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Original research article

Effect of quinolinic acid – A uremic toxin from tryptophan metabolism – On hemostatic profile in rat and mouse thrombosis models

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ABSTRACT

Purpose: We aimed to determine the effect of quinolinic acid (QA) on hemostasis in rat and mouse models of thrombosis.

Material and methods: Wistar rats (male, n = 72) received QA dissolved in drinking water in doses of 3, 10, 30 mg/kg or pure drinking water (vehicle control group -VEH) for 14 days. On the 14th day of the experiment the effect of QA on hemostasis was evaluated using electrically induced arterial thrombosis model. The following parameters were measured: thrombus weight, hematology, thromboelastometric (ROTEM) parameters, TXA_2 and 6-keto-$PGF_{1\alpha}$ concentration, coagulation and fibrinolytic markers activity and concentration.

GFP mice (male, n = 30) were assigned to the group receiving QA (30 mg/kg) or VEH for 14 days and to the group receiving: single intravenous dose of QA (30 mg/kg) or VEH or the same dose of QA and anti-CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1) antibody conjugated with Alexa Fluor 647. The effect of QA on hemostasis was evaluated in the model of laser-induced injury of mesentery vein using intravital confocal microscopy.

Results: Administering QA for 14 days resulted in a divergent, depending on dose, increase in concentration of active form of tPA and PAI-1 and concentration of total PAI-1 and PAP complexes in rats' plasma. In turn, administering QA for 14 days in mice revealed its prothrombotic activity, while single-dose IV administration revealed its antithrombotic activity, through the up-regulation of PECAM-1 expression.

Conclusions: We demonstrated the first evidence for the opposite biological effects of QA on hemostasis in rat and mouse thrombosis models.

1. Introduction

Quinolinic acid (QA) is one of the most important metabolites of tryptophan degradation in the kynurenine pathway [1] in many cell types and organs, including the central nervous system (CNS) and peripheral organs [2]. Under physiological conditions, QA is produced from the precursor 2-amino-3-carboxymuconic-6-semialdehyde (ACMS) at equilibrium with picolinic acid [3]. However, when its competing enzyme 2-amino-3-carboxymuconic-6-semialdehyde decarboxylase (ACMSD) is saturated, inactive, or absent, ACMS is non-enzymatically converted into QA [4].

Next, QA is broken down by quinolinate phosphoribosyl transferase (QPRT) to essential co-factor nicotinic acid mononucleotide (NAD^+) [5]. Under inflammatory conditions, the kynurenine pathway is stimulated both in the brain and in the periphery [6]. In both cases, elevated levels of QA can lead, acutely or chronically, to toxic effects via several mechanisms. Within the CNS, QA biological activity is primarily associated with endogenous cytotoxicity by its activation of N-methyl-D-aspartate (NMDA) receptors and mitochondrial impairment, but also involves additional targets that could be independent of its agonist activity, resulting in cellular energetic dysfunction, oxidative stress, inflammation, and cell death [7]. QA-induced toxicity in the brain is responsible for the development and progression of many neurodegenerative diseases [8].

In the periphery, QA is produced in the liver, kidney, circulating monocytes, and monocyte-derived macrophages (MDMs) or endothelial cells (EC) [9,10]. Likewise, QA-induced toxicity in the periphery is associated with inflammation, leading to multiple organ pathologies

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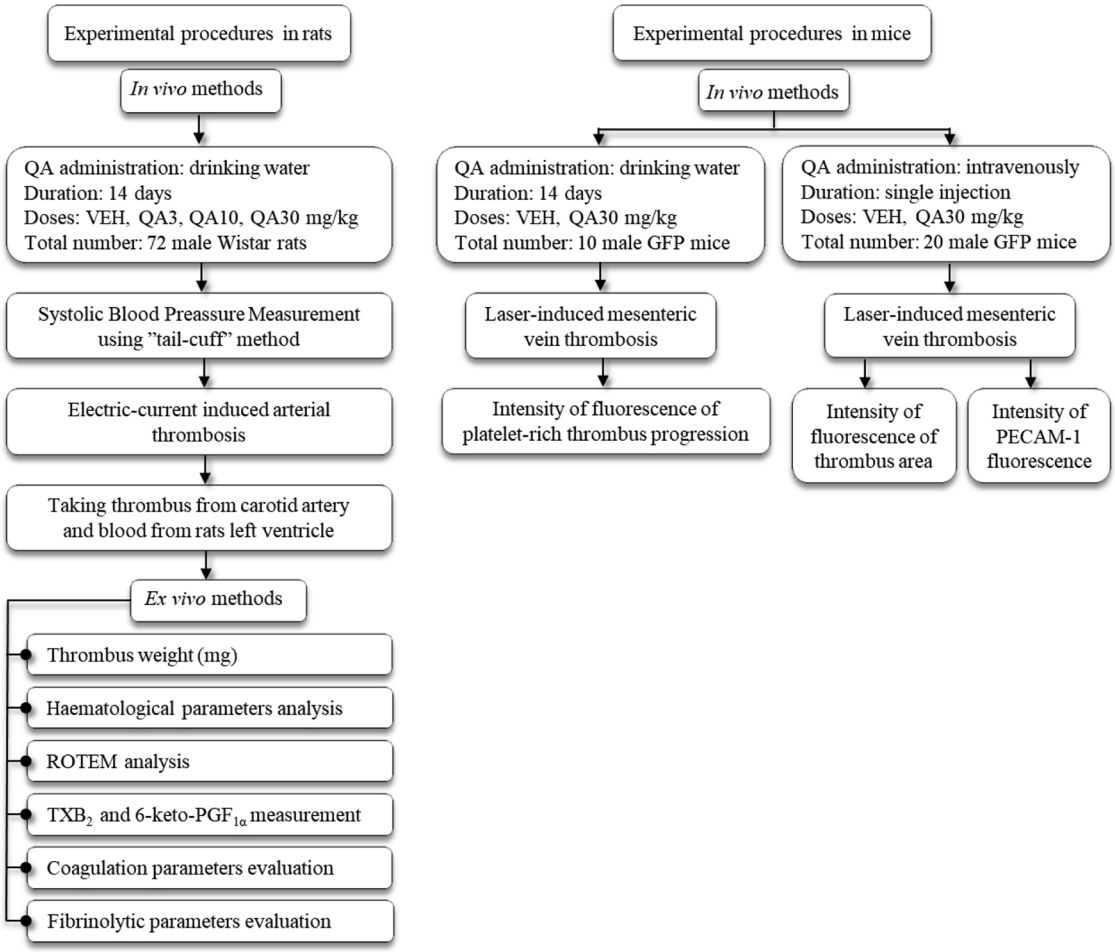

Fig. 1. Schematic presentation of experimental procedures in rats and mice.

