

Quinapril decreases antifibrinolytic and prooxidative potential of propofol in arterial thrombosis in hypertensive rats

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Abstract

Angiotensin converting enzyme inhibitors and propofol both exert hypotensive action and may affect hemostasis. We investigated the influence of quinapril and propofol on hemodynamics and hemostasis in renal-hypertensive rats with induced arterial thrombosis. Two-kidney, one clip hypertensive rats were treated with quinapril (3.0 mg/kg for 10 days), and then received propofol infusion (15 mg/kg/h) during ongoing arterial thrombosis. The hemodynamic and hemostatic parameters were assayed. Quinapril exerted a hypotensive effect increasing after propofol infusion. Quinapril showed an antithrombotic effect with the platelet adhesion reduction, fibrinolysis enhancement and oxidative stress reduction. Propofol did not influence thrombosis; however, it inhibited fibrinolysis and showed prooxidative action. The effect of propofol on fibrinolysis and oxidative stress was significantly lower in quinapril-pretreated rats. Mortality was increased among rats treated with both drugs together. Our study demonstrates that pretreatment with quinapril reduced the adverse effects of propofol on hemostasis. Unfortunately, co-administration of both drugs potentiated hypotension in rats, which corresponds to higher mortality.

Keywords

Quinapril, propofol, rat, thrombosis, hypertension

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Introduction

Angiotensin-converting enzyme inhibitors (ACE-Is), widely used hypotensive drugs, reveal antithrombotic, endothelium-dependent activity even at non-hypotensive doses. We have previously shown antithrombotic action of ACE-Is in arterial and venous thrombosis in normotensive rats and this action was significantly greater in young rats than in adult and old rats.^{1–4} This antithrombotic activity of ACE-Is depends on the inhibition of angiotensin II (Ang II) formation, bradykinin (Bk) degradation, and potentiation of Ang-(1-7) formation.⁵ A decrease in the Ang II level supports functions of antithrombotic endothelium and decreases platelet activation.⁶ In turn, increased Bk levels, via type II G-protein-coupled Bk receptors, activate calmodulin and in consequence increase the production of such antithrombotic molecules as prostacyclin (PGI₂) or

tissue-type plasminogen activator (t-PA).⁷ Furthermore, ACE-Is increase Ang-(1-7) production, probably due to the activity of such enzymes as ACE 2 or endopeptidases, which are not susceptible to inhibition by ACE-Is.⁸ Ang-(1-7) activating its own G-protein coupled receptors,

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Mas₁₋₇, increases the production of nitric oxide (NO) and PGI₂ in endothelial cells.⁹ Moreover, ACE-Is affect hemostasis also through the inhibition of platelet and erythrocyte aggregation, and down regulation of glycoprotein IIb/IIIa complex on the platelet surface.⁹⁻¹¹ ACE-Is also cause a decrease in serum and aortic ACE activity, PAI-1 protein level, and tissue factor (TF) activity.^{12,13} These favorable endothelial effects of ACE-Is have been confirmed in human studies and could explain reduced cardiovascular mortality caused by these drugs.¹⁴⁻¹⁷

Many patients treated with ACE-Is undergo general anesthesia with propofol (Pro), an intravenous anesthetic. Among its advantages directly in anesthesia, Pro also increases endothelium-dependent NO release.¹⁸ Mainly for this reason, Pro causes vasodilatation, resulting in clinically relevant hypotension.¹⁹ Indeed, our previous observations in hypertensive patients treated with ACE-Is showed that Pro given for induction of general anesthesia significantly decreased blood pressure (BP), but precise mechanism of this interaction is still unknown and needs further investigations.²⁰

Pro also activates the kinin-kallikrein system in plasma and acts as an L-type calcium channel antagonist decreasing peripheral vascular resistance.^{21,22} *In vitro* and *ex vivo* data suggest that Pro exerts antiplatelet and anti-inflammatory effects.²³⁻²⁶ Moreover, Pro inhibits platelet thromboxane synthesis and NO production in leukocytes, stimulating the activity of guanyl cyclase in platelets.²⁷ In our preliminary study, we also observed NO release from endothelial cells, which indicates the significant involvement of endothelium in the effect of Pro on hemostasis.²⁸

Because of the structural similarity to vitamin E, Pro could also regulate oxidative stress.²⁹ Moreover, since the chemical structure of the Pro molecule (a simple phenol substituted with two isopropyl groups in the ortho position) resembles the structure of acetylsalicylic acid it may potentially influence hemostasis.³⁰

There are also some clinical studies about thrombotic complications of the deep brachial veins during Pro treatment, although the mechanism has not yet been examined.^{31,32} However, data concerning the effect of simultaneous administration of Pro and ACE-Is on hemostasis are lacking.

Therefore, the aim of the current study was to assess the influence of quinapril (Quin) and Pro on the process of arterial thrombosis, primary hemostasis, NO bioavailability, oxidative stress and hemodynamic parameters in two-kidney, one-clip (2K1C) hypertensive rats.

Materials and methods

Drugs and reagents

Pro (Plofed 1%, Polfa S.A., Poland), Quin (Accupro, Pfizer, Germany), lipofundin (MCT/LCT 10%, Braun, Germany),

gum arabic (Pharma Cosmetic, Poland), pentobarbital (Vetbutal, Biovet, Poland), collagen type I (Collagen, Chronolog, USA), and heparin (Heparinum, Polfa, Poland) were used in the study. Bovine albumin, apyrase, HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) were delivered by Sigma-Aldrich (Poland). Calcium chloride, EDTA, glucose, magnesium chloride, sodium chloride, potassium chloride, sodium bicarbonate, sodium phosphate, Tris buffer, and trisodium citrate were provided by Polish Chemicals Reagents (Poland). Rabbit anti-rat TAFI monoclonal antibody (ImmunoKontakt AMS Biotechnology, Germany), rat active PAI-1 ELISA kit (Innovative Research, USA), rat active t-PA ELISA kit (Innovative Research, USA), hydrogen peroxide colorimetric detection kit (Assay Designs, USA), correlate assay nitric oxide NO-2/NO-3 assay kit (Assay Designs, USA), MDA adducts ELISA kit (Cell Biolabs, USA), Trizol (Invitrogen Life Technologies, USA), qPCRTM Mastermix, SYBR Green I (Eurogentec Seraing, Belgium), Oligotex Kit (Qiagen, USA), and TaqMan reverse transcription reagents kit (Applied Biosystems, USA) were also used.

