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ORIGINAL ARTICLE

Microcirculatory effects of sildenafil in experimental testicular torsion in rats

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Abstract

Purpose Investigate the short-term effect of sildenafil on microcirculation, especially the velocity, the pattern of the flow and the recruitment of the leukocyte in postcapillaries.

Methods In male Sprague–Dawley rats, the microcirculatory consequences of 60 min experimental testicular torsion, followed by 240 min of reperfusion, were examined. Using fluorescence intravital microscopy, changes in red blood cell velocity in post-capillary venules and rolling as well as adhesion of leukocytes in the postcapillary venules were examined before the torsion and every hour during the reperfusion period. Sildenafil was given 10 min prior to reperfusion (iv 0.7 mg/kg, $n=6$), while control animals received saline vehicle ($n=5$).

Results The characteristic flow motion disappeared in the affected testicular during the torsion. Red blood cell velocity values were dramatically decreased (by > 50%) and both rolling and adhesion of leukocytes increased during the reperfusion phase. Sildenafil treatment resulted in significantly higher red blood cell velocity values during the entire reperfusion period, but exerted only a temporary positive effect on the plost-ischaemic leukocyte–endothelial interactions.

Conclusions Intraoperative administration of sildenafil during surgical detorsion may provide marked testicular microperfusion benefits, but failed to influence the overall leukocyte-driven microcirculatory inflammatory reactions.

Keywords Phosphodiesterase-5-inhibitor (PDE-5 inhibitor) · Testicle · Testicle torsion · Microcirculation · Sildenafil · Rat

Introduction

Testicular torsion (TT) is one of the most common indications for emergency surgery amongst children, adolescents and young adults. The prevalence of torsion may affect 3.8–4.5 men out of 100,000 who are admitted to hospital with sudden testicular pain [1, 2]. Prompt diagnosis and treatment are essential, as cell-hypoxia-induced damage increases over time [2–4].

Successful surgical intervention can be achieved in 90–100% of cases, if performed within 4–8 h from the onset of complaints; however, the overall success rate decreases drastically over time (within 12 h 50%, and 24 only 10%) [5]. Significant microcirculation impairment and permanent hypoxia can result in irreversible damage, starting from the sixth hour of the pain onset [2]. As described previously by Gandhi et al., administration of sildenafil can improve the microcirculation of the testicle by limiting hypoxia-related irreversible cell death, improving surgical outcomes. Furthermore, the ischaemia/reperfusion (I/R) injury of the testicle can also be directly decreased through the simultaneous administration of antioxidants [6].

Testicular damage (during torsion/detorision) can arise from two separate mechanisms. Firstly, torsion of the spermatic cord can dramatically reduce or stop testicular blood in-flow, causing permanent hypoxaemia and subsequent cell death. After the spermatic cord obstruction, the tissue will not be able to receive any oxygen. So in these tissues the hypoxia inflicts a reduced level of ATP. Without the ATP, the indispensable processes will not able to work, so elicit

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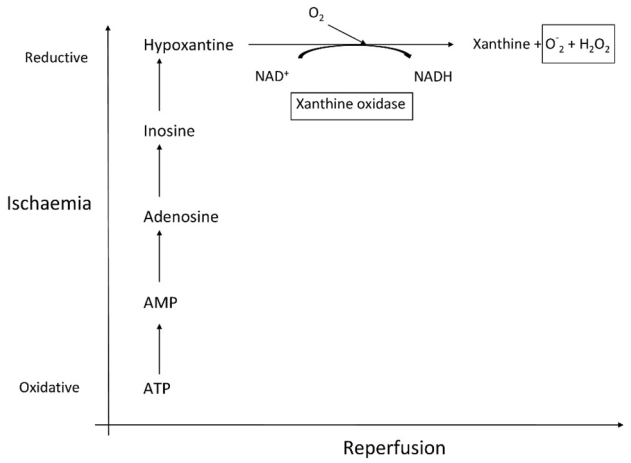

Fig. 1 The mechanisms of ROS production by xanthine oxidase in tissues exposed to ischemia and reperfusion. At the time of ischemia, ATP is transformed to hypoxantine. Then, at reperfusion, xanthine oxidase catalysis hypoxanthine and the restored tissue O_2 to both superoxide (O_2^-) and hydrogen peroxide (H_2O_2) [26]