[6,11]. QA is markedly accumulated in the blood and cells of patients with chronic kidney disease (CKD) due to both inflammation and impaired kidney function [12]. Furthermore, QA promotes atherosclerosis in CKD by activating oxidative stress in endothelial and vascular smooth muscle cells and leukocytes [13].

Experimental studies indicated QA contribution to cardiovascular diseases, especially an increased concentration of QA was linked to carotid atherosclerosis [14] and endothelial dysfunction [15] in patients with impaired renal function. More recently, a number of opposite biological activities of QA have been described, including its contribution to hypercoagulability [16,17] or hyperfibrinolysis [18] in patients with chronic renal failure. Coagulation disorders with either episodes of severe bleeding or thrombosis represent an important cause of morbidity and mortality among CKD patients [19].

Since we were most interested in establishing whether blood coagulation and fibrinolysis parameters were sensitive to QA, we undertook these studies to investigate the influence of QA on the hemostatic profile in two animal models of thrombosis *in vivo*.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Wistar Cmdb:Wi) 6 weeks old, weighting 190–200 g and green fluorescent protein (GFP)-expressing transgenic mice 4 weeks old, weighting 19–20 g (derived from Jax®Mice strain 003291, C57BL/6-Tg(CAG-EGFP)1Osb/J) were purchased from the Centre of Experimental Medicine, Medical University of Bialystok (Poland), Poland. Rats and mice were grouped in individually ventilated cages (IVC) and maintained under specific pathogen free (SPF) conditions in an environment-controlled room (12 h light/ 12 h dark cycle, 21 ± 2 °C, 55% relative humidity, 15 air changes/h) and had access to sterilized tap water and standard rat or mice chow *ad libitum*.

2.2. Ethical issues

All experimental procedures involving animals were approved by the Local Bioethics Committee on Animal Testing at the Medical Univerisity of Bialystok, Poland (Permit Number: 33/2013, 31/2015, 125/2015) and were conducted in accordance with the institutional guidelines, which are in compliance with national and international laws, including EU Directive 2010/63/EU for animal experiments and the *Guidelines for the Care and the Use of Animals in Biomedical Research* (Giles, 1987). The 3R rule ("Replacement, Reduction and Refinement") was respected in the study.

2.3. Chemicals

Bioreagents used in the experiment, such as QA, calcium chloride ($CaCl_2$) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (Germany). Agents used in the experiment, including pentobarbital, ketamine and xylazine were purchased from Biowet (Poland), and acetylsalicylic acid (ASA) was purchased from Sigma-Aldrich, Germany. Ready-to-use kits for blood cell count were purchased from HoribaABX (Germany), ELISA kits for rat plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator (t-PA), urokinase-type plasminogen activator receptor (uPAR), plasmin-antiplasmin complex

(PAP-complex) were purchased from Innovative Research (USA), in turn thromboxane B_2 (TXB_2) ELISA kit was purchased from ENZO Life Science (USA), and 6-keto-prostaglandin F1-alpha(6-keto-$PGF_{1\alpha}$) ELISA kit was purchased from Cayman (USA). Reagents used to measure prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (Fg) levels were purchased from HemosIL Instrumentation Laboratory, USA. Anti-CD31 (platelet endothelial cell adhesion molecule, PECAM-1) antibody conjugated to Alexa Fluor 647 was purchased from Abcam (UK).

2.4. Experimental procedures in rats

2.4.1. Experimental design

Since we were most interested in establishing whether hemostasis parameters were sensitive to QA, we first examined its activity in rats. Wistar rats were randomly assigned to the vehicle control group (VEH, n = 18), comprising of rats receiving pure drinking water, and to the QA group (n = 18 rats/group), comprising of rats receiving QA dissolved in drinking water in doses of 3, 10, 30 mg/kg of body weight per day for 14 days. To ensure the reliability and to check the repeatability of the experiment results, we conducted the experiment twice. The experimental procedures are illustrated in Fig. 1. One day before the induction of arterial thrombosis, the rats underwent monitoring of blood pressure (BP) using the tail-cuff method. At the day of the experiment, the rats were anesthetized with pentobarbital (50 mg/kg) and subjected to electrical arterial injury. The formed thrombus was dissected and blood samples were collected into anticoagulant 3.13% trisodium citrate. The volume of blood needed for hematological analysis, platelet activation (TXB_2) and ROTEM (thromboelastometry) analysis was taken from the sample. Plasma was prepared by centrifuging the blood at 3500 × g for 20 min at room temperature.

2.4.2. Rationale for dose level selection in rats

We aimed to investigate if 14 days of QA administration and dose-related exposure will affect hemostasis. Because there was no relevant literature available, we selected doses in logarithmic dose sequences of 3, 10, and 30 mg/kg. In preliminary experiments with rats, we determined that the average daily consumption of water (both with or without QA) did not exceed 30 ml/day/rat weighting 190–200 g (under the same experimental conditions for 2 rats housed in one cage). According to these calculations, all QA concentrations were prepared "*ex tempore*" in drinking water each day of the 14 days of the experiment and were adjusted to the rats' average daily water consumption and body weight.

The rats were observed daily for survival, behavioral and clinical signs of toxicity (vocalization, aggression, personal hygiene, posture, appearance of eyes, ears, tail, fur, paws and urine or feces).

2.4.3. Measurement of blood pressure in rats

One day before electrical arterial injury, the rats underwent BP monitoring to examine if QA affected systolic BP (SBP). SBP was measured using the tail-cuff method (Non-invasive Blood Pressure System, Panlab, Harvard Apparatus, USA) in conscious rats [20]. Each value was calculated as the average of three consecutive readings.

