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Sildenafil Postconditioning in a Rat Model of Ventricular Fibrillation/ Resuscitation

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Abstract

Objective: To evaluate the multi-organ postconditioning potential of sildenafil during resuscitation. Circulatory arrest/resuscitation induces ischemia/reperfusion (I/R) injury in all organs. I/R injury can be reduced using postconditioning during reperfusion. Sildenafil has proven strong single-organ postconditioning properties.

Methods: Ventricular fibrillation (VF) was induced and left untreated for 6 min in anesthetized adult male Wistar rats. During resuscitation, placebo (n=10) or intravenous sildenafil 0.2 mg/kg (n=10) was administered. Troponin-i release, lactate release, blood gases and hemodynamic parameters were assessed. After 3 h of reperfusion, rats were euthanized; brain and heart were removed for infarct staining with triphenyltetrazolium chloride. Urinary kidney injury molecule (KIM-1) was assessed. Data expressed as median (interquartile range), $p<0.05$ significant.

Results: Resuscitation/defibrillation resulted in return of spontaneous circulation in all rats. Compared with the sildenafil group, the control group showed higher overall troponin release (Control 50 (32-60) *versus* Sildenafil 16 (12-42) h·microg/L, p=0.032) and higher left ventricular infarct percentage (Control 19 (16-24) *versus* Sildenafil 16 (12-18)%, p=0.039). There was no significant difference between the groups with respect to mortality, hemodynamic recovery, cerebral cortex infarct percentage, lactate release, blood gas values and urinary KIM-1 release. Three rats in the sildenafil group developed pulmonary edema *versus* none in the control group.

Conclusions: Sildenafil postconditioning during resuscitation significantly reduces cardiac injury but does not affect mortality, cerebral and renal injury after resuscitation.

Keywords: Circulatory arrest; Ventricular fibrillation; Resuscitation; Postconditioning

Abbreviations: ANOVA: Analysis of Variance; AUC: Area Under the Release Curve; CPR: Cardiopulmonary Resuscitation; ECG: Electrocardiogram; IQR: Interquartile Range; I/R: Ischemia/ Reperfusion; KIM-1: Kidney Injury Molecule-1; LV: Left Ventricle/ Ventricular; NaCl: Sodium Chloride; PEEP: Positive End-Expiratory Pressure; ROSC: Return of Spontaneous Circulation; RV: Right Ventricle/Ventricular; TTC: Triphenyltetrazolium Chloride; VF: Ventricular Fibrillation.

Introduction

Sudden cardiac arrest is a frequent cause of death and results in significant sequelae in survivors. During cardiac arrest, ischemia of all organs occurs, followed by reperfusion in case of successful resuscitation. All organ systems are subject to hypoxia during circulatory arrest, but some organs such as the heart and brain are more susceptible to injury than others and their dysfunction frequently results in death or significant impairment, even after successful resuscitation [1].

I/R injury can be reduced using preconditioning [2] and postconditioning [3], by applying various stimuli either before or immediately after the ischemic event, respectively. Postconditioning [3] could offer wide clinical applications since unexpected I/R injury can be mitigated using postconditioning triggers. Sildenafil is one of the most well-known postconditioning triggers. In single-organ animal studies, sildenafil has been shown to strongly protect against I/R injury in organs such as the heart [4-9], brain [10] and kidney [11]. The protective effects of sildenafil are explained by the fact that sildenafil is a phosphodiesterase-5 inhibitor [12] and thus reduces cGMP breakdown, increasing intracellular cGMP to cardioprotective levels [6]. Sildenafil also opens the mitochondrial ATP-sensitive K+ channel [8,9], which is an important step in the protective molecular signaling cascade [8].

Recently, sildenafil preconditioning in piglets before induction of VF, reduced post-resuscitation cardiac dysfunction and improved cardiac metabolism [13,14]. However, the postconditioning effects of sildenafil on the heart, brain and kidney after resuscitation have never been explored. Given the amount of I/R injury in the whole body after resuscitation and the promising results of single-organ postconditioning with sildenafil, we set out to evaluate the postconditioning potential of sildenafil to reduce I/R injury in multiple organs in a rat model of ventricular fibrillation/resuscitation.