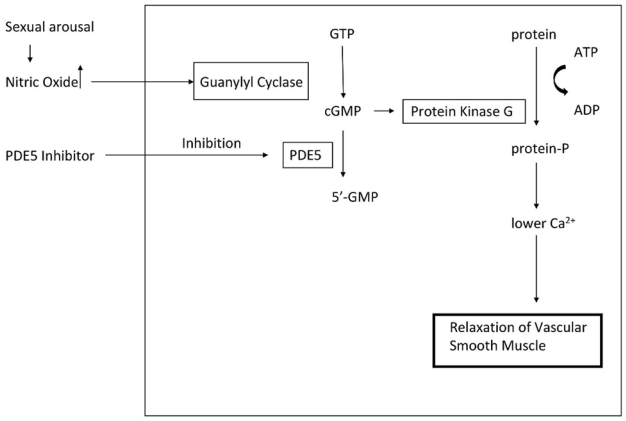

Fig. 2 The mechanism of PDE5 inhibitor. Sexual arousal causes enhanced level of NO, which stimulate the guanylyl cyclase to convert GTP to cGMP. PDE5 inhibitor, inhibit the PDE5 enzyme, which is responsible for the catabolism of cGMP to 5′-GMP. The increased level of cGMP activates the protein kinase G, which decreases the Ca^{2+} level. As a result of this, smooth muscle relaxation and subsequently erection are formed

a cell death. It has long been known that the length of the hypoxaemia and the seriousness of the cell damage change in inverse proportion [7]. Secondly, reactive oxygen species (ROS) can cause deterioration after reperfusion (reperfusion damage). Right after the blood flow reestablishment, the ROS formation was started (Fig. 1). At a relevant concentration of ROS, it can cause the damage in the post-ischaemic tissues including endothelial barrier dysfunction, increased expression of endothelial cell adhesion molecules, enhanced leukocyte–endothelial cell adhesion and increased production of inflammatory mediators (e.g., platelet activating factor) [7, 8]. Nicotinamide adenine dinucleotide phosphate (NADPH) is the key enzyme catalyzing the formation of ROS and it is released by leukocytes [9]. The endothelial cell (EC) monolayer simulating various microvascular alteration, including an enhanced ROS production, increases the leukocyte adhesivity to the EC and damages the barrier function of the EC [7].

Microcirculation monitoring is generally performed by Intravital Video Microscope (IVM), in vitro, and Orthogonal Polarisation Spectral Imaging can be applied, in vivo, as an alternative option [10].

In our current study, we aimed to examine the effect of sildenafil (Fig. 2) on microcirculation by measuring the recruitment of the leukocyte and red blood cell velocity (RBCV), and the pattern of the flow in post-capillaries examined with IVM, before and after I/R damage in rat testicles.

Materials and methods

In our current study, post-capillary RBCV, flow pattern and leukocyte recruitment have been analyzed in rat testicles, after I/R damage. Inflammatory markers and oxidative stress parameters were not been examined because they have been already investigated in other studies.

The appropriate National Institutes of Health Guidelines were applied which are approved by the Animal Welfare Committee of University of Szeged [11].

Study groups

60 min TT was performed in male Sprague–Dawley rats (weight 250 ± 10 g, $n = 11$), randomized into two subgroups. Sildenafil was administered, in a single dose of 0.7 mg/kg (dissolved in 1 ml/kg saline, iv), 10 min prior to reperfusion (torsion + SIL group, $n = 5$), while control animals received saline vehicle (1 ml/kg saline iv + torsion, $n = 6$). The testicular microcirculation was monitored by fluorescence IVM (Figs. 3, 4) before the experimental torsion, right after, and hourly, during the 4 h reperfusion period.

Surgical exposure

Sodium pentobarbital (45 mg/kg) was introduced intraperitoneally for initiating anesthesia. Then, the trachea was isolated and cannulated (for intubation, maintaining the homeostatic body oxygen level). Jugular vein was also isolated and cannulated (infusing Ringer lactate at the rate of