2.4.4. Induction of thrombosis in rats

Anesthetized rats were placed in a supine position on a heated (37 °C) operating table. Arterial thrombosis was induced by electrical stimulation of the right common carotid artery as previously described [21,22]. Briefly, the artery was exposed and placed on a stainless steel L-shaped wire (anode). The cathode was attached subcutaneously to the hindlimb. A piece of parafilm M (Bemis, EU) was inserted under the artery for electrical isolation. Artery stimulation (1 mA) took 10 min. The anode was removed after termination of stimulation. For the next 45 min, thrombus developed *in vivo* and then the formed thrombus was dissected with the segment of the common carotid artery and completely removed, dried at room temperature, and weighted after 24 h.

2.4.5. Hematological analysis in rats

Blood was collected from the animals by cardiac puncture into anticoagulant 3.13% trisodium citrate (volume ratio 10:1).Blood cells count, including platelets (PLT), white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume(MCV), mean corpuscular hemoglobin(MCH), mean corpuscular hemoglobin concentration (MCHC) was performed in automated hematology analyzer (Scil Horiba Vet ABC Plus, Horiba, Germany) immediately after blood drawing.

2.4.6. Measurement of dynamic TXB_2 generationin rats

TXB_2 is a stable metabolite of platelet-derived thromboxane A_2 (TXA_2). The protocol for dynamic TXB_2 generation (stirring-stimulated platelet activation) in citrated whole blood samples was performed as described previously [23]. Whole blood samples were diluted with 0.9% NaCl at a volume ratio 1:1 and stirred in aggregation cuvettes at 1000 rpm and 37 °C. 60 min after stirring, samples of mixed blood were drawn off and mixed with a solution of ASA (final concentration: 500 mM) at volume ratio 1:1. Then the samples were stirred once more at 1000 rpm, 4 °C, 15 min. TXB_2 level in so-obtained plasma was measured using standard procedure and ready-to-use ELISA kit in microplate reader ELx808 (BioTek Instruments, Inc., United States).

2.4.7. Measurement of 6-keto-$PGF_{1\alpha}$ in rats

6-keto-$PGF_{1\alpha}$ is a stable hydrolysis product of endothelium-derived prostacyclin2 (PGI_2). 6-keto-$PGF_{1\alpha}$ levels were measured in rat plasma, using standard procedure and ready-to-use ELISA kit in microplate reader ELx808 (BioTek Instruments, Inc., United States).

2.4.8. Coagulation parameters and fibrin generation in rats

Coagulation parameters including PT, APTT, TT and Fg levels (Clauss method) were determined in rat plasma according to the kit manufacturer's instructions using the Coag-Chrom 3003 Coagulometer and standard reagents (Bio-ksel, Poland).

Fibrin generation was determined using the method described previously [24,25] and modified by us [26]. Fibrin generation was initiated by recalcination of rat plasma samples directly in microplate wells with $CaCl_2$ (36 mmol/L) dissolved in Tris buffer (66 mmol/LTris, 130 mmol/L NaCl; pH = 7.4, 37 °C). Fibrin generation was determined by measuring (microplate reader ELx808, BioTek Instruments, Inc., United States) increases of its optical density in 1-min intervals over 15 min and was expressed as changes of optical density (OD) and area under the curve (AUC).

2.4.9. Thromboelastometry analysis (ROTEM) in rats

Thromboelastometry analysis was performed using the ROTEM system (Tem International GmbH, Mannheim, Germany). ROTEM analyses were performed in whole blood *ex vivo,* according to the method described elsewhere [27,28]. An oscillating pin was suspended into a stationary cup that contained a whole blood sample. As the blood clots, fibrin strands link the pin to the cup. Clot strength was determined by measuring the amplitude of pin rotation, which increases proportionally with clot strength. The strength is dependent on fibrin polymerization and crosslinking, and platelet-fibrin interactions. Assessing the ROTEM analysis is performed along the time axis (from left to right) and provides information about all stages of the coagulation process which are described as: clotting time (CT), clot formation time (CFT), alpha angle (α), and maximum clot firmness (MCF). All the above-mentioned variables can be ordered as follows: CT – as the variable indicating clot formation (time from beginning of the test to the beginning of the fibrin polymerization process), CFT - provides information on the kinetics of clot formation, α – the angle showing the dynamics of clot formation, MCF – a parameter reflecting the strength

of the formed clot to resist the pin oscillation (displays clot stabilization). Coagulation was initiated with calcium chloride (12 mmol/L) in samples with blood taken from the control and the QA group and examined *ex vivo* with the NATEM test.

2.4.10. Fibrinolysis parameters in rats

Total plasma concentration of uPAR and PAP complexes, as well as total plasma concentration and concentration of active form of t-PA and PAI-1 were determined by ELISA techniques, at 25 °C using a microplate reader (ELx808, BioTek Instruments, Inc., United States) according to the manufacturer's instructions.

2.4.11. Statistical analysis of results in rats experiment

The data were analyzed using GraphPad Prism 6 (La Jolla, CA, USA). Graphic design presentation of the results was performed using GraphPad Prism 6.Normally distributed data were analyzed using an unpaired *t*-test and shown as MEAN ± SEM. Non-Gaussian data were presented as median (with maximum and minimum values) and analyzed using the non-parametric Mann-Whitney test. A value of $P < 0.05$ was considered as statistically significant.

2.5. Experimental procedures in mice

2.5.1. Experimental design

GFP mice were randomly assigned to a 14-day group receiving QA in drinking water and an acute group receiving QA intravenously. The 14-day group comprised of a control group (VEH) receiving pure drinking water, and QA group receiving QA dissolved in drinking water (30 mg/kg per day for 14 days). In turn, the acute group comprised of control mice (VEH) receiving a single IV injection of 0.9% NaCl, or the QA group receiving a single IV injection of QA at a dose of 30 mg/kg. GFP mice assigned to the 14-day VEH group (n = 5) or QA group (n = 5) underwent laser-induced thrombosis to measure fluorescence intensity of platelet-rich thrombus progression during 5 min of intravital recording and visualization. GFP mice assigned to the acute experiment underwent laser-induced thrombosis to measure fluorescence intensity of the thrombus area or PECAM-1 expression 10 min after IV injection of QA (n = 10) or VEH (n = 10). Experimental procedures are presented in Fig. 1.