Animals

Male Wistar rats (180–250 g) were used in this study. The animals were housed in a room with a 12 h light/dark cycle, and were given tap water and fed standard rat chow. The animals' health status was monitored throughout the experiments by a health surveillance program according to Federation of European Laboratory Animal Science Associations guidelines and Guidelines for the Care and Use of Animals in Biomedical Research.³³ All the procedures involving animals and their care were approved by Local Ethical Committee on Animal Testing at the Medical University of Bialystok (Permit Number: 2004/27).

Induction of renovascular hypertension

Rats were anesthetized with pentobarbital (40 mg/kg, i.p.). Two-kidney, one-clip (2K1C) renovascular hypertension was induced by a partial, standardized clipping of the left renal artery.³⁴ After 6 weeks, to verify hypertension development the systolic and diastolic BP (SBP/DBP) were measured using the "tail cuff" method (LE 5001 Non-invasive Blood Pressure System, Panlab, Spain) in conscious rats.³⁵ Each value was the average of three consecutive readings. Only rats with BP higher than 140/90 mmHg were used in the further experiments evaluating the effect of Quin and Pro on hemostasis and hemodynamics.

Drug administration

Quin (3.0 mg/kg) or adequate volume of 5% aqueous gum arabic solution was administered *per os* for 10 days. The experiments were done on the 11th day after treatment initiation. During the study, the animals received intravenous

infusion of Pro (15 mg/kg/h) or lipofundin (solvent for Pro) at the same dose. The animals were divided into four groups: Group I – 5% aqueous gum arabic solution + lipofundin (Veh); Group II – Quin + lipofundin (Quin); Group III – 5% aqueous gum arabic solution + Pro (Pro); Group IV – Quin + Pro (Quin+Pro).

Indirect BP measurement after 10 days of treatment

The SBP and DBP in conscious rats were measured after 10 days of treatment again, according to the method described above.³⁵ Each value was the average of three consecutive readings.

Arterial thrombosis model

Arterial thrombosis induction was performed according to the method described previously.^{36,37} Rats were introduced into anesthesia with pentobarbital (40 mg/kg intraperitoneally) and then fixed on the operating table. Anesthesia was maintained with an additional dose of pentobarbital (20 mg/kg) intraperitoneally 40 min after the first dose, at a level such that painful stimuli and surgery did not evoke noticeable motor or cardiovascular responses. Anesthetic condition remained unchanged throughout the study.

Thrombosis was induced secondarily to electrical stimulation and endothelial injury with a current, as previously described.^{36,37} The left common carotid artery was then separated from the surrounding tissue along the length of at least 20 mm. A stainless, hook shaped, steel electrode was inserted under the left carotid artery. Under the electrode, a tiny piece of “M” parafilm (5 mm × 20 mm) was inserted for electrical isolation and the hook of the electrode was in contact with the artery. The second electrode was inserted subcutaneously in the abdominal region. Both electrodes were connected to a circuit with a constant current generator. A Doppler flow probe (1 mm-diameter, Hugo Sachs Elektronik – Harvard Apparatus GmbH, Germany) was placed in contact with the exposed artery downstream of the electrode and connected to a blood flowmeter (The HSE-TRANSONIC Transit Time Flowmeter, Germany). Blood flow was monitored continuously during thrombus formation.

After 5 min stabilization, the baseline blood flow was determined. Pro was administered into the femoral vein by a continuous infusion (Constant-Rate Infusion Pump, Kwapisz, Poland) 15 min before the induction of arterial thrombosis and was continued for 100 min, to the end of the experiment. The control animals obtained lipofundin in the same way and volume.

Fifteen minutes after the start of Pro infusion, thrombosis was induced by electrical stimulation (2 mA/5 min) and a hemostatic clamp delivered to the outer surface of the left common carotid artery (between 20 and 25 min). The hemostatic clamp was placed between the electrode and Doppler

flow probe, and the degree of thrombotic occlusion was expressed by a decrease in carotid blood flow. Carotid patency status was monitored continuously for 80 min after the cessation of electrical stimulation and hemostatic clamp. The carotid patency status was expressed according to the following classifications: (1) persistent occlusion – no reflow; (2) cyclic reflow – cyclic reflow and reocclusion after initial reflow; (3) persistent patency – a persistent flow without reocclusion after the initial reflow. The patency status of the carotid artery for the 100 min observation was schematically represented for each animal (see Figure 2(c)). Representative examples of original recordings of blood flow from selected animals with persistent occlusion, cyclic reflow, and persistent patency are shown in Figure 2(d) to (f)

At the end of the study (100 min after Pro administration), blood samples were taken for hemostatic analysis, the aorta was taken for enzyme analysis, and the thrombus was collected at room temperature for 24 h and weighed. All animals were euthanized by exsanguination at the end of experiments under deep anesthesia.

Measurement of hemodynamic parameters

The SBP and DBP were measured in anesthetized rats receiving Pro or lipofundin directly through a cannula filled with heparin solution (150 IU/mL) placed in the left common carotid artery connected to a pressure transducer (Gabarith 1DT-XX, Poland) and an apparatus for pressure measurement (HSE-TRANSONIC, TAM-A, Germany). The carotid blood flow was measured by placing a flow probe on the right carotid artery connected to a blood flowmeter (HSE-TRANSONIC, TTFM, Germany). Both the upper limbs and the right lower limb were connected to electrodes for ECG measurement (HSE-TRANSONIC, ECGA, Germany). Heart rate was calculated from the ECG. In these animals, arterial thrombosis was not induced.