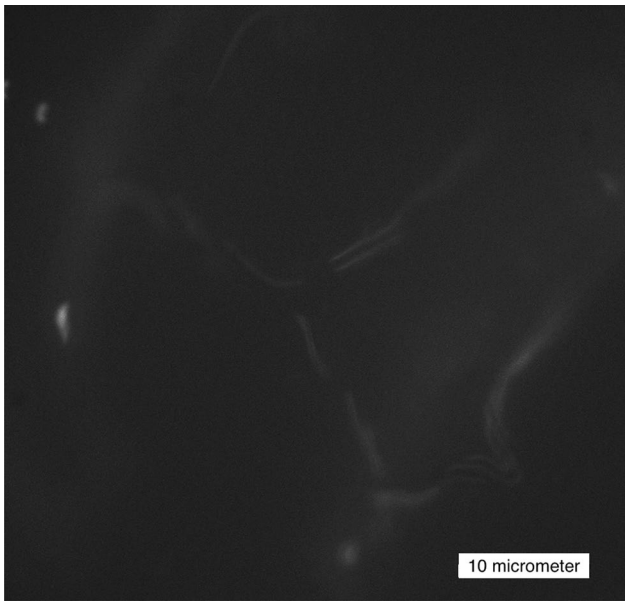

Fig. 3 Isothiocyanate labelled red blood cells detected by IVM

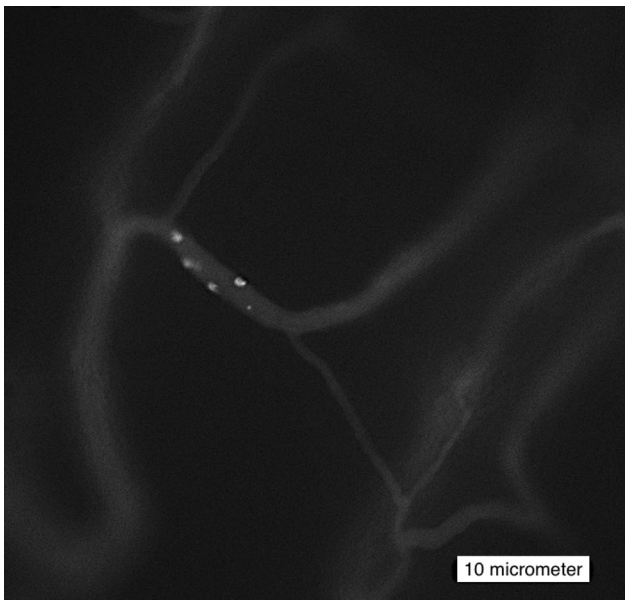

Fig. 4 Rhodamine-6G labelled leukocytes detected by IVM

10 ml/kg/h, maintaining homeostatic volume stability, and administering pentobarbital at a rate of 5 mg/kg, maintaining the anesthesia and allowing sildenafil or saline injection of the appropriate groups). The carotid artery was isolated for central blood-pressure monitoring (Experiment Ltd., Budapest, Hungary). Thereafter, testicular isolation was carried out by lateral incision and weaning from the epididymis preserving the vessels, tunica albuginea and spermatic cord. Finally, to mimic testicular torsion, the testicle was twisted 720° clockwise, maintaining the hypoxia for 60 min.

Testicular microcirculation monitoring

After the 60 min of torsion, fluorescein isothiocyanate-labeled erythrocytes (0.2 ml intravenously, Sigma–Aldrich) staining red blood cell and rhodamine-6G (0.2% in 0.1 ml iv, Sigma–Aldrich) staining leukocytes were injected. Thereafter, the testicles were isolated and examined by Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, and Acroplan 20× water immersion objective (Carl Zeiss GmbH, Jena, Germany), and recorded by charge-coupled device video camera (AVT HORN-BC 12, Aalen, Germany) connected to a personal computer [11]. A minimum of five post-capillary venules was analyzed in each subject, in order to evaluate RBCV and the presence of the pulsatile pattern and leukocyte–endothelial cell interactions (rolling and adherence of leukocytes). Leukocytes rolling was defined as being at least 40% slower compared to red blood cell movement. Leukocyte adherence was defined as attachment to the vessel wall for at least 30 s.

Statistical analysis

Differences between groups were compared by two-way ANOVA test. All statistical analyses were performed with SigmaStat® for Windows® (Jandel Corporation, San Rafael, CA, USA).