2.5.2. Rationale for dose level selection in mice

In preliminary 14-day experiments with mice, we determined that the average daily consumption of water (with or without QA) does not exceed 5 ml/day/mouse weighting 19–20 g (under the same experimental conditions for 2 mice housed in one cage). QA concentrations in drinking water were adjusted to the average daily water consumption and body weight of the mice.

During 14 days of QA administration in drinking water, the mice were observed daily for survival, behavioral and clinical signs of toxicity (vocalization, aggression, personal hygiene, posture, appearance of eyes, ears, tail, fur, paws and urine or feces).

2.5.3. Induction of thrombosis in mice: intravital experiment

Intravital fluorescence confocal microscopy imagining was explored and modified as the basis of the protocol described by Falati et al. [29]. On the day of the confocal experiment, the mice were anesthetized with intraperitoneal ketamine and xylazine (120 mg/kg and 12.5 mg/kg, respectively).Mice received VEH (0.1 mL of 0.9% NaCl) or QA (30 mg/kg in 0.1 mL of 0.9% NaCl) alone or the same dose of QA and anti-CD31 (PECAM-1) antibody conjugated to Alexa Fluor 647 (0.05 mL of 0.9% NaCl) into the left femoral vein 10 min prior to laser injury of the mesentery vein. Next, a midline laparotomy incision was made, and then the mesentery of the ileum was pulled out of the abdomen and draped over a plastic mound. The mesentery vein was placed under an objective (W Plan-Apochromat 20×/1.0 water immersion objective, Carl Zeiss Microscopy GmbH, Germany) and identified. The mesentery was continuously perfused with 37 °C-warmed saline to prevent the vessels from drying. Endothelial injury was induced by pulsed 532 nm argon-ion laser beam (Ablate, 3iL33, Intelligent Imaging Innovations, Inc., USA) aimed through a microscope objective lens. During the experiment, GFP was excited by a 488 nm laser (LaserStack 488 nm, 3iL33, Intelligent Imaging Innovations-3I, Denver, CO, USA) to visualize the thrombus area or development (green emission), in turn PECAM-1 was excited by a 647 nm laser (LaserStack 647 nm, 3iL33, Intelligent Imaging Innovations-3I, Denver, CO, USA) to visualize PECAM-1 expression (red emission). Thrombus progression and PECAM-1 expression was registered for 5 min with the Confocal Scanner Unit CSU-X1 (Yokogawa Electric Corporation, Japan) in one focal plane (2D imaging) corresponding with the largest area of the thrombus. At most, three thrombi were induced in one mouse at particular time-points.

2.5.4. Image analysis

The results and images were analyzed with SlideBook6 (Intelligent Imaging Innovations, Inc., US). Relative changes in the fluorescence intensity of GFP platelet-rich thrombus after laser injury were analyzed during 5 min of recording. The thrombus area (μm^2) was analyzed 10 min after induction of thrombosis recorded for 5 min and was represented as a sum of fluorescent regions encircled every 15 s during recording. PECAM-1 intensity of fluorescence was calculated 10 min after induction of thrombosis recorded for 5 min as ratio of PECAM-1 to GFP fluorescence and was represented as a sum of fluorescent regions encircled every 15 s during 5 min of recording.

3. Results

3.1. General characterization of rats

QA did not affect water, food intake, and body weight parameters during 14 days of the experiment in rats. The average 14-day water intake in VEH rats (797.8 ± 31.54) was compared with the rats receiving QA in doses of 3 mg/kg (810.6 ± 18.75), 10 mg/kg (799.4 ± 33.95) and 30 mg/kg (804.7 ± 41.71). The average 14-day food intake in VEH rats (642.0 ± 18.37) did not differ from rats receiving QA in doses of 3 mg/kg (632.0 ± 23.62), 10 mg/kg (646.0 ± 22.63), and 30 mg/kg (618.6 ± 14.88). The average 14-day weight gain in VEH rats (70.89 ± 4.00) was compared with the rats receiving QA in doses of 3 mg/kg (67.78 ± 3.50), 10 mg/kg (67.83 ± 2.96), and 30 mg/kg (63.89 ± 2.78). Data are summarized in Table 1.

During 14 days of administration in drinking water, QA did not exert toxicological effects in the examined rats with respect to survival and behavioral and clinical observations.

Table 1
Effect of 14 days of quinolinic acid administration in drinking water on average 14-day water and food intake, and average 14-day weight gain in rats.

Parameter	VEH	QA 3 mg/kg/24 h	QA 10 mg/kg/24 h	QA 30 mg/kg/24 h
Water intake [ml]	797.8 ± 31.54	810.6 ± 18.75	799.4 ± 33.95	804.7 ± 41.71
Food intake [g]	642.0 ± 18.37	632.0 ± 23.62	646.0 ± 22.63	618.6 ± 14.88
Body weight [g]	70.89 ± 4.00	67.78 ± 3.50	67.83 ± 2.96	63.89 ± 2.78

Table 2
Effect of 14 days of quinolinic acid administration in drinking water on hemodynamic parameters, hematology parameters, and biochemical parameters in rats.

Parameter	VEH	QA 3 mg/kg/24 h	QA 10 mg/kg/24 h	QA 30 mg/kg/24 h
Hemodynamic parameters				
Systolic [mmHg]	161.0 ± 17.5	166.9 ± 16.2	174.5 ± 13.2	165.6 ± 21.3
Hematology parameters				
PLT [10^3/mm^3]	543.5 ± 8.45	548.5 ± 13.97	528.2 ± 10.62	529.3 ± 11.85
WBC [10^3/mm^3]	2.1 (1.4–3.4)	2.2 (1.7–3.7)	2.25 (1.6–3.2)	2.3 (1.7–2.9)
RBC [10^6/mm^3]	7.1 (6.1–7.9)	7.3 (6.8–7.6)	7.3 (5.9–7.8)	7.3 (6.7–8.0)
HGB [g/dL]	13.3 (11.6–14.4)	13.6 (12.7–14.3)	13.4 (7.6–14.1)	13.5 (12.5–14.2)
HCT [%]	40.7 (35.3–45)	41.7 (38.9–43.9)	41.7 (33.4–44.3)	41.9 (38.1–44.4)
MCV [μm^3]	58 (56–61)	58 (56–61)	57 (55–59)	57 (55–59)
MCH [pg]	18.78 ± 0.19	18.82 ± 0.13	18.59 ± 0.11	18.45 ± 0.10
MCHC [g/dL]	32.4 (31.4–33.3)	32.7 (31.8–33.4)	32.5 (31.8–34.2)	32.5 (31.4–33.2)
Biochemical parameters				
TXB2 [ng/mL]	47.48 ± 3.24	53.67 ± 6.58	51.01 ± 3.97	55.72 ± 4.36
6-keto-PGF$_{1\alpha}$[ng/mL]	320.6 ± 25.02	310.6 ± 19.99	355.5 ± 28.03	288.6 ± 14.41

