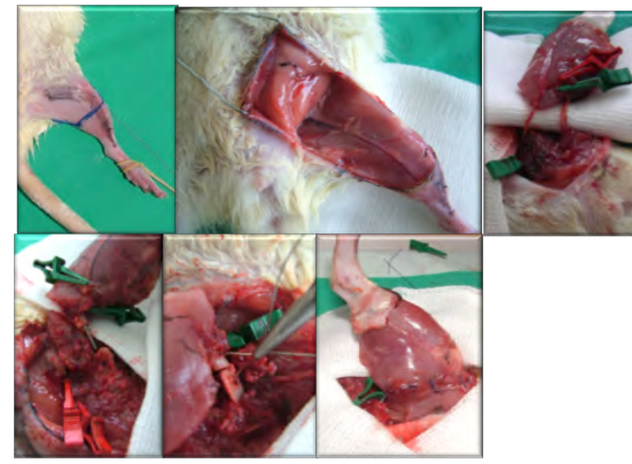


Figure 1. Surgical steps of the first part of the study. Femoral and sciatic nerves remained intact.

Bone fixation, muscle repair, and subsequent anastomosis of femoral arteries and veins were performed; the ischemic period from the amputation to the opening of the arterial clamps after revascularization was 180 minutes. Anastomoses were performed by the same surgeon under operating microscope with a 25x magnification. A total of 8 separate sutures were used in end-to-end fashion with 10/0 Prolene for each vascular anastomosis.

The subjects in the control and study groups were followed for a total of 7 days. 10 mg/kg/day sildenafil citrate was administered to subjects of the study group and 1 cc of saline solution was administered to the subjects of the control group as placebo; both were applied intraperitoneally for a 7 day period (5,6). Pictures of all subjects were taken on the 1st, 3rd, 5th, and 7th days postoperatively and potential necrosis and their levels on replanted legs were detected. Subjects having no ischemia or necrosis on the replanted leg were scored as 0, subjects having partial necrosis were scored as 1, and subjects having total necrosis were scored as 2. The results were statistically evaluated.

For the second part of the study, the aim was to investigate the effects of sildenafil citrate on ischemia-reperfusion damage on the tissue level of the control (n=7) and study (n=7) groups. The right hind legs of subjects from the control and study groups were incompletely amputated at the inguinal level and their femoral arteries, veins, and nerves remained intact. Ischemia was initiated by putting two vascular clamps to each femoral artery and vein during amputation. Subsequently, 10 mg/kg/day sildenafil citrate was administered to the study group and 1 cc saline solution was administered to the control group, intraperitoneally as a single dose. It was performed just after the clamping. At the end of a 180-minute (3-hour) ischemic period, reperfusion was obtained by opening the clamps. Tissue samples were taken from femoral vein and gastrocnemius muscle at the 4th hour of reperfusion for histological and biochemical analyses. The subjects were sacrificed after taking intracardiac blood samples.

The femoral vein and gastrocnemius muscle samples for histological assays were kept in 10% formalin. Direct microscopic and immunohistochemical assays were performed on samples of femoral veins and muscles. For direct microscopic assay, the tissue samples were dyed with Hemotoxylin-Eosin (H&E) and Masson's trichrome stains and muscle samples were scored for necrosis, loss of striation, edema, leukocyte infiltration, and nucleus centralization, indicating the severity of inflammation caused by ischemia-reperfusion (7,8); whereas the femoral vein samples were scored for endothelial damage, tunica media damage, and presence of thrombus in lumens (9-11).

For immunohistochemical analysis the presence of satellite cells indicating muscle regeneration was evaluated by immunohistochemical marking of PCNA (proliferating cell nuclear antigen) protein in muscle tissue samples. Furthermore, angiopoietin (Ang-1) and vascular endothelial growth factor (VEGF) immune dyes were applied to femoral vein tissue samples (12,13) to interpret endothelial cell survival and angiogenesis. Catalase levels were analyzed with ELISA in blood samples.