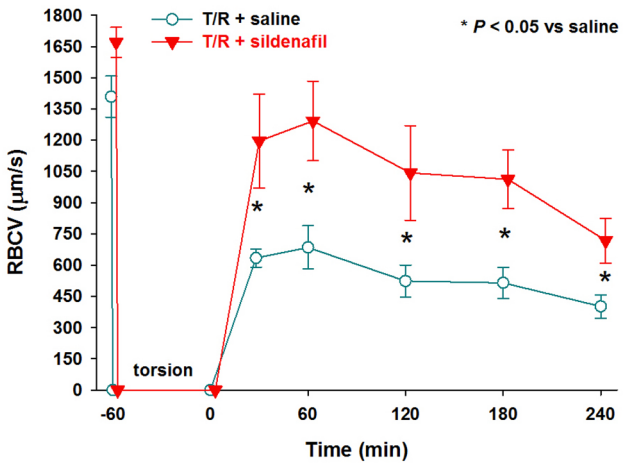

Fig. 5 Effect of sildenafil on the testicular torsion-induced deterioration of the RBCV during reperfusion (*$p < 0.05$, two-way ANOVA test)

Results

Red blood cell

Testicular torsion resulted in a complete cessation of microcirculatory perfusion in the testicular capillaries (Fig. 5). Reperfusion was associated with marked deterioration of RBCV (all of the post-ischaemic values were lower than those of baseline, in both groups, not shown) ($p < 0.05$, measured by two-way ANOVA test). After detorsion, the blood flow came back in a pulsatile pattern in the sildenafil-treated group. The RBCV was measured in the high flow periods, when the pulsatile pattern could be observed. Sildenafil treatment resulted in significant elevation of RBCV data every hour (as compared to saline) ($p < 0.05$, measured by two-way ANOVA test), but values did not regain the original velocity baseline.

Leukocyte rolling and adherence

There was a marked increase during reperfusion in both leukocyte rolling (Fig. 6) and adherence (Fig. 7) in the testicular post-capillary values. Sildenafil exerted only a temporary positive effect at the onset of reperfusion. In the sildenafil-treated group, rolling leukocytes only in the 30th minute and the adherent leukocytes only in the 60th minute showed significantly better results when compared to the saline group. These results do not show us significantly better results in overall.

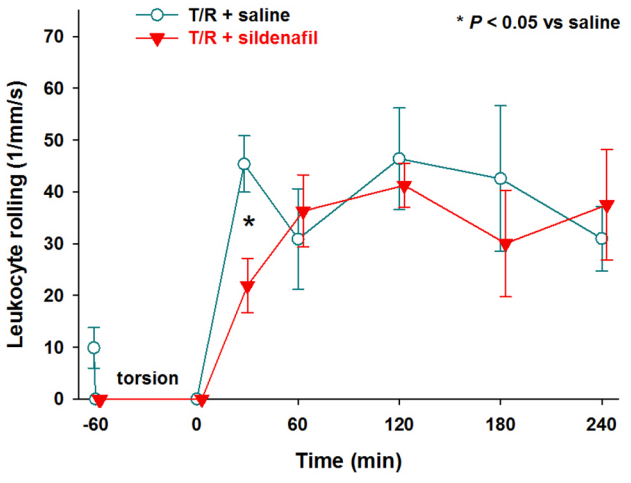

Fig. 6 Effect of sildenafill on the testicular torsion-induced leukocyte rolling formation during reperfusion (*$p < 0.05$, two-way ANOVA test)

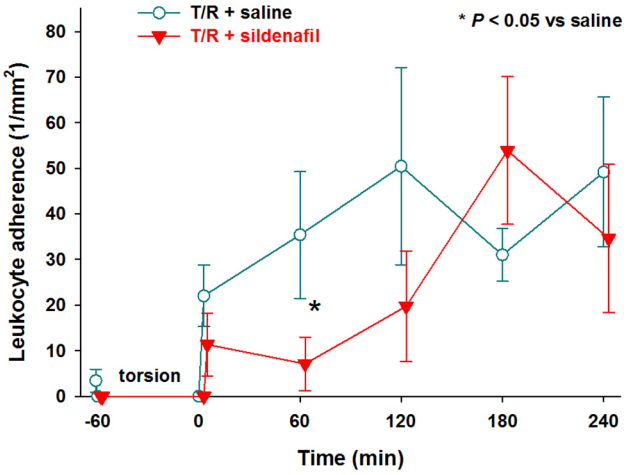

Fig. 7 Temporary positive effect of sildenafil on the testicular torsion-induced increase in the sticking form of leukocyte-endothelial interactions during reperfusion (*$p < 0.05$, two-way ANOVA test)

Discussion

In this current study, we examined the effect of sildenafil on microcirculation (Figs. 5, 6, 7) during and after I/R injury in rat testicles.