Methods

Animals and ethical committee approval

Twenty male Wistar rats were allocated either to a control (n=10, placebo) or an intervention group (n=10, sildenafil postconditioning). The research protocol was approved by the Maastricht University Animal Ethics Committee (project DEC 2010-166), and performed in accordance with institutional and national guidelines.

Animal preparation

The rat ventricular fibrillation/resuscitation model was based on several published rat resuscitation models from other research groups [15-18]. Buprenorphine (0.02 mg/kg) was administered subcutaneously as analgesic and pentobarbital (60 mg/kg) was administered intraperitonially for induction of anesthesia. Core temperature was measured rectally and maintained at 37.0°C by a servo-controlled heating plate and lamp. All surgical procedures were performed under aseptic conditions. Tracheostomy was performed and all rats were mechanically ventilated (neonatal ventilator Babylog 9000, Dräger, Telford, PA, USA) with humidified 37.0°C air (60 breaths/min, positive end-expiratory pressure PEEP 0 mbar, FiO_2 0.40, tidal volume initially 3.5 ml with peak inspiratory pressure adjusted to maintain normocapnia). The right femoral vein and artery were cannulated for administration of fluids/drugs, and for blood pressure measurement/blood sampling, respectively. Arterial infusion of heparin (1 U/ml in 0.9% saline, 1 ml/h) and venous infusion of saline (NaCl 0.9% 1 ml/h) and pentobarbital (4 mg/kg/h) were administered continuously. The left superior caval vein was exposed and cannulated with a bipolar 4F pacing lead (Pacel, St. Jude Medical, St. Paul, MN, USA), which was advanced into the coronary sinus [19]. In rats, the left superior caval vein drains into the coronary sinus to reach the right atrium, enabling electrical stimulation close to the LV to induce ventricular fibrillation (VF). Electrocardiography (ECG) was performed by placing 4 limb needle electrodes.

Induction of ventricular fibrillation, resuscitation, reperfusion

Intravenous vecuronium bromide (0.3 mg/kg) was given in order to suppress muscle tremor during electrical stimulation. Mechanical ventilation and pentobarbital maintenance were interrupted, and VF was induced with 50 Hz, block wave current of 9V (Philips PM 5132 Function Generator, Philips Radios, Germany) on the bipolar pacing lead in the coronary sinus. Circulatory arrest was confirmed by a sudden arterial blood pressure drop to <15 mmHg. After one minute of stimulation at 9V, the current was reduced to 6V for one min, followed by one minute of stimulation at 3V, to be stopped after a total of 3 min of electrical stimulation. Three minutes of stimulation were necessary in rats to prevent spontaneous conversion to sinus rhythm, as described previously in rat VF models [16]. VF was confirmed on the ECG after cessation of external electrical stimulation. VF was left untreated and continued for another 3 min, making total circulatory arrest time 6 min, after which cardiopulmonary resuscitation (CPR) was started. Mechanical ventilation was resumed (5 ml tidal volume, FiO_2 1.00), manual chest compressions were administered at 200/min, and 20 microg/kg adrenaline was administered intravenously. After one minute of CPR the rats were defibrillated with 5J using infantile internal defibrillation paddles placed on the lateral external chest walls (Lifepak 9 Physio Control, Redmond, WA, USA). If VF persisted, CPR was continued for another minute before the next defibrillation attempt. This was repeated during maximum 5 min. When diastolic blood pressure during CPR was <40 mmHg, an additional intravenous 10 microg/kg adrenalin bolus was administered. When diastolic blood pressure after return of spontaneous circulation (ROSC) remained <50 mmHg, continuous intravenous adrenaline perfusion (1.6 microg/kg/minute) was started, the dose of which was increased when diastolic blood pressure remained <50 mmhg.