All data obtained from measurements and scorings were evaluated with MINITAB 14. For all variables, the Shapiro Wilk Normality Test was performed. Since the variables of the study were not normally distributed, the Kruskal-Wallis Test was used for evaluation. For the variables differing between the groups, the Hollander Wolfe Multiple Comparisons Test was applied.

Results

Macroscopic Evaluation:

In the first part of the study, four subjects were diagnosed with total hind leg necrosis and two subjects were diagnosed with partial necrosis in the 7th day results of the control group. The leg of one control subject was completely healthy. No necrosis or ischemia finding was detected in the study group at the end of the follow up (Figure 2, Figure 3).

The results of the study group was statistically and significantly different compared to the control group (p<0.05).



Figure 2. Macroscopic view of the control group.

Histological Evaluation:

When the histological scoring of muscle tissue samples were statistically evaluated; the variables of necrosis, loss of striation, and leukocyte infiltration were significantly lower in the study group (p<0.001, p<0.05, and p<0.05 respectively). Nucleus centralization was not extensive in either group and there was no difference between groups in terms of edema and nucleus centralization variables (Figure 4,5).

When the histological scoring of vein tissue samples were statistically evaluated, tunica media damage was significantly higher in the control group compared to the study group (p<0.05). Although endothelial damage and the presence of intraluminal thrombus was higher in the control group than the study group, the difference was statistically insignificant.

Immunohistochemical Evaluation:

For PCNA immune-staining of gastrocnemius muscle tissues a low number of PCNA (+) cells was detected in the control group. On the other hand, a high number of PCNA (+) cells was detected in the study group, statistically different from the control group (p<0.05).

For Ang-1 immune-staining of femoral vein samples, a slight staining was detected in the control group, while there was moderate level of staining in the study group, a statistically significant difference (p<0.05) (Figure 6 A-F).

For VEGF immune-staining of femoral vein samples, moderate



Figure 3. Macroscopic view of the sildenafil citrate group.

Parameters	Control	Study	
Necrosis	++++++	+	p< 0.001
Striation loss	+++++	+	p<0.05
Nukleus centralization	+	++	p˃ 0.05
Leukocyte infiltration	+++++	+	p<0.05
Edema	+	++	p˃0.05

Figure 4. Histologic parameters of the muscle tissue after replantation.

level of staining was detected in all groups and there was no difference between the groups (Figure 7 A-C).