The major findings were as follows:

- Sildenafil significantly improves the microcirculatory blood flow.
- Sildenafil has minor effect on leukocytes recruitment or the inflammatory cascade.

Clinical diagnosis of testicular torsion is mainly driven by accurate anamnesis (sudden and severe pain located in the testicle); however, physical examination and confirmation of impaired blood flow with Doppler ultrasound are also essential [3]. Physical examination usually applied as rotating the testicle to the correct position, manually, from the inside to outside (testicles are most often torquated medially); however, one-third of cases are twisted laterally [9] requiring the opposite of the aforementioned approach. In the case of manual detorsion failure, acute surgery should be performed as appropriate to rotate the testicle to the correct position restoring arterial flow. Reperfusion damage, however, can occur, which may cause further tissue impairment. Leukocytes are essential in the reperfusion, as they release NADPH oxidase, catalyzing the molecular oxygen transformation to ROS (Fig. 1) with subsequent cell death [12–15]. Infertility and azoospermia my occur in the long term [16], mainly driven by impaired sperm cell morphology and the presence of anti-sperm antibodies (not sperm cell motility abnormalities) after surgery [16].

The beneficial effects of sildenafil have been proven after I/R or other type of injury in different organs.

In a recent study, Behmengurt et al. investigated, in rats, the acute cardioprotective effect of a phosphodiesterase 5 (PDE5) inhibitor by activating the mitochondrial large-conductance Ca^{2+}-sensitive potassium channels. They used 30 min of ischaemia, then 60 min of reperfusion. They introduced the sildenafil 10 min prior to ischaemia. In their control animals, infarct size was $52 \pm 8\%$, compared to the sildenafil-treated group where the infarct size decreased to $35 \pm 6\%$ ($p < 0.05$ vs. control) [17].

Ockaili et al. reported decreased area of infarcted myocardium after myocardial infarction. They introduced the PDE5 inhibitor (0.7 mg/kg, iv) 30 min prior to 30 min of heart ischeamia and 3 h of reperfusion, in rabbits. In the control group, the infarct size was $33.8 \pm 1.7\%$; this reduced to $10.8 \pm 0.9\%$ a (68% reduction) ($p < 0.05$) [18].

Kolettis et al. investigated sildenafil dosages [low dose (0.7 mg/kg)], (high dose (1.4 mg/kg)) on an I/R (20 min/45 min) injured heart. They examined the following parameters: post-ischaemic recovery and hypercontracture. Low dose sildenafil showed better results ($75.1 \pm 2.4\%$, $p = 0.0069$), compared to both the control group ($62.9 \pm 2\%$) and the high dose sildenafil group ($69.1 \pm 2.1\%$), in post-ischaemic recovery, and found no difference in hypercontracture between the groups [19].

High doses of PDE5 inhibitor diminish the mucosal lesions on indomethacin-pretreated rats stomach. In the study mentioned above, a group of rats received 2 mg/kg (PDE5I-2), and another group received 10 mg/kg (PDE5I-10) PDE5 inhibitor. The gastric mucosal lesions (GML) count and area were examined. In the PDE5I-10 group, the GML count was significantly lower ($1.25 \pm 1.38\%$, $p < 0.05$) compared to the control group ($6.25 \pm 3.49\%$, $p < 0.05$) and the GML area was significantly lower as well (0.75 ± 0.88–$21 \pm 12.35\%$, $p < 0.001$) [20].

In testicle, similarly good results are achieved.

Zavras et al. examined the effect of erythropoietin (EPO) (1000 IU/kg) and sildenafil (0.7 mg/kg), compared to the control group which received no drugs. In the 60th minute of ischaemia, they received the drugs inraperitoneally. 30 min later, the ischaemia stopped. After 24 h of reperfusion, they carried out histopathological examination on the testicles. Both the EPO (main grade: 3.24, range: 3.05–3.45) and the sildenafil group (mean grade: 2.69, range: 2.4–2.9) had significantly better results than the control group (mean grade: 3.81, range: 3.65–4, $p = 0.0002$ and $p = 0.00000009$), but the sildenafil group had significantly better results compared with the EPO-treated group ($p = 0.0002$) [21].