VEH – control group; QA – quinolinic acid; PLT – platelets; WBC - white blood cells; RBC - red blood cells; HGB – hemoglobin; HCT – hematocrit; MCV - mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; TXB_2 _ thromboxane B_2; 6-keto-PGF$_{1\alpha}$ - 6-keto prostaglandin F$_{1\alpha}$.

3.2. Measuring blood pressure in rats

SBP was recorded using the non-invasive tail-cuff method in conscious rats one day before arterial thrombosis induction. SBP measurement provided a direct indication of the effect of 14-day QA administration on cardiovascular function. QA had no effect on SBP parameters in VEH rats (161.0 ± 17.5) versus rats receiving QA at all examined doses 3 mg/kg (166.9 ± 16.2), 10 mg/kg (174.5 ± 13.2), and 30 mg/kg (165.6 ± 21.3). Data are summarized in Table2.

3.3. Inducing thrombosis in rats

Arterial thrombosis was induced using electrical stimulation of the carotid artery. As a result of stimulation, platelet-rich thrombus was formed at the site of the injured artery with exposed collagen fibers. QA (3; 10; 30 mg/kg) administered rats did not develop statistically significant larger thrombus (1.12 ± 0.05; 0.93 ± 0.04; 1.06 ± 0.05, respectively) compared with the VEH group (1.03 ± 0.05) in a model of electrically-induced carotid artery thrombosis. Data are summarized in Table3.

3.4. Hematological analysis in rats

Hematological parameters were assessed to determine if QA had any pathogenic effect in the cardiovascular system. None of the tested QA doses (3; 10; 30 mg/kg) had changed the hematological parameters compared to VEH rats. Hematological data are summarized in Table 2.

3.5. Measuring dynamic TXB2 generation in rats

$TXB_2$is a stable metabolite of TXA_2, which is a potent platelet agonist released from platelet upon activation and aggregation. TXB_2 concentrations were measured to examine if QA had an impact on platelet activation and aggregation after 14 days of QA administration. TXB_2 concentration did not differ statistically significantly in the plasma of VEH rats (47.48 ± 3.24) compared with QA administered rats at all examined doses of 3 mg/kg (53.67 ± 6.58), 10 mg/kg (51.01 ± 3.97), and 30 mg/kg (55.72 ± 4.36). Data are summarized in Table 2.

Table 3
Effect of 14 days of quinolinic acid administration in drinking water on arterial thrombosis, coagulation parameters and fibrin generation, thromboelastometry parameters and fibrinolytic parameters in rats.

Parameter	VEH	QA 3 mg/kg/24 h	QA 10 mg/kg/24 h	QA 30 mg/kg/24 h
Arterial thrombosis				
Thrombus weight [mg]	1.03 ± 0.05	1.12 ± 0.05	0.93 ± 0.04	1.06 ± 0.05
Coagulation parameters and fibrin generation				
PT [s]	9.9 (9–10.9)	9.7 (9.3–10.6)	9.8 (9–10.9)	9.8 (9–10.7)
QUICK [%]	97.88 ± 2.33	96.59 ± 1.8	97.35 ± 1.7	96.76 ± 2.0
aPTT[s]	25.3(15.4–37.4)	24.6 (16.8–37.8)	25.1 (15.5–38.7)	22.7 (18.4–37.4)
TT [s]	28.46 ± 0.87	28.24 ± 0.74	27.97 ± 0.79	28.52 ± 0.65
Fibrinogen [g/l]	1.26 (0.83–1.52)	1.25 (1.06–1.36)	1.24 (1.05–1.41)	1.22 (0.89–1.32)
Fibrin generation [AUC]	4298 ± 125.2	4081 ± 139.9	4283 ± 117.8	4233 ± 112.5
Fibrin generation [OD]	2865 ± 83.45	2781 ± 76.16	2855 ± 78.54	2822 ± 74.99
Thromboelastometry analyses				
CT [s]	232.1 ± 11.14	229.9 ± 12.86	237.0 ± 11.11	272.4 ± 17.69
CFT [s]	70.07 ± 4.64	63.12 ± 4.24	66.27 ± 3.03	74.41 ± 4.47
α [angle]	77.13 ± 0.74	78.06 ± 0.72	77.67 ± 0.48	76.24 ± 0.77
MCF [mm]	73.06 ± 0.87	72.18 ± 0.86	72.94 ± 0.98	71.63 ± 0.70
Fibrinolytic parameters				
tPA total [ng/mL]	0.74 ± 0.05	0.79 ± 0.05	0.70 ± 0.03	0.74 ± 0.05
uPAR [pg/ml]	1.17 ± 0.06	1.15 ± 0.06	1.21 ± 0.05	1.19 ± 0.04

VEH – control group; QA – quinolinic acid; PT – prothrombin time; aPTT– activated partial thromboplastin time; TT – thrombin time; AUC – area under the curve; OD – optical density; CT – clotting time; CFT – clot formation time; α – Alpha angle; MCF – maximum clot firmness; tPA – tissue plasminogen activator; uPAR – urokinase-type plasminogen activator receptor.