The blood flow was measured downstream from the site of electrical stimulation and monitored continuously from the beginning of the study until its end.

Primary hemostasis: template bleeding time

At the end of the experiment, prior to the removal of the arterial thrombus, the template bleeding time (BT) was measured, according to Dejana et al.³⁸ A standardized device was applied longitudinally on the dorsal part of the tail between 6 and 9 cm from the tip, taking care to avoid the large veins. Immediately after injury, the tail was placed into a cylinder with isotonic saline at 37°C and bleeding time was measured from the moment the tail was surgically cut until bleeding completely stopped (i.e. lack of bleeding for <30 s).

Blood and aorta collection

At the end of the experiments, blood samples were drawn from the right ventricle of the heart. Whole blood and

plasma were received and deep-frozen (-80°C) for further investigations. To obtain plasma, the blood was mixed with 3.13% trisodium citrate in a volume ratio of 9:1 and centrifuged for 20 min at $3500\times g$, at 4°C . At the end of the experiments (only arterial model of thrombosis), the aortas were obtained from rats and deep-frozen (-80°C).

Platelet adhesion to fibrillary collagen *ex vivo*

Platelet adhesion to fibrillary collagen was assayed *ex vivo* according to Mant et al.³⁹ The washed platelet suspension was prepared from blood as previously described.⁴⁰ The final concentration of platelets in washed platelet samples was 3×10^5 platelets/ μL . The 250 μL of washed platelet samples were incubated in an Elvi 840 aggregometer at 37°C and stirred at 900 rpm with EDTA (13 μL ; 100 mM) to prevent platelet aggregation. After 5 min pre-incubation, collagen (12 μL) was added and platelets were further incubated for 10 min. Samples of the suspension were obtained before and 15 min after adding the collagen, then platelets were counted in a hemocytometer after dilution with the Unopette system. The index of adhering platelets was calculated using the formula [(platelet count before adding the collagen – platelet count after adding the collagen)/platelet count before adding the collagen] $\times 100\%$.

Determination of fibrinolysis

To determine whether fibrinolysis could be changed after Quin and/or Pro administration, t-PA, PAI-1 and TAFI plasma levels were determined by enzyme immunoassays according to manufacturer's directions. PAI-1 mRNA levels were measured in whole blood using the real-time polymerase chain reaction (PCR) technique, as previously described.^{40,41}

Determination of plasma nitric oxide level

To establish the influence of Quin and Pro treatment on NO bioavailability, plasma NO metabolite levels were measured. Since NO is an extremely labile molecule and decomposes rapidly in biological solutions to nitrite (NO_2^-) and nitrate (NO_3^-), these stable metabolites of NO were analyzed in plasma as indirect markers for NO plasma bioavailability. In our study, the NO plasma level was measured colorimetrically as $\text{NO}_2^-/\text{NO}_3^-$ concentration with a commercially available kit.

Determination of aorta NOS mRNA level

To determine an influence of Quin and/or Pro administration on nitric oxide synthase (NOS) activity, eNOS and iNOS mRNA levels were measured in the aorta using the real-time PCR technique, as previously described.^{40,41} The following PCR primers were designed using software Primer Express (Applied Biosystems): 5'-CATCGGCG TGCTGCGGGATCAG-3' and 5'-GGGCTGTTGGTGTG

TGAGCCGG-3' and 5'-CCAACAATGGCAACATCA GG-3' and 5'-TCGTGCTTGCCATCACTCC-3', specific for mRNA of eNOS and iNOS respectively. The reaction for each probe was conducted in the same conditions as a multiplex PCR with two pairs of primers (eNOS and iNOS) at the same time. The amount of eNOS and iNOS mRNA was quantified.

Oxidative stress parameters

To determine the influence of Quin and Pro treatment on the oxidative stress parameters, plasma concentrations of hydrogen peroxide (H_2O_2), malonyl dialdehyde (MDA), and enzymatic expression of superoxide dismutase (SOD) and oxidase NADPH in aortic rings were assayed.

H_2O_2 and MDA levels were assayed in plasma with commercially available kits. To evaluate the influence of Quin and/or Pro administration on oxidative stress in the aorta, amounts of mRNA of NADPH oxidase and SOD were measured in rat aortas using the real-time PCR technique, as previously described.^{40,41}

Statistical analysis

The data are shown as mean \pm SEM. The incidence of occlusion in carotid artery was calculated by Fisher's exact test. Two-tail Mann-Whitney tests were used consistently throughout the study to test whether the mean of a variable differs between two groups. A p -value < 0.05 was considered significant.

Comparisons between the experimental rat groups and the saline-treated placebo rat group were performed using one-way analysis of variance (ANOVA) or Kruskal-Wallis test, followed by post-hoc Dunnett's or Dunn's tests, depending on data distribution. A p -value < 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, USA).

Results

Hemodynamics

A 10-day Quin treatment (3 mg/kg) showed a significant ($p < 0.05$) decrease in BP (systolic and diastolic) as compared to Veh rats (Table 1). Pro alone and added to Quin treated rats showed a notable ($p < 0.05$) decrease in SBP and DBP at 100 min (T_{100}) of infusion in comparison to the onset of the experiment (T_0). Changes in BP did not result in the acceleration of the heart rate in all animal groups.

Arterial thrombosis

The initial blood flow was comparable in all study groups. A gradual decrease in the blood flow was observed in all the groups (Figure 1(a)). The difference between the final (T_{100}) and the initial carotid blood flow

Table 1. Hemodynamic parameters before and after 100 min Pro administration.