Postconditioning intervention

After 6 min of circulatory arrest, the postconditioning intervention consisted of 1 ml of NaCl 0.9% injected intravenously in the control group, and 0.2 mg/kg sildenafil (Revatio sildenafil citrate solution for intravenous injection, Pfizer Limited, Sandwich, Kent, UK) with NaCl 0.9% to reach 1 ml volume intravenously in the sildenafil postconditioning group. In previous animal cardiac pre/postconditioning studies, sildenafil was protective in a dose range between 0.01 mg/kg and 1.4 mg/kg [4-9]. The 0.2 mg/kg sildenafil dose was chosen after dose-finding studies in our model showed that 0.8 mg/kg resulted in pulmonary edema in 3 out of 4 of experiments.

Measurements

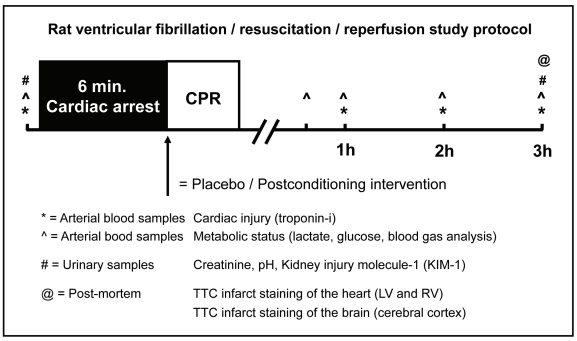

Figure 1: Rat ventricular fibrillation/resuscitation/reperfusion study protocol. CPR: cardiopulmonary resuscitation, TTC: triphenyltetrazolium chloride.

During the experiments, ECG, systemic blood pressure (femoral artery), rectal temperature, tidal volume, and ventilator settings were continuously monitored. Figure 1 depicts the study protocol with timing of the measurements. Arterial blood gas analysis was performed (to adjust ventilator settings and as measure of metabolic derangement) using a portable I-stat device (Abbott Point of Care, Princeton, NJ, USA). Furthermore, troponin i (as measure of cardiac injury, I-stat device, Abbott), and lactate (as indicator of tissue

perfusion, Lactate Scout EKF diagnostics, Magdeburg, Germany) were assessed serially (Figure 1).

Area under the release curve (AUC) of troponin i was calculated for each rat using the trapezoidal rule [20].

Urinary samples were obtained at baseline and post-mortem for analysis of kidney-injury molecule (KIM-1, as measure of renal injury) normalized to urinary creatinine level, as performed previously [21].

After 180 min of reperfusion, the rats were euthanized (intravenous 200 mg/kg pentobarbital injection). Heart and brain were removed, stored overnight at -20°C, subsequently cut into 2 mm slices and stained with 1% triphenyltetrazolium chloride (TTC) for 15 minutes at 37°C. The slices were put in 4% buffered formalin solution for 24 h. Digitized planimetry was subsequently performed (Leica Qwin, Leica Microsystems, Heerbrugg, Switzerland) by a blinded investigator (HA). Planimetry data are expressed as infarct (white, non-stained by TTC) percentage of the area at risk (viable tissue, brick-red by TTC staining). As reperfusion injury develops over time, only samples from rats that survived >120 min after resuscitation were analyzed.

Statistical analysis

Null hypothesis was that there would be no difference in troponin release between control and sildenafil group. Statistical analyses were performed using SigmaPlot software version 13 (Systat Software Inc., San Jose, CA, USA). Differences with $p<0.05$ were considered significant. Incidence of mortality and pulmonary edema data were analyzed using Fisher's exact test. Analysis showed that several data were not distributed normally (Shapiro-Wilk test). Therefore, all data are expressed as median, interquartile range Q1-Q3 (IQR). For data that were not distributed normally (Shapiro-Wilk test) and/or that failed the equal variance test (Brown-Forsythe), ANOVA on ranks was performed (Kruskal-Wallis) where appropriate. Variables measured at one time point were analyzed using one way analysis of variance (ANOVA), variables measured consecutively were analyzed using repeated measures ANOVA. When overall repeated measures ANOVA found a p-value <0.05, further analysis with pairwise multiple comparison procedures was performed (Holm-Sidak).