3.6. Measuring 6-keto-PGF$_{1\alpha}$in rats

6-keto-PGF$_{1\alpha}$ is a stable hydrolysis product of PGI$_2$. Measuring 6-keto-PGF$_{1\alpha}$ concentrations was aimed to examine if QA had an effect on vascular PGI$_2$ production. PGI$_2$ has directly opposing actions to TXA$_2$, and both are responsible for thromboxane-prostaglandin balance, reflecting the current thrombotic state. There were no statistically significant changes in 6-keto-PGF$_{1\alpha}$ concentration in the plasma of VEH rats (320.6 ± 25.02) versus QA administered rats in doses of 3 mg/kg (310.6 ± 19.99), 10 mg/kg (355.5 ± 28.03), and 30 mg/kg (288.6 ± 14.41). Data are summarized in Table 2.

3.7. Coagulation parameters and fibrin generation in rats

Standard clotting assays in plasma did not detect statistically significant differences in starting times of clotting. PT time was comparable in VEH rats (9.9 (9–10.9)) and QA (3; 10; 30 mg/kg) administered rats (9.7 (9.3–10.6); 9.8 (9–10.9); 9.8 (9–10.7), respectively). Similarly, both APTT time in VEH rats (25.3(15.4–37.4)) and QA (3; 10; 30 mg/kg) administered rats (24.6 (16.8–37.8); 25.1 (15.5–38.7); 22.7 (18.4–37.4), respectively) as well as TT time in VEH rats (28.46 ± 0.87) and QA (3; 10; 30 mg/kg) administered rats (28.24 ± 0.74; 27.97 ± 0.79; 28.52 ± 0.65, respectively) did not show statistical significance. Also, Fg concentration, which depends on plasma coagulation properties, had comparable values in VEH rats (1.26 (0.83–1.52) and all other groups of rats administered with QA *i.e.*3 mg/kg (1.25 (1.06–1.36)), 10 mg/kg (1.24 (1.05–1.41)) and 30 mg/kg (1.22 (0.89–1.32)).

Fibrin generation assay, dependent on time and clotting factors concentration, was also unaffected by QA. No statistically significant differences were found regarding fibrin generation between VEH rats compared with QA (3; 10; 30 mg/kg) administered rats, as a result of AUC (4298 ± 125.2 *vs.* 4081 ± 139.9; 4283 ± 117.8; 4233 ± 112.5, respectively) as well as changes of the OD in recalcinated rats plasma (2865 ± 83.45 *vs.* 2781 ± 76.16; 2855 ± 78.54; 2822 ± 74.99, respectively). Data are summarized in Table 3.

3.8. Thromboelastometry analyses (ROTEM) in rats

Thromboelastometry parameters were analyzed in the whole blood of rats. ROTEM provides information on the whole kinetics, including the dynamics of clot development and its stabilization, that reflects *in vivo* hemostasis. No statistically significant differences were found between VEH rats versus QA 3, 10 and 30 mg/kg administered rats regarding the following ROTEM parameters: CT (232.1 ± 11.14 *vs.* 229.9 ± 12.86; 237.0 ± 11.11; 272.4 ± 17.69, respectively); CFT (70.07 ± 4.64 *vs.* 63.12 ± 4.24; 66.27 ± 3.03; 74.41 ± 4.47, respectively); α angle (77.13 ± 0.74 *vs.* 78.06 ± 0.72; 77.67 ± 0.48; 76.24 ± 0.77) as well as MCF (73.06 ± 0.87 *vs.* 72.18 ± 0.86; 72.94 ± 0.98; 71.63 ± 0.70, respectively). Data are summarized in Table 3.

3.9. Fibrinolytic parameters in rats

Fibrinolytic parameters are responsible for plasma fibrinolytic activity and along with coagulation parameters regulate the thrombosis process. Fourteen days of QA administration in drinking water led to a statistically significant increase in the following parameters: concentration of active form of tPA in rats receiving QA 3 mg/kg compared with VEH rats (0.57 ± 0.02 *vs.* 0.47 ± 0.02; $p < 0.001$***; Fig. 2A.); total PAI-1 plasma concentration in rats receiving QA 10 and 30 mg/kg compared with the VEH group (1.02 ± 0.08 and 1.08 ± 0.08 *vs.*0.82 ± 0.04, respectively; $p < 0.05$*; Fig. 2B.); concentration of active form of PAI-1 in rats receiving QA 30 mg/kg compared with the VEH group (0.70 ± 0.05 *vs.*0.52 ± 0.07; $p < 0.05$*; Fig. 2C.); as well as total PAP complexes plasma concentration in rats receiving QA 3, 10 and 30 mg/kg compared with VEH (1.81 (1.25–2.75), 1.81 (1.18–2.91) and 1.96 (1.25–2.83) *vs.* 1.45 (1.12–2.26), respectively; $p < 0.05$*; Fig. 2D.). No statistically significant differences were found regarding total tPA plasma concentration between VEH rats (0.74 ± 0.05) and QA (3 mg/kg; 10 mg/kg and 30 mg/kg) treated rats (0.79 ± 0.05; 0.70 ± 0.03; 0.74 ± 0.05, respectively) as well as none of the examined doses of QA had affected total uPAR plasma concentration compared with the VEH rats (1.15 ± 0.06; 1.21 ± 0.05; 1.19 ± 0.04 *vs.* 1.17 ± 0.06; Table 3).

3.10. General characterization of mice

During 14 days of administration in drinking water, QA did not exert toxicological effects in the examined rats with respect to survival, behavioral and clinical observations. The examined dose of QA did not affect water, food intake, and body weight parameters during 14 days of the experiment in mice (data not shown).

3.11. Inducing thrombosis in mice: intravital experiment

The laser-induced mesenteric vein thrombosis model produces platelet-rich thrombus at the site of the injured vessel. We observed an opposing effect of QA on thrombus formation, which was dependent on the duration of QA administration. Namely, 14 days of QA (30 mg/kg) administration in drinking water resulted in increased fluorescence intensity of platelet-rich thrombus progression in GFP mouse mesenteric vein *vs.* the VEH group (39.73 ± 5.95 *vs.* 15.20 ± 0.46; $p < 0.001$***; Fig. 3). In turn, IV administration of QA (30 mg/kg) resulted in decreased fluorescence intensity of the platelet-rich thrombus area 10 min after the laser injury of mouse mesenteric vein *vs.* the VEH group (1,470,000 ± 339,427 *vs.* 4,397,000 ± 458,551; $p < 0.01$**; Fig. 4). Moreover, the antithrombotic effect of QA given at the same scheme and dose was confirmed once more by increased intensity of the ratio of PECAM-1 to GFP fluorescence of platelet-rich thrombus in GFP mice *vs.* the VEH group (24.92 ± 1.72 *vs.* 17.79 ± 0.03; $p < 0.01$**; Fig. 5).