Group	n	Heart rate (bpm)			Systolic blood pressure (mmHg)			Diastolic blood pressure (mmHg)		
		T_0	T_{100}	$\Delta T (T_{100} - T_0)$	T_0	T_{100}	$\Delta T (T_{100} - T_0)$	T_0	T_{100}	$\Delta T (T_{100} - T_0)$
Veh	6	424 ± 17	418 ± 15	-6	143 ± 8	137 ± 7	-6	112 ± 6	102 ± 6	-10
Quin	6	433 ± 14	393 ± 11 ^Δ	-40	123 ± 5*	113 ± 7*	-10	98 ± 4*	88 ± 4*	-10
Pro	5	419 ± 19	400 ± 27	-19	148 ± 5	118 ± 7 ^Δ	-30	110 ± 5	90 ± 6 ^Δ	-20
Quin+Pro	5	419 ± 23	409 ± 5	-10	128 ± 9*	104 ± 8* ^Δ	-24	99 ± 4*	78 ± 3 ^{Δ*}	-21

Veh – control with lipofundin infusion, Quin – rats treated with quinapril and lipofundin infusion, Pro – control with propofol infusion, Quin+Pro – rats treated with quinapril and propofol infusion, T_0 – beginning of the experiment, T_{100} – end of the experiment, after 100 min flow registration. Data are expressed as mean ± SEM * $p < 0.05$ vs Veh; ^Δ $p < 0.05$ T_{100} vs T_0 .

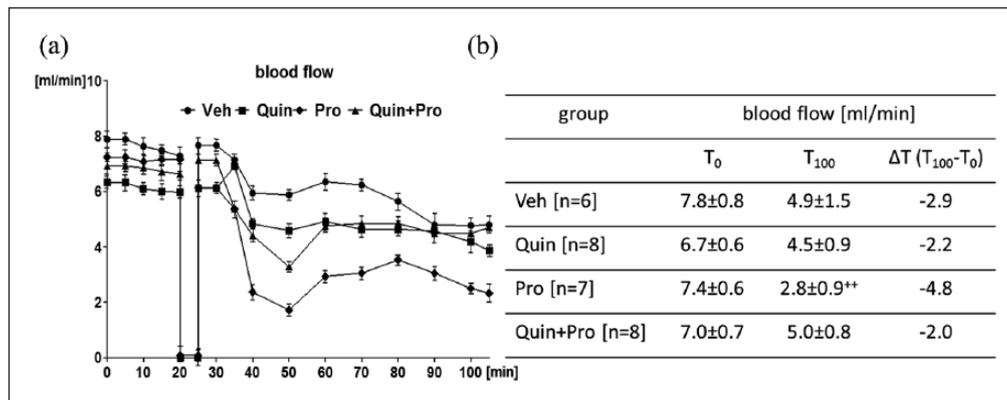


Figure 1. Changes in the carotid blood flow (a) with the values of initial (T_0) and final (T_{100}) carotid blood flow (b) determined in Veh, Quin, Pro, and Quin+Pro rats subjected to electrical stimulation and clamping of the carotid artery between 20–25 min of the experiment. The lines represent the course of blood flow registered for 100 min after administration of Pro or Quin. T_0 – beginning of the experiment, T_{100} – end of the experiment, after 100 min flow registration. Data are expressed as mean ± SEM. ++ $p < 0.01$ vs T_0 .

(T_0) assumed as ΔT was the highest in the Pro group (2.8±0.9 vs 7.4±0.6 mL/min, ΔT -4.8 mL/min; $p < 0.01$) (Figure 1(b)). Furthermore, the final blood flow after Pro infusion was the lowest in comparison to Veh and Quin treated rats ($p < 0.05$) (Figure 1(b)).

In the Quin group, the longest time to primary vessel occlusion (32±0.9 min, Figure 2(a)), the lowest thrombus weight (0.3±0.17 mg, $p < 0.05$, Figure 2(b)), and the highest persistent patency (62%, Figure 2(c)) were noted. The incidence of persistent occlusion or cyclic reflow in the Quin group was similar to the Veh-treated rats (37% vs 33%). The administration of Pro increased the incidence rates of cyclic reflow: Pro alone by 34% and added to Quin by 27%, when compared to Veh (Figure 2(c)).

Pro alone did not change significantly thrombus weight in comparison to Veh (1.0±0.3 mg vs 0.8±0.21 mg), and added to Quin reduced its benefit effect on thrombus weight (1.0±0.3 mg vs 0.3±0.17 mg, $p < 0.05$) (Figure 2(b)).

The primary hemostasis

As shown in Figure 3(a), collagen-induced platelet adhesion was attenuated by Quin (20±4.4%, $p < 0.05$). Both

Pro and Quin significantly decreased platelet adhesion to collagen in comparison to Veh and Pro alone (16±2.0% vs 30±2.5% and 26±2.0%, respectively, $p < 0.05$) (Figure 3(a)). Any changes in BT were observed; only Quin tended to prolong BT (Figure 3(b)).

Plasma fibrinolytic status

Quin greatly enhanced fibrinolysis through increased t-PA expression ($p < 0.05$) and decreased PAI-1 expression ($p < 0.05$), when administered alone (Table 2). The antifibrinolytic response exerted by Pro was partially abolished by Quin ($p < 0.05$ in t-PA, $p < 0.01$ in PAI-1) (Table 2).

The strongest increase in TAFI was induced by Pro infusion in the rats that did not previously receive Quin ($p < 0.01$), as compared to Veh. Pretreatment with Quin decreased Pro-induced TAFI plasma concentration ($p < 0.05$) in comparison to Pro alone (Table 2).

NO bioavailability

The 10-day Quin treatment caused a decrease in plasma NO_2/NO_3 concentration (Figure 4(a)) and reduction in aortic iNOS mRNA expression (Figure 4(b)), as compared to