Results

Resuscitation and survival

All rats remained in VF after 3 minutes of electrical stimulation. ROSC was reached in all rats. There were no differences between the control and sildenafil groups with respect to body weight, mortality, number of defibrillation shocks, time from VF induction to ROSC, number of adrenalin administrations, and maximum dosage of adrenalin perfusion in the reperfusion phase, maximum peak inspiratory pressure and survival time during reperfusion (Table 1). Of note, 1 rat in the sildenafil group spontaneously converted to sinus rhythm during CPR without a defibrillation shock. In the control group, 8/10 rats survived to 3 hours of reperfusion after VF (deaths at 116 and 125 minutes), *versus* 7/10 in the sildenafil group (deaths at 30, 111 and 150 minutes). All three premature deaths in the sildenafil group developed pulmonary edema (defined as foamy endotracheal tube excretion+PaO_2(mmHg)/FiO_2ratio <200) *versus* 0/10 in the control group.

Characteristic & outcome measure	Control group n=10	Sildenafil group n=10	Statistical test performed	p-value control *vs.* sildenafil group
Body weight (g)	406, 379-431	410, 400-437	ANOVA[#]	0.343
n defibrillation shocks	1, 1-2	1, 1-1	ANOVA[#] on ranks	0.210
Time from VF[*] to ROSC[†] (sec.)	503, 450-563	480, 471-513	ANOVA[#] on ranks	0.446
Mortality[‡] (n)	2	3	Fisher's exact	1.000
n adrenalin boluses during CPR§	2, 2-3	2, 2-3	ANOVA[#]	0.806
Maximum adrenalin (ml/h)	0.5, 0.5-1.1	1.0, 0.5-2.8	ANOVA[#] on ranks	0.180
Maximum PIP[‖] (mbar)	20, 19-22	20, 17-30	ANOVA[#] on ranks	0.819
Survival time[¶] (min.) during reperfusion	178, 163-180	175, 140-182	ANOVA[#] on ranks	0.704
Pulmonary edema (n)	0	3	Fisher's exact	0.211

Table 1: Group characteristics and general outcome measures. Data expressed as median, interquartile range Q1-Q3, n: number; [*]VF: ventricular fibrillation; [†]ROSC: return of spontaneous circulation; [‡]Mortality: number of deaths before 180 min of reperfusion; §CPR: cardiopulmonary resuscitation; [‖]PIP: peak inspiratory pressure; [¶]Survival time: minutes from VF induction to death or euthanasia; [#]ANOVA: analysis of variance.

Cardiac injury, hemodynamic recovery

Overall troponin i release, (area under the release curve, AUC) was significantly higher in the control group (median 50, IQR 32-60 h·microg/L) as compared with the sildenafil group (16, 12-42 h·microg/L, $p=0.032$). There were no significant differences between the control and sildenafil groups in troponin i release at any individual corresponding time point (Figure 2). As compared with baseline, troponin release was higher at every post-resuscitation time point (60, 120 and 180 min) in the control group, but only at 60 min after resuscitation in the sildenafil group (Figure 2).