4. Discussion

In the present study, we show that QA can modulate hemostasis, and its effect depends on the administered dose, time, and route of administration. The effect of QA on hemostasis was analyzed in two experimental models of thrombosis. The first one involved electrical stimulation of rat artery and the second one involved laser stimulation of mouse mesenteric vein. In both of these models, hemostatic parameters make a major contribution to the development of thrombosis [30], thus both are useful to determine the activity of coagulation and fibrinolysis in the presence of the investigated agent. We also used both models of thrombosis due to advantages of using different species, which in turn allows to measure different spectrum of biological parameters. Namely, blood and plasma samples collected from rats give enough volumes to measure broad spectrum of various parameters, including cell counts or protein levels and activity. In turn, mice, giving too little volumes of blood samples for such biochemical analysis, are an ideal animal model dedicated to confocal microscopy and allow to measure kinetics of thrombus formation, thrombus area and expression of selected markers simultaneously in real time, using relatively smaller amounts (adjusted to mice body weight) of ready to use antibodies labeled with fluorescent dye. The limitation of both models is that we cannot compare the investigated parameters in both species, nevertheless still we can have an insight into the parameters of thrombosis which rely on cell-plasma or cell-cell interactions. So far, QA has not been investigated in this regard, therefore we examined its effect on hemostasis after 14 days of administration in drinking water in rats and mice, as well as after a single IV administration in mice.

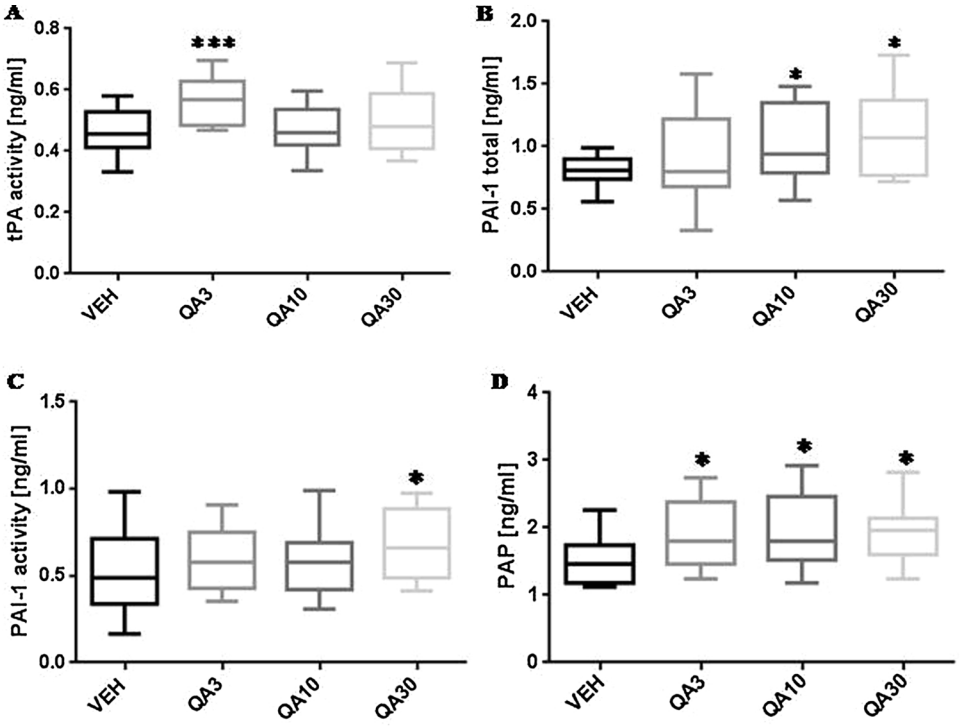

Fig. 2. Effect of 14-day QA administration in drinking water on [A] tPA (tissue plasminogen activator) activity; [B] PAI-1 (plasminogen activator inhibitor type 1) concentration; [C] PAI-1 activity; [D] PAP (plasmin-antiplasmin complex) concentration in rats. VEH – control group; QA3, QA10, QA30 – doses of quinolinic acid (mg/kg/24 h); $p < 0.001$*** compared with VEH; $p < 0.05$* compared with VEH.

Since we were most interested in establishing whether blood coagulation and fibrinolysis parameters were sensitive to QA, first we tested QA in a 14-day experiment. In our study QA did not induce changes in BP or hematological parameters, which allowed us to investigate the direct effects of QA on hemostasis after 14 days of administration in drinking water using a model of electrically-induced arterial thrombosis in rats. This model was well-characterized in the study by Schumacher et al. [21] and it combines both platelet and fibrin-rich thrombus formation, involving the activation of coagulation and fibrinolysis. We found no differences between the 14-day group administered with QA and the VEH group in terms of thrombus weight (which will be discussed below).

Next, we tested platelet activity and the hemostatic profile of rats using a wide-range of assays. QA was devoid of statistically significant effects on TXB_2 and 6-keto-$PGF_{1\alpha}$ plasma concentration, which suggested that QA did not affect the balance between prothrombotic platelet-derived $TXA_2$and antithrombotic vascular-derived PGI_2. Furthermore, QA administration had no impact on standard coagulation screening tests and ROTEM (thromboelastometry) analyses. Clotting assays evaluating the extrinsic pathway of coagulation such as PT, and intrinsic pathway of coagulation, such as APTT, or TT, designed to assess fibrin formation from fibrinogen as well as fibrin concentration in rat plasma, were not affected by QA in a model of electrically-induced thrombosis. These results also corresponded with unchanged parameters of fibrin generation in QA plasma, calculated as AUC or OD, compared with VEH rats. Similarly, ROTEM parameters which