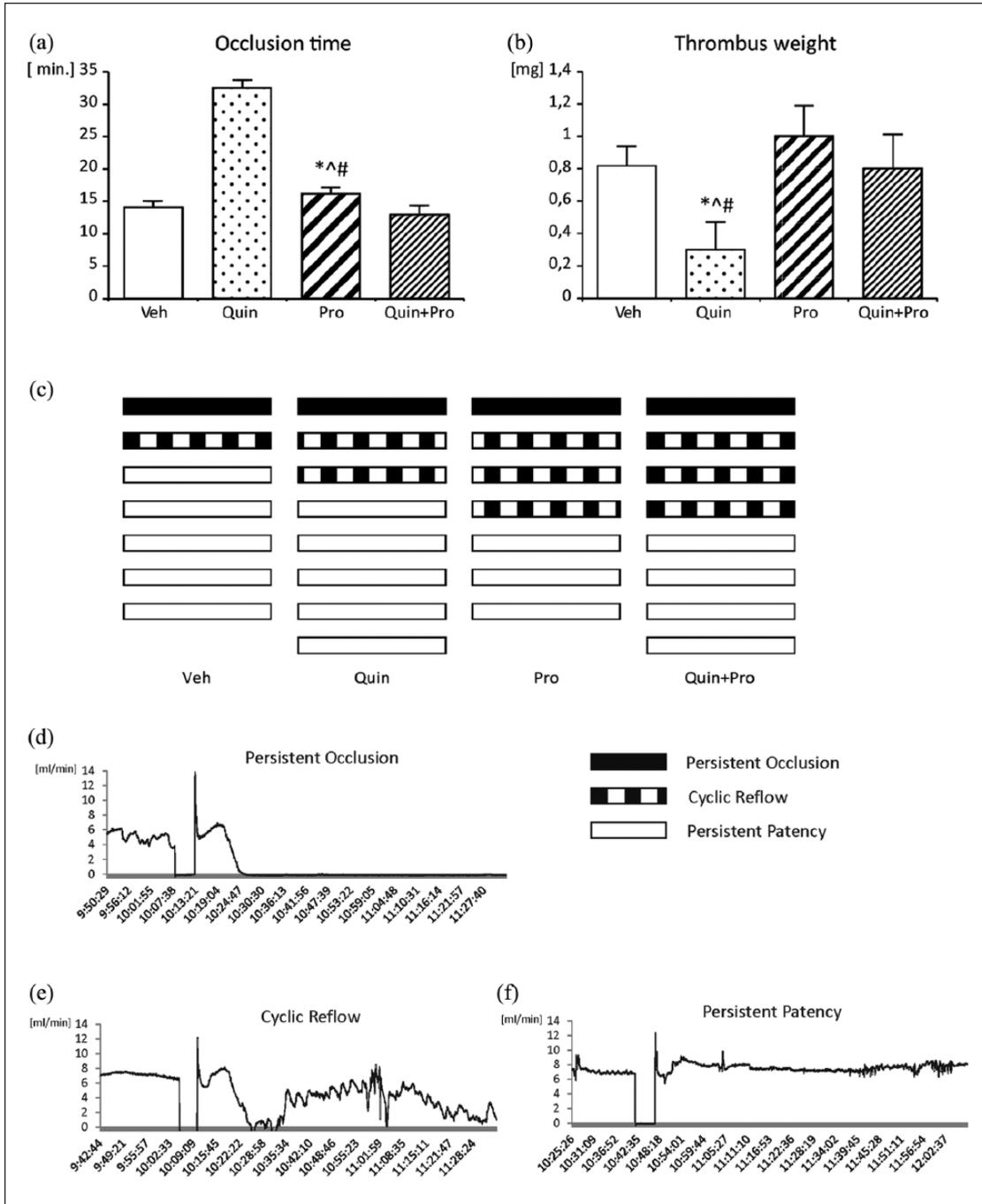


Figure 2. The effect of Quin and Pro treatment on occlusion time (a) and dry thrombus weight (b). Schematic representation of the patency status of the carotid artery in individual animals for the 100 min observation (c). Representative examples of original recordings of blood flow from selected animals with persistent occlusion (d), cyclic reflow (e), and persistent patency (f). Data are expressed as mean \pm SEM. *^#*p* <0.05 Pro vs Veh, Quin, Quin+Pro, respectively.

Veh ($7.88 \pm 0.06 \mu\text{M/L}$ vs $8.76 \pm 0.08 \mu\text{M/L}$, $p < 0.05$ and $0.52 \pm 0.03 \ 2^{-\Delta\Delta\text{ct}}$ vs $0.78 \pm 0.05 \ 2^{-\Delta\Delta\text{ct}}$, $p < 0.01$; respectively). The increase in plasma NO metabolite level in the Pro group ($11.35 \pm 0.43 \mu\text{M/L}$ vs $8.76 \pm 0.08 \mu\text{M/L}$; $p < 0.01$ vs Veh) was paralleled by an increase of iNOS mRNA levels in the aortic rings ($1.29 \pm 0.04 \ 2^{-\Delta\Delta\text{ct}}$ vs 0.78 ± 0.05

$2^{-\Delta\Delta\text{ct}}$, $p < 0.01$ vs Veh) (Figure 4(a) and (b)). During co-administration of Quin and Pro, we still observed an increase in iNOS activity and plasma NO₂/NO₃ concentration in comparison to Quin ($p < 0.001$) (Figure 4(a) and (b)). The expression of eNOS was comparable in all study groups.

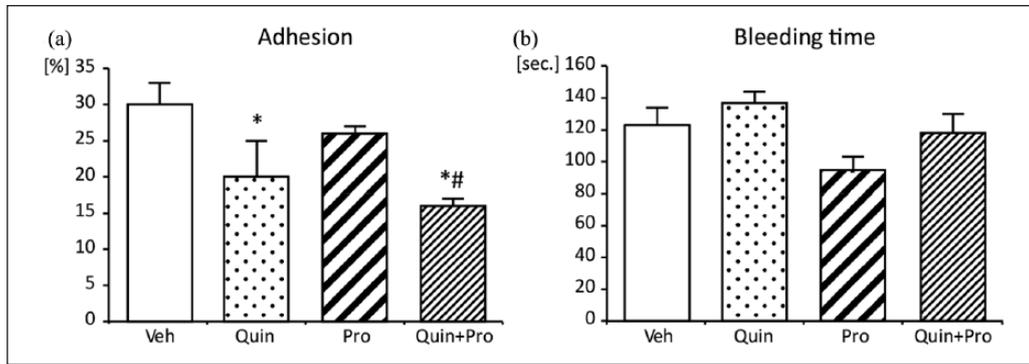


Figure 3. Effect of Quin and Pro treatment on platelet adhesion *ex vivo* (a) and bleeding time (b). Data are expressed as mean \pm SEM. * $p < 0.05$ vs Veh; # $p < 0.05$ vs Pro.