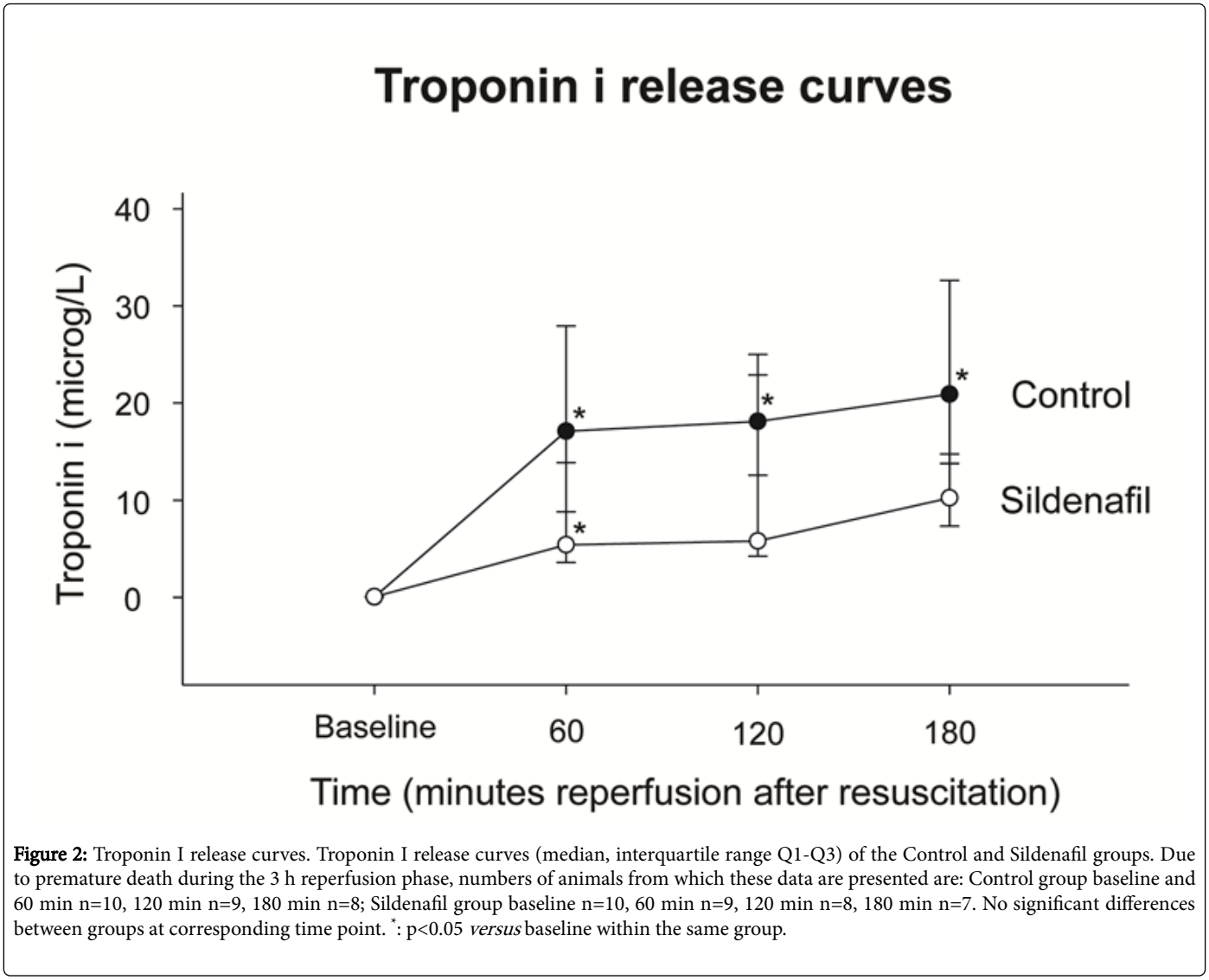

Figure 2: Troponin I release curves. Troponin I release curves (median, interquartile range Q1-Q3) of the Control and Sildenafil groups. Due to premature death during the 3 h reperfusion phase, numbers of animals from which these data are presented are: Control group baseline and 60 min n=10, 120 min n=9, 180 min n=8; Sildenafil group baseline n=10, 60 min n=9, 120 min n=8, 180 min n=7. No significant differences between groups at corresponding time point. *: $p<0.05$ *versus* baseline within the same group.

TTC staining of the LV showed a significantly larger infarct size in the control group (19, 16-24%) as compared with the sildenafil group (16, 12-18%, p=0.039). Blood pressures dropped after sildenafil administration and recovered more during further reperfusion as compared to the control group, but this difference between the groups did not reach the level of statistical significance (Figure 3).