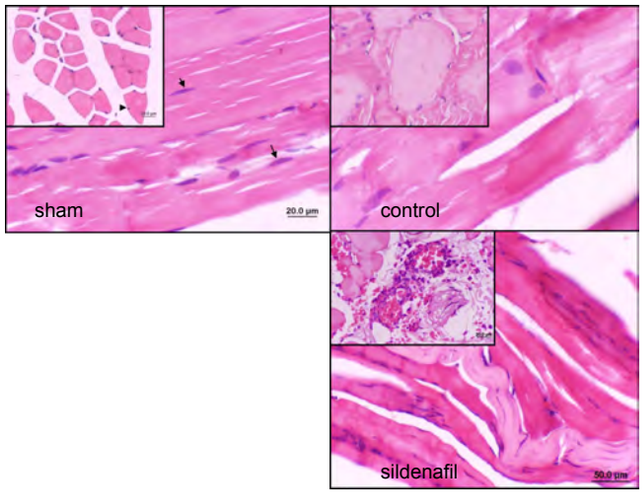


Figure 5. Microscopic image of the striation loss of the groups

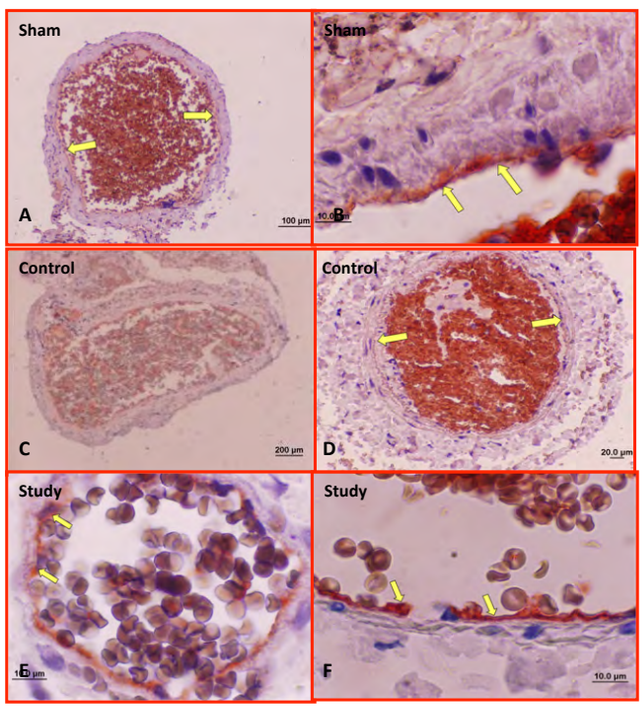


Figure 6. Ang-1 for the sham group applied light microscopic image of the femoral vein. Faint staining of endothelial cells (arrow) (bar: A:100 µm, B: 10,0 µm) (Ang-1). C,D: Ang-1 for the control group, light microscopy image of the femoral vein. Faint staining of endothelial cells (arrow) (bar: C: 200µm, D: 20.0µm) (Ang-1). E,F: Ang-1 for the study group applied light microscopic image of the femoral vein. Intense staining of endothelial cells (arrow) (bar: 10.0µm) (Ang-1) (A,B).

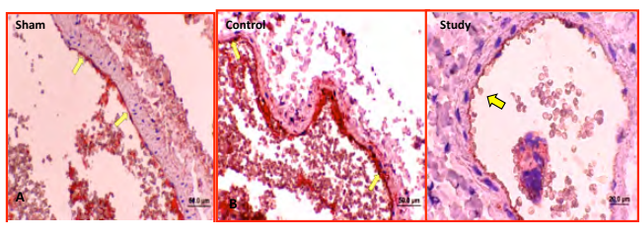


Figure 7. Light microscopic image of the femoral vein of the VEGF in the sham group. Faint staining of endothelial cells (arrow) (bar: 50.0µm) (VEGF)(A), Light microscopic image of the femoral vein of the control group, faint staining of endothelial cells (arrow) (VEGF). (bar: 20.0µm)(B), Light microscopic image of the femoral vein of the study group of VEGF. Faint staining of endothelial cells (arrow) (bar: 20.0µm) (VEGF)(C).

Biochemical Evaluation:

Catalase level was significantly higher in blood samples of the study group compared to other groups ($p<0.05$).

Discussion

The rat hind leg replantation model was used in this study since numerous research studies have been performed with this model and its anatomy is well-known. The ischemia time changes are reported as between 2-6 hours for hind leg ischemia-reperfusion models in the literature (14,15). After two hours of ischemia, minimal ultrastructural damages occur. In order to see histological changes, 4 hours of ischemia is necessary. Irreversible muscle damage occurs after 6 hours of ischemia. In our study, the critical ischemia time was determined to be 3 hours, both to observe some histologic changes and to see if they are effected in some way from sildenafil. When the macroscopic results of the study were evaluated, it was thought that sildenafil citrate significantly enhanced survival. Because all of the surgical procedure was performed by the same surgeon, the difference between groups cannot be attributed to the surgeon's skills; the survival success in the study group may be attributed to effects of sildenafil citrate at both the vascular and tissue levels. Vascular smooth muscle is under the control of endothelial nitric oxide (NO) which is synthesized by NO synthase. NO's main action is to activate the soluble guanylyl cyclase to produce cGMP. A very potent inhibitor of PDE-5, sildenafil citrate, blocks the degradation of cGMP to 5'GMP. The amplification of cGMP signaling enables relaxation of vascular smooth muscle activity by causing relaxation of smooth muscle cells through potassium (K+) channels. However, PDE-5 selectively hydrolyses cGMP and causes vascular resistance. PDE-5 is mostly present in vascular smooth muscle, intestine, heart, platelets, placenta, and chondrocytes, making them putative targets for sildenafil (1). The number one factor in the success of microsurgery is the prevention of vasoconstriction which is induced by several factors such as hypothermia, blood loss, and desiccation of the operative field. Sildenafil is thought to relieve the vasoconstriction by potentiating the cGMP concentration.