Table 2. *Ex vivo* plasma fibrinolytic parameters at the end of arterial thrombosis induction.

	t-PA (ng/mL)	PAI-I (ng/mL)	TAFI (μ g/mL)
Veh (n = 6)	7.02 \pm 0.18	4.83 \pm 0.15	4.34 \pm 0.02
Quin (n = 7)	7.94 \pm 0.14 ^{###}	4.22 \pm 0.05 ^{###}	4.54 \pm 0.07
Pro (n = 6)	5.68 \pm 0.24 ^{***^}	7.13 \pm 0.2 ^{***^}	5.29 \pm 0.03 ^{**}
Quin+Pro (n = 8)	6.74 \pm 0.06 ^{#^}	5.41 \pm 0.07 ^{*###^}	4.54 \pm 0.03 ^{###}

* $p < 0.05$, ** $p < 0.01$ vs Veh, # $p < 0.05$, ### $p < 0.01$, #### $p < 0.001$ vs Pro, ^^ $p < 0.01$, ^^ $p < 0.001$ vs Quin.

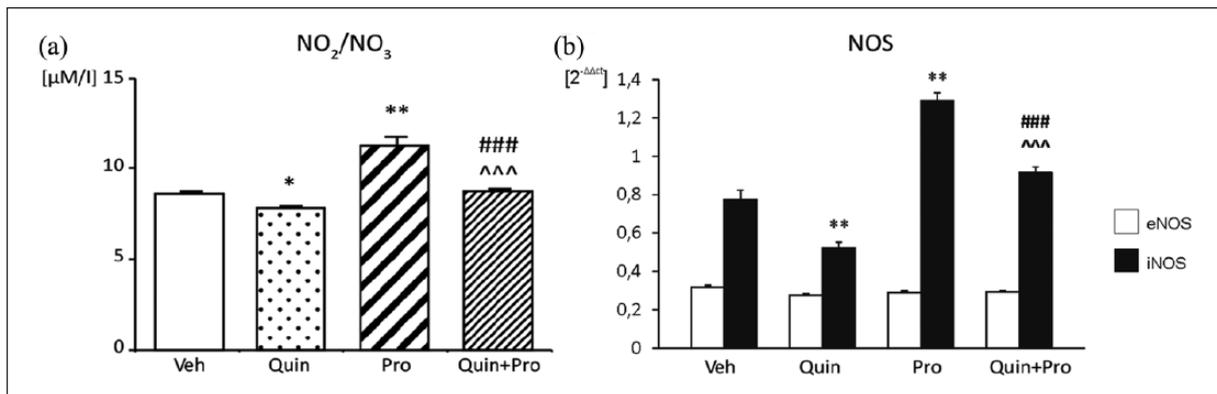


Figure 4. Effect of Quin and Pro treatment on NO_2/NO_3 plasma concentration (a) and eNOS and iNOS mRNA level in aorta (b). Data are expressed as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$ vs Veh; ### $p < 0.001$ vs Pro; ^^ $p < 0.001$ vs Quin.

Oxidative stress

Quin and Pro co-administration did not change the expression of SOD and NADPH oxidase in comparison to Veh, whereas Quin alone significantly decreased SOD ($p < 0.05$) and NADPH oxidase expression ($p < 0.001$) and prevented their increase caused by Pro ($p < 0.01$) (Figure 5(a) and (b)). Quin treatment did not change H_2O_2 concentration (102 \pm 3 ng/mL) (Figure 5(c)), while MDA concentration was decreased as compared to Veh (0.23 \pm 0.005 vs 0.29 \pm 0.004 pM/mg protein, $p < 0.01$) (Figure 5(d)). Pro infusion increased both H_2O_2 and MDA concentration (176.3 \pm 5.3, $p < 0.01$ vs Veh;

0.407 \pm 0.003, $p < 0.01$ vs Veh). Quin markedly attenuated prooxidative potency of Pro when administered jointly, in comparison to Pro alone (H_2O_2 117.2 \pm 1.9 mg/ml, MDA 0.295 \pm 0.007 pM/mg protein, $p < 0.001$) (Figure 5(c) and (d)).

Animal mortality

Interestingly, Pro added to Quin caused the highest mortality (38%, 3/8 animals) in rats. The animals died 52 \pm 9 min after the start of the experiment. We also registered one death (13%, 1/8) in the Quin group in minute 45 of the experiment.