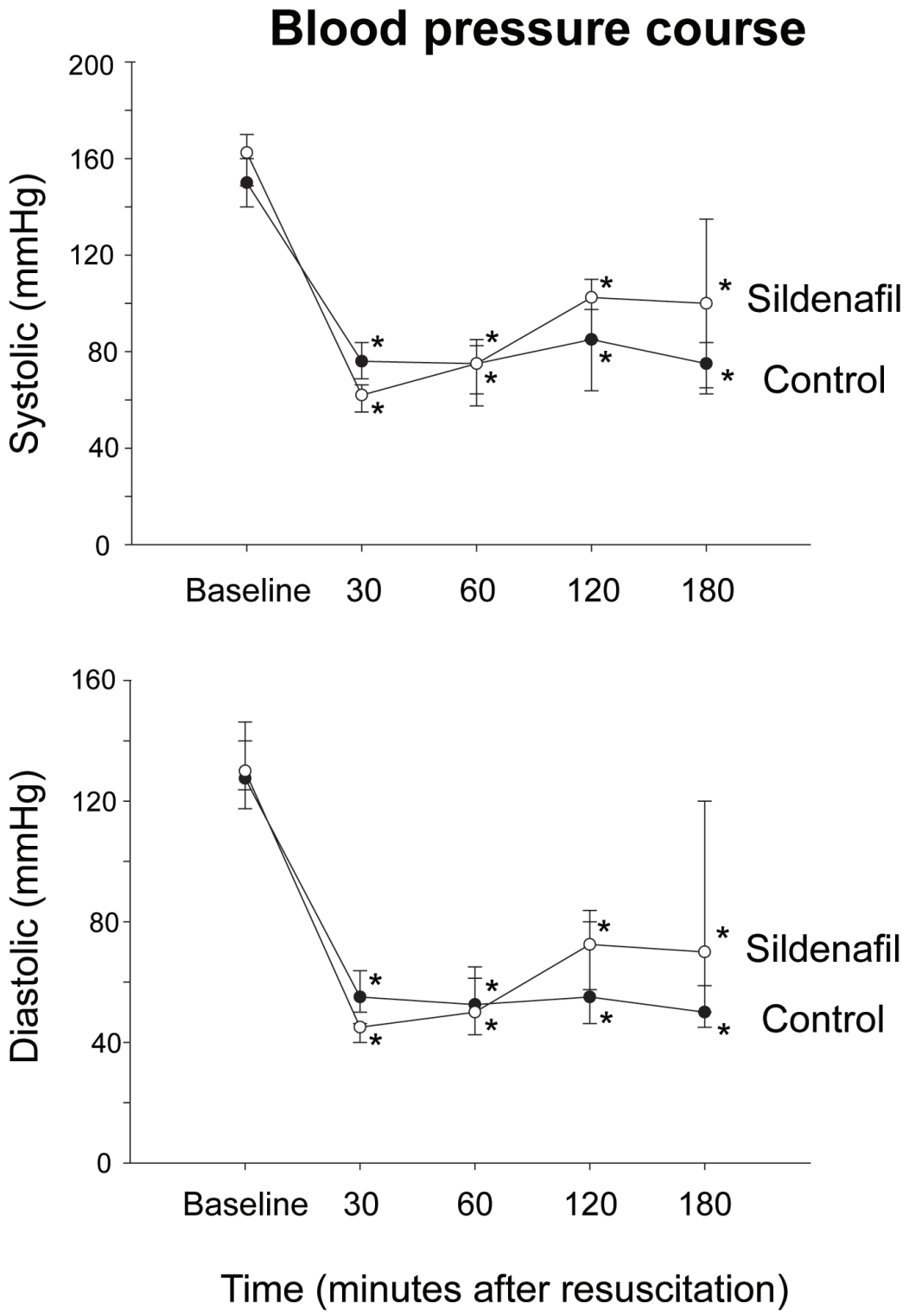

Figure 3: Blood pressure course of the Control and Sildenafil groups. Upper panel: systolic blood pressure, lower panel: diastolic blood pressure. Due to premature death during the 3 h reperfusion phase, number of animals from which these data are presented are: Control group baseline, 30 and 60 min n=10, 120 min n=9, 180 min n=8; Sildenafil group baseline and 30 min n=10, 60 min n=9, 120 min n=8, 180 min n=7. No significant differences between the groups at corresponding time point. *: $p<0.05$ *versus* baseline within the same group.

Cerebral injury

TTC staining of the cerebral cortex showed similar infarct percentages in the control (25, 14-48%) and sildenafil groups (23, 15-44%, p=0.438).

Renal injury

Urinary KIM-1/creatinin after 3 h of reperfusion was 1.23 (0.60-2.10) mg/g in the control group *versus* 0.60 (0.50-0.97) mg/g in the sildenafil group, p=0.110 between groups. There were no significant differences in urine pH, urine creatinine level, absolute KIM-1 and KIM-1/creatinine between the control and sildenafil groups at corresponding time points (Supplementary Table 1).