According to the histological evaluation results, sildenafil citrate significantly reduced the leukocyte infiltration, loss of striation, and the necrosis ratio in the ischemic hind legs of the subjects, whereas it did not show any difference regarding oedema and nucleus centralization. Cellular or intercellular oedema, inflammatory infiltration, and alterations in striation bands are some of the histopathological changes of muscle in response to ischemia. According to Akar et al., while striation loss is a minor and initial change after ischemia, necrosis and nucleus centralization represent a more serious and advanced injury (7). In contrast, Zhang et al. consider loss of striation in muscle to be irreversible muscle damage by (16). In any case, sildenafil restrained the ischemic damage in the muscle. In many ischemia reperfusion studies, sildenafil was demonstrated to attenuate the injury by decreasing leukocyte infiltration (8,17).

The major cause of replant loss after replantation is thrombus formation at the anastomosis. Veins are more susceptible to thrombus formation after microsurgery because the flow is slower. Endothelial damage is an initiator factor in thrombus formation. Regardless of the initial stimulus, suppressing the

platelet activation may inhibit thrombus formation. Platelets contain three types of PDE isoenzymes: PDE2, PDE3, and PDE5. Platelet PDE5 is inhibited by sildenafil but the Gresele at al. study was unable to prove that sildenafil can inhibit collagen-induced thrombosis alone (3). But, Berkels et al. showed that sildenafil significantly prolonged bleeding time in healthy men (18). Based on the results of the first phase of the study, we were expecting to see statistical difference on the femoral vein samples between the control and study groups with regard to luminal thrombus presence and endothelial damage. Thrombus, endothelium damage, and tunica media damage were detected in the control group in the histological evaluation of the femoral vein samples. In contrast, a minimum level of endothelium and tunica media damage was present in the study group, while no thrombus was detected. Sildenafil's effects on platelet aggregation is not yet clearly defined in the literature, but we believe that its ability to augment nitric oxide synthase levels may account for inhibition of thrombus formation by increasing the amount of a strong antiaggregant, nitric oxide (19).

The satellite cells are located under the basal lamina and are in charge of creating new muscle fibers in response to any damage. They proliferate after acute injury and repair the muscular tissue considerably. PCNA is used to indicate the activation of satellite cells. PCNA (+) staining was shown to be higher in the study group in immunohistochemical analysis supporting the histological data indicating recovery after injury. Armstrong et al. assessed the apoptosis by evaluating caspase-3 expression in soleus muscle after ischemia reperfusion in the rat hind leg ischemia model. The apoptotic rate in the sildenafil-treated group was significantly lower than the control group after 6 hours ischemia-24 hours reperfusion cycle. From a different perspective, but parallel to our results, they concluded that sildenafil citrate administration after the onset of ischemic injury reduced the cellular damage (17)