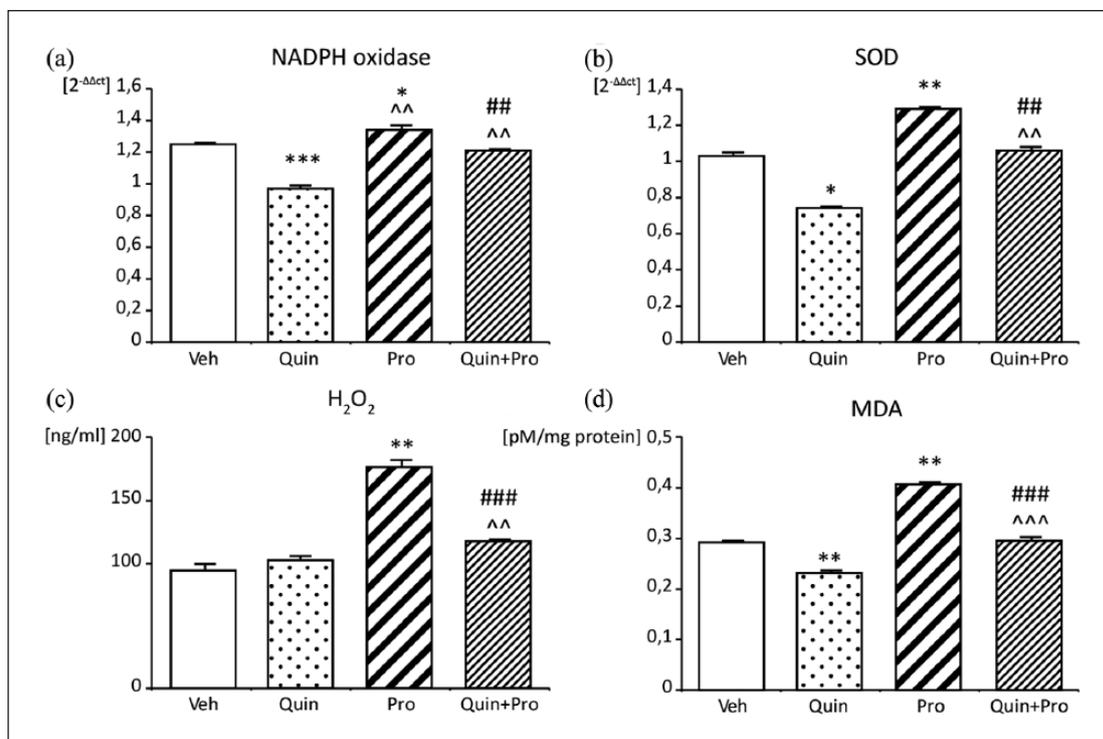


Figure 5. Effect of Quin and Pro treatment on NADPH oxidase in aorta (a), superoxide dismutase (SOD) in aorta (b), H₂O₂ plasma concentration (c), and malonyl dialdehyde (MDA) plasma concentration (d). Data are expressed as mean ± SEM. **p*<0.05, ***p*<0.01 vs Veh; ###*p*<0.01, ####*p*<0.001 vs Pro; ^^*p*<0.01, ^^*p*<0.001 vs Quin.

Discussion

We showed previously that in hypertensive patients treated with ACE-Is, propofol given for induction of general anesthesia significantly decreased BP. This observation may indicate the additive effect of ACE-Is and intravenous anesthetic, but precise mechanism of this interaction is not clarified.²⁰ Moreover, there are some data indicating antithrombotic effect of ACE-Is and very few suggesting the effect of Pro on hemostasis. However, data concerning the effect of simultaneous administration of Pro and ACE-Is on hemostasis are still lacking.

In the current study, the effects of Quin and Pro on hemostasis and hemodynamics were evaluated. We performed experiments in the model of renovascular hypertensive rats (2K1C). This model of hypertension is mediated by the increased activities of both systemic and tissues (heart, aorta, lung, and kidney) rennin-angiotensin-aldosterone system (RAAS) and is recommended for the evaluation of chemicals affecting RAAS such as ACE-Is, AT₁-As or RAAS peptides.^{42,43} Bearing in mind that the arterial thrombosis is a dynamic and a very complex process we used a wide panel of tests evaluating both the role of platelets, as well as coagulation and fibrinolysis to find the mechanism(s) of Quin and Pro action in hemostasis in the arterial model of thrombosis.

Previously, we proved the antithrombotic activity of ACE-Is in venous and arterial thrombosis in normotensive rats and demonstrated the involvement of NO and PGI₂ in this phenomenon.¹⁻³ Presently, we observed the antithrombotic activity and hypotensive effect of Quin in renovascular hypertensive rats, developing hypertension on the basis of temporary over-activation of the RAAS accompanied by harmful biochemical changes and structural damages to the endothelium and vascular wall, involving changes in the activity of various enzymes, inflammation as well as platelet activation.⁴⁴ Thus, the mechanisms involved seem to be at least partially different to those in normotensive rats.

Our results demonstrate that Quin was effective in decreasing platelet adhesion to collagen *ex vivo*. According to literature data, in Quin-treated animals platelet activation could be inhibited by NO, PGI₂ as well as by CYP2C9-dependent endothelium-derived hyperpolarizing factor (EDHF).⁴⁵⁻⁴⁸ Interestingly, in our Quin-treated rats, the plasma NO level and aortic iNOS expression were reduced, but aortic eNOS expression was unchanged. Although it was documented in experimental and clinical studies that ACE-Is increase expression of eNOS consequently leading to NO bioavailability augmentation, we think that the antithrombotic and antiplatelet effect of Quin observed in our hypertensive rats is mediated via downregulated iNOS

and oxidative stress reduction, than by eNOS up-regulation and NO bioavailability increase. Moreover, data concerning the regulation of eNOS expression in hypertensive rats are not consistent, since decreased, increased and unchanged eNOS expression have been reported.^{49–53} Therefore, the NO bioavailability in hypertension may not be directly affected by eNOS expression. Chou et al. showed that in spontaneously hypertensive rats (SHR) the activity and expression of eNOS is reduced, whereas the expression of iNOS is markedly increased (due to increased oxidative stress), thus the sum of NO production may be unchanged or even enhanced.⁵⁴ The increased expression of iNOS in SHR was paralleled by augmented NO₂/NO₃ plasma level. Therefore, the higher basal NO₂/NO₃ levels in SHR were most likely mediated by iNOS rather than by eNOS. What is more, the exaggerated iNOS expression was attenuated with 2 weeks treatment of Quin. The authors suggested that the mechanism by which Quin reduced iNOS expression might be associated with the reduction of superoxide production. Similarly, Bachetti et al. reported that Quin downregulated iNOS with a possible TNF- α -mediated mechanism in normotensive rats.⁵⁵ Our previous studies showed that the antithrombotic effect of Quin is not fully dependent on eNOS activity in hypertensive rats, while in normotensive rats this dependence seems to be the predominant mechanism of the NO level increase.^{1,3} Therefore, in our study NO seemed not to be the leading vasorelaxant factor during chronic Quin treatment. Similarly to EDHF-dependent platelet inhibition after ACE-I treatment, literature data confirm the involvement of EDHF-dependent relaxation in the improvement of endothelial function. Moreover, as expected, the prolongation of bleeding time was not significant since vasorelaxation and BP lowering after Quin led to bleeding time shortening due to decreased blood flow, but not due to the inhibition of thrombosis. The observed here Quin-induced reduction in aortic iNOS expression was accompanied by the reduction of superoxide production. Quin administration to 2K1C rats decreased the level of MDA in plasma and NADPH oxidase activity in aortic rings. It seems that the antioxidant function of Quin is modified through lowering Ang II formation or promoting Ang-(1-7) production, which reduces the increase in ROS and phosphorylation of c-Src kinase by Ang II in intact endothelial cells.^{56–58} This thesis is supported by our previous results in HUVECs, where oxidative stress and NO bioavailability reduction was observed after Quin.²⁸