Blood gas analyses and metabolic status

There were no differences between the groups in blood gas values, paO_2/FiO_2 (as a measure of acute lung injury) and lactate content between the groups (Supplementary Table 2).

Discussion

The main finding of this study was that in our rat model of severe multi-organ I/R injury due to cardiac arrest and resuscitation, sildenafil postconditioning significantly reduced I/R injury in the heart. There was no sildenafil-effect on I/R injury in brain and kidneys, nor did sildenafil reduce mortality after resuscitation.

Protection by sildenafil in the resuscitation setting

The novelty of the current study is that the cardiac postconditioning property of sildenafil is maintained in the setting of cardiac arrest/resuscitation. Recently, sildenafil preconditioning in piglets before induction of VF, also reduced post-resuscitation cardiac dysfunction and improved cardiac metabolism [13,14]. Our findings are in line with several animal studies in which sildenafil reduced cardiac I/R injury after only the heart had been regionally ischemic [4-9]. The observed protection is especially notable because the conditions after resuscitation are different from those after the usually applied occlusion/reperfusion of the supplying coronary artery, major differences being generalized acidosis, metabolic derangements, and overall reduced cardiac pump function.

The failure of sildenafil to reduce I/R injury in brain and kidney despite positive single organ I/R studies could be explained by the fact that the applied dose was too low. The dosage reported to be protective for the kidneys (0.15 mg/kg [11]) is close to the range applied in our study. The high sildenafil dosages needed for cerebral protection (4-32 mg/kg [10], 2-5 mg/kg [22]) were not assessed in our study.

The lack of observed neuro-protection could also be caused by the relatively short reperfusion time of 3 h. Although cerebral injury is already evident by TTC staining at 1.5 h of reperfusion in a rat model of stroke [23], up to 24 h of reperfusion are required to express maximal injury [23].

To our knowledge, there are no previous cardiac arrest/resuscitation postconditioning studies that compared the effects of the postconditioning trigger on cardiac, cerebral and renal injury.

Clinical implications

The finding in the present study that sildenafil reduces damage to the heart after resuscitation raises the question whether such protection may also be achieved in man. Given the major impact of cardiac arrest on patients, the application of sildenafil postconditioning could be beneficial to humans with ventricular fibrillation.

In our study, sildenafil was administered intravenously. While this access route is certainly a possibility in patients with circulatory arrest/resuscitation, also nasal administration may be considered, as studies have shown that sildenafil is absorbed rapidly after intranasal administration [24].

To the best of our knowledge this study is the first demonstrating the efficacy of pharmacological postconditioning during resuscitation. This proof-of-principle may also be extended to other interventions that appear promising based on single organ studies, such as cyclosporine postconditioning [25], inhalation of argon [18] or hydrogen [26].

Limitations

Sildenafil blood levels were not determined in this study, limiting assessment of adequate dosing of the postconditioning drug. However, the blood pressure course in sildenafil-treated rats is compatible with adequate drug levels. Our rats needed inotropic support after CPR and neurological function was impaired, hindering awakening after the 3 hours reperfusion period. For ethical reasons all animals in the study were euthanized at 3 h of reperfusion. Lungs weights were not assessed at sacrifice, therefore, pulmonary edema as defined in this manuscript could not be compared with lung weights.

The occurrence of pulmonary edema in 3/10 sildenafil-treated rats is a contraindication in the clinical setting. However, this side effect is most likely caused by the combined use of pentobarbital and sildenafil in rats [27]. After all, sildenafil is being used extensively in adults with erectile dysfunction, and in patients with pulmonary hypertension and under all these conditions, pulmonary edema has not been described as a side-effect. Clearly, prior to any clinical trial, a study should be performed with larger animals, and with other anesthetics. We also recommend to evaluate higher doses of sildenafil, in order to assess the potential for neuro-protection and nephro-protection of sildenafil postconditioning.

Conclusions

Sildenafil postconditioning during resuscitation from VF significantly reduces cardiac I/R injury, whereas it does not protect the brain and kidneys.

Acknowledgments

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