Angiogenesis is a process requiring growth factors such as VEGF and angiopoietin that affect the proliferation and differentiation of endothelial cells (20). VEGF is the main factor that initiates the accumulation and proliferation of endothelial cells; it induces endothelial cell mitosis and promotion of capillary sprouting (2,21). The angiopoietins are responsible for the formation of new blood vessels from proliferating endothelial cells as Ang-1 and Ang-2. They function by binding their physiologic cell surface receptors, Tie-1 and Tie-2. Vidalavur et al. demonstrated that sildenafil strongly induced VEGF, Tie-1, and Tie-2 in human coronary arteriolar endothelial cells in vitro and concluded that it is a very potent pro-angiogenic factor (2). Many other studies state that sildenafil citrate significantly induces VEGF and Ang-1 in ischemic myocardium tissue. The neoangiogenetic and angioprotective effects of Ang-1 and VEGF on cardiac tissues are also emphasized (19,22). In a study on Ang-1's effect on neurovascular regeneration after stroke, it is stated that Ang-1 represses perivascular inflammation and decreases ischemia-reperfusion damage by protecting the stability of vascular endothelium and preventing the disruption of permeability (25). In the present study, it was investigated whether sildenafil citrate might induce Ang-1 and VEGF levels in peripheral endothelium, which is similar to myocardial and neural tissues. In the Vidalavur et al. study, sildenafil mediated the tube formation of endothelial cells in low doses, but in higher doses endothelial cells showed more ring-like structures, indicating that sildenafil triggered Ang-1 expression (2).

In our study, through immunohistochemical evaluation Ang-1 protein staining of the study group was found to be significant when compared to the control group. The femoral vein was stained moderately, at the same level, for VEGF immunohistochemically in both groups. But Ang-1 staining was statistically different in the study group than in the control group.. VEGF is known to be up-regulated by tissue hypoxia and it is significantly elevated in ischemic muscles after arterial occlusion. We were unable to show a difference in VEGF levels between ischemic and sildenafil-treated ischemic in femoral vein samples; muscle sample evaluation might deliver different VEGF levels.

Catalase activity is used as a marker of enzymatic defense against reactive oxygen species. Catalase is frequently used by cells to rapidly catalyze the decomposition of harmful hydrogen peroxide into less-reactive gaseous oxygen and water molecules. After ischemia reperfusion injury, the catalase levels are expected to be decreased (24). In the present study, biochemically high level of catalase in the sildenafil-treated ischemic hind leg group demonstrates that sildenafil citrate may have an antioxidant effect. On the other hand, an interesting finding revealed by Urao et al. suggested that endogenous endothelial cell-derived H2O2 plays a critical role in reparative neovascularization in response to ischemia by upregulating adhesion molecules and activating eNOS in endothelial cells and that high levels of catalase are shown to be antiangiogenic by degrading H2O2 (25). Although it is known that an excess amount of H2O2 in pathological conditions has a negative impact on endothelial function, neovascularization and tissue repair, Yun et al. have shown that optimal levels of H2O2 are required for signaling and for normal biological function (26).

In addition, Urao et al. found that VEGF level is markedly reduced in the ischemic tissue in catalase transgenic mice (25). The overexpression of catalase resulted in disorganized microvasculature formation and caused a substantial decrease in the number of microvessels. They explained that endothelial H2O2 can regulate endothelial functions independent of VEGF. It is clear that sildenafil treatment did not affect VEGF levels. Either the increased levels of catalase caused by sildenafil suppress VEGF or another mechanism is responsible for static VEGF levels despite an increase in Ang-1.

In conclusion, our study demonstrated that sildenafil citrate has significantly increased the survival of the replant in the ischemic rat hind leg replantation model. The mechanism of increased hind leg survival by sildenafil may mainly rely on its vasodilatory effect provided by smooth muscle relaxation or partly on the protective effect against ischemic reperfusion injury and antioxidant activity. The present study was unable to show any antithrombotic activity of sildenafil. Further investigation of this important topic with a larger number of subjects is necessary because the c-GMP-NO pathway is a critical intracellular inhibitory messenger that interferes with platelet activation signaling.

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Competing interests

The authors declare that they have no competing interests.

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