Our observations also support the concept that antithrombotic properties of Quin depend on its fibrinolytic activity, including a significant decrease in PAI-1 concentration and function and an increase in euglobulin clot lysis time in the mechanisms dependent on the inhibition of Ang II synthesis, as well as a Bk-dependent increase in t-PA concentration, which is a potent stimulus in the human and animal vasculature.^{2,59–65}

For the first time, the influence of Pro on the coagulation process in hypertensive rats was evaluated. Pro alone did not affect thrombus weight. However, it decreased occlusion time as well as incidents of persistent vascular patency, probably by its antifibrinolytic and prooxidative action.

In the present study, Pro alone did not significantly influence platelet adhesion to collagen *ex vivo*, although it significantly increased the expression of aortic iNOS and NO metabolite levels in plasma. On the other hand, De La Cruz et al. showed that both erythrocytes and leukocytes increased the anti-aggregatory effect of Pro in the mechanism dependent on leukocyte production of NO.²⁷ In our study, platelet adhesion was assessed separately in the platelet-poor plasma deprived of leukocytes and RBC. Therefore, in the absence of leukocyte-secreted NO, Pro did not affect platelet adhesion. On the other hand, Chung et al. observed, that Pro in concentrations required for sedation and general anesthesia had no inhibitory effect on platelet aggregation after 3 h of incubation.²³

We used Pro in hypertensive rats with intensified oxidative stress. In face of increase in NADPH oxidase activity, NO as well may be converted into superoxide anions, e.g. peroxynitrite (ONOO⁻).^{66,67} Taken together, the upregulation in aortic iNOS expression, with the increase of NO plasma level may be a source of ROS, which is in line with H₂O₂ and MDA increase after Pro treatment. Pro also increased SOD activity in aortic rings and H₂O₂ production. On the other hand, higher SOD activity catalyzed dismutation of strongly toxic superoxide anion to less toxic H₂O₂. Thus, it seems that the rise in SOD activity protects NO from degradation by superoxide anion and increases the concentration of H₂O₂, which is believed to be a member of EDHF.⁶⁸

For the first time we proved that Pro has a strong antifibrinolytic activity in the animal model *ex vivo*. This effect may considerably contribute to the increased incidence of thrombus formation despite elevated NO bioavailability after Pro. On the other hand, intensification of oxidative stress by Pro resulting in endothelial dysfunction may lead to further impairment of the fibrinolytic potential. This thesis is supported by our previous results in HUVEC cultures, where t-PA reduction, PAI-1, oxidative stress, and NO synthesis potentiation also occurred after Pro.²⁸

The dose of Pro used in our study reached plasma concentration that allows rat anesthesia (2.8 μ g/mL) and is clinically relevant, since it has been estimated that plasma concentration in humans ranges 2–10 μ g/mL.^{20,69} Pro significantly reduced SBP and DBP. In our opinion, this is a combined effect of myocardial depression (SBP-dependent), decreased sympathetic activity, direct endothelium-derived vascular relaxation (DBP-dependent), activation of the kinin–kallikrein system, and increased NO bioavailability.^{18,70–72}

We observed that the beneficial effect of Quin on thrombus weight was abolished after Pro infusion, but it was not higher than in the Veh group. This can be explained by an unfavorable influence of Pro on the mechanisms involved in thrombus formation (fibrinolysis and oxidative stress), and a cumulative antihypertensive effect of both drugs. Pro also decreased the number of rats with patent flow as compared to the Quin pre-treated animals, although this effect was still weaker than the one caused by Pro alone. Surprisingly, the two drugs administered jointly had a stronger inhibitory effect on platelet adhesion than Quin alone. The mechanism that potentiates platelet adhesion in the presence of both drugs should be further investigated. There was also no change in BT after Quin and Pro in comparison to Veh. We can explain this strong hypotension and vasodilatation after both drugs, because according to our observations these hemodynamic changes slowed down blood flow, which resulted in BT shortening.

The important finding in our study was an increase in animal mortality (by 38%) in the group treated with both drugs. Previously, we and others observed more episodes of hypotension in patients treated with Pro after therapy with ACE-Is that may be associated with the endothelium-derived EDHF release and additional NO production after Pro administration.^{20,70} Higher mortality of animals after Pro may be also the result of ventilation impairment with a considerable effect on central chemoreceptor sensitivity.⁷³ Also a significant fall in hemoglobin concentration and hematocrit was observed 30 min after induction of anesthesia in patients who received increasing doses of Pro.^{74,75} Summing up, it seems that in our model the final effects of Quin, Pro and both drugs given together on arterial thrombosis comprise the influence of drugs on fibrinolysis, primary hemostasis and oxidative stress as well as systemic hemodynamic parameters.

Conclusion

The data presented here may offer an additional explanation of Quin antithrombotic activity in renal-hypertensive rats. Our study demonstrates beneficial effects of Quin on platelet adhesion and fibrinolysis. Pro alone did not influence the thrombotic process, although it showed strong antifibrinolytic and prooxidative actions. Interestingly, pretreatment with Quin decreased the unfavorable effects of Pro in hemostasis, but caused hemodynamic disorders.

Perspectives

Despite the fact that patients receiving both ACE-Is and Pro may suffer from hypotension, ACE-Is prevent hemostasis disorders caused by Pro. Considering all these problems, we suggest that the most beneficial action is not to eliminate the dose of Quin before surgery but to prevent the occurrence of intraoperative hypotension.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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