In another study, Beheshtian et al. examined laboratory parameters—after introducing sildenafil to rats in the 30th min, intraperitoneally, (total of 60 min of torsion)—after 4 h of reperfusion and the germ cell apoptosis after 24 h of reperfusion. The group which received sildenafil had significantly better results compared to the group which did not receive the drug (MDA: 148.81 ± 17.97–169.69 ± 14.66, $p < 0.05$, CAT: 299.46 ± 37.11–235.85 ± 24.09, $p < 0.05$, SOD: 1797.34 ± 126.05–1505.58 ± 154.44, $p < 0.05$, apoptotic nuclei: 4.83 ± 2.96–9.25 ± 3.26, $p < 0.05$) [22].

Yildiz et al. investigated the effect of double doses (1.4 mg/kg) of sildenafil after I/R injury in the testicle. In their study, they induce 2 h of torsion, then 2 h of reperfusion. In the 60th minute of torsion, they introduce the sildenafil (0.7 mg/kg and 1.4 mg/kg). After the second hour of reperfusion, they examined the laboratory parameters [23, 24]. They found significantly better results in the group where the rats received 0.7 mg/ml sildenafil (group 3) compared to the groups where the rats received 1.4 mg/ml sildenafil (group 4) and the control group (group 2) (GSH (μmol/g protein): 1.26 ± 0.42 (group 3) -1.01 ± 0.1 (group 4) -1.03 ± 0.15 (group 2) $p < 0.05$, MDA (μmol/g protein): 0.64 ± 0.04 (group 3) -0.77 ± 0.12 (group 4), 0.84 ± 0.02 (group 2) $p < 0.01$, NO (μmol/g tissue) 31.03 ± 0.46 (group 3) -33.51 ± 0.27 (group 4) -33.48 ± 0.61 (group 2) $p < 0.01$) [24].

Istanbulluoglu et al. examined orally administered PDA5-Inhibitor (vardenafil), in pigs, after I/R injury of their testicle. They created 2 h of torsion. 45 min prior to the end of the torsion, they introduced the vardenafil (0.4 mg/kg) orally. After 8 h of reperfusion, they investigated the germ cell apoptosis through the apoptosis protease-activating factor (APAF-1) in the testicles. They found no significantly better results between the vardenafil-treated group (VTG) compared to the control group (CG) (APAF-1: 10 ± 4.08 (VTG)–8 ± 2.44 (CG), $p > 0.05$) [25]. These results may be due to the small amount of the PDE5 inhibitor used.

All the above-mentioned articles prove the significantly good effect of the sildenafil on testilculars after I/R damage, even though the ischaemia then the reperfusion cause a huge damage to the tissue.

In our study, significantly better results were obtained in the sildenafil-treated group, compared to the control group, in microcirculatory parameters and in the blood flow pattern ($p < 0.05$, Fig. 5) after 1 h of torsion. Minor significant differences were found between the groups in the number of leukocytes (Figs. 6, 7), which means that the sildenafil had no overall effect on the acute inflammation of the reperfusion phase.

Our study has several limitations. The number of rats included was relatively small and long-term follow-up did not occur. Oral administration of the sildenafil, which would be more true-to-life, was not tried. One study was found where the authors tried orally administrated PDE5 inhibitor, unsuccessfully, since the dose was insufficient. Further study should be carried out on larger sample sizes and using different methods of administration of drugs. Sildenafil was

used in Sprague–Dawley rats; however, the mechanism of action of sildenafil in microcirculation changes in humans with TT is still not clearly understood.

Conclusion

Intravenous sildenafil administration improves microcirculation in the testicle, providing significantly lower ischaemic burden on testicular cells and improves short- and long-term surgical outcomes. Sildenafil has a minor influence on leukocyte-driven microcirculatory inflammatory reactions in the acute phase of the reperfusion state.

Author contributions MO Data collection, data analysis, manuscript writing, AS Project development, manuscript editing, ÁMF Data analysis, GD Data analysis, ZB Project development, manuscript editing

Compliance with ethical standards

Disclosure of potential conflicts of interest M Oroszi: Nothing to disclose, A Szabó: Nothing to disclose, ÁM Fehér: Nothing to disclose, G Deák: Nothing to disclose, Z Bajory: Pfizer Compound Transfer Program supported for us the substance (just the substance) for experiment for free.

Research involving human participants and/or animal Our research involves just rats (Sprague–Dawley). It was animal experiments.

Informed consent We applied the appropriate National Institutes of Health Guidelines approved by the Animal Welfare Committee of University of Szeged.

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