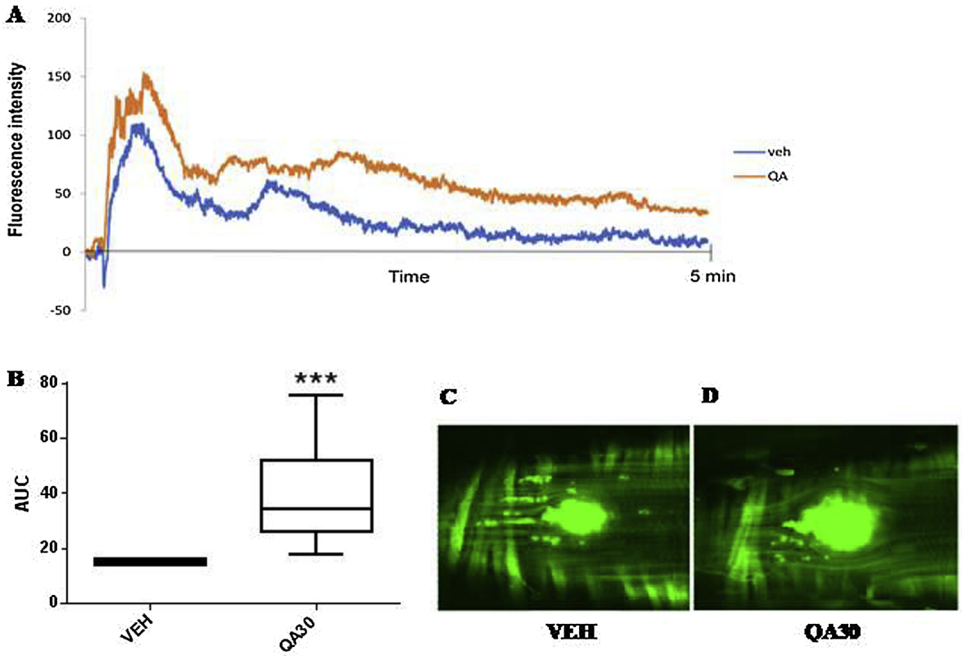

Fig. 3. Effect of 14-day QA administration in drinking water on GFP platelet-rich thrombus formation represented as [A] relative fluorescence intensity and [B] area under the curve (AUC) of the intensity of fluorescence of the thrombus formed at the site of the laser-injured mesenteric vein in GFP mouse. Intensity of fluorescence was recorded during 5 min of thrombus formation using confocal microscopy. [C, D] representative images of the GFP platelet-rich thrombus (green) formed in a mouse mesenteric vein. VEH – control group; QA30 – dose of quinolinic acid (mg/kg/24 h); $p < 0.001$*** compared with VEH. Water immersion, x200.

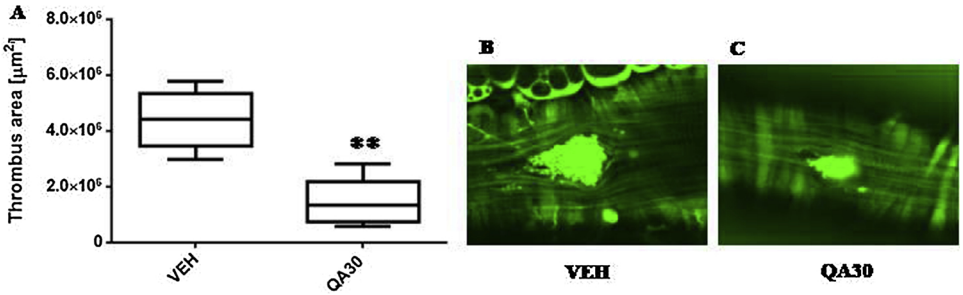

Fig. 4. Effect of intravenous QA administration on [A] area of GFP platelet-rich thrombus formed at the site of laser-injured mesenteric vein in mice. Intensity of fluorescence was recorded 10 min after QA administration for 5 min. [B] representative images of the GFP platelet-rich thrombus (green) formed in a mouse mesenteric vein. VEH – control group; QA30 – dose of quinolinic acid (mg/kg); $p < 0.01$** compared with VEH. Water immersion, x200.

determine the contributions of platelets and plasma factors to fibrin clot formation in the whole blood also proved that all stages of clot formation, beginning from time of plasma clotting factor activation (CT), their influence on the dynamic of clot formation (α angle, CFT) and its final stabilization (MCF) were not changed by QA at all examined doses.

According to the data presented above, 14 days of QA administration had no impact on coagulation parameters activation, however it had a contradictory effect on fibrinolytic profile in rats. Firstly, the results showed that QA at the lowest dose (3 mg/kg) increased the concentration of active form of tPA, and at the highest dose (30 mg/kg) it increased concentration of active form of PAI-1 and also at the highest doses (10 and 30 mg/kg) it increased total PAI-1 plasma concentration, while at all examined doses QA increased total PAP complexes concentration in rat thrombotic plasma. Secondly, QA at the lowest dose exerted pronounced pro-fibrinolytic activity (increasing tPA activity), while at the higher doses QA was devoid of statistically significant effects on tPA activity as well as tPA and uPAR concentration. This discrepancy between antigen and activity levels can be attributed to the fact that tPA is the principle activator of plasminogen within vascular thrombolysis [31], whereas uPA has a major function in tissue-related proteolysis and plays a secondary role to tPA in clot lysis [32]. tPA is further directed into the inhibitory pathway by PAI-1, or it can, alongside plasminogen, bind to fibrin.

According to our results described above, QA at higher doses increased PAI-1 activity and concentration in rat plasma. PAI-1 is the most efficient inhibitor of tPA in plasma and normally there is a regulated balance between the formation of fibrin and its subsequent removal. Based on these results, we can conclude that 14 days of QA administration displayed divergent, depending on the dose, effects on the activity of circulating activators and inhibitors of fibrinolysis. Moreover, we can assume that QA also had an impact on plasmin activation and deactivation at the fibrin site through the following sequence: during hemostasis, tPA - if not inhibited by PAI-1, activates plasminogen to plasmin, which then degrade fibrin into fibrin degradation products (FDPs) or undergo inactivation by alpha2-antiplasmin (α2AP).

Both α2AP and PAI-1 have a crucial role in reducing plasmin production and activity and thus inhibiting fibrinolysis [33]. On the contrary, it was previously reported that the presence of PAP complexes in the circulation provides evidence of the activation of plasminogen to plasmin by tPA *in vivo* [34]. Our results are in accordance with this data. Namely, QA evidently increased tPA activity, which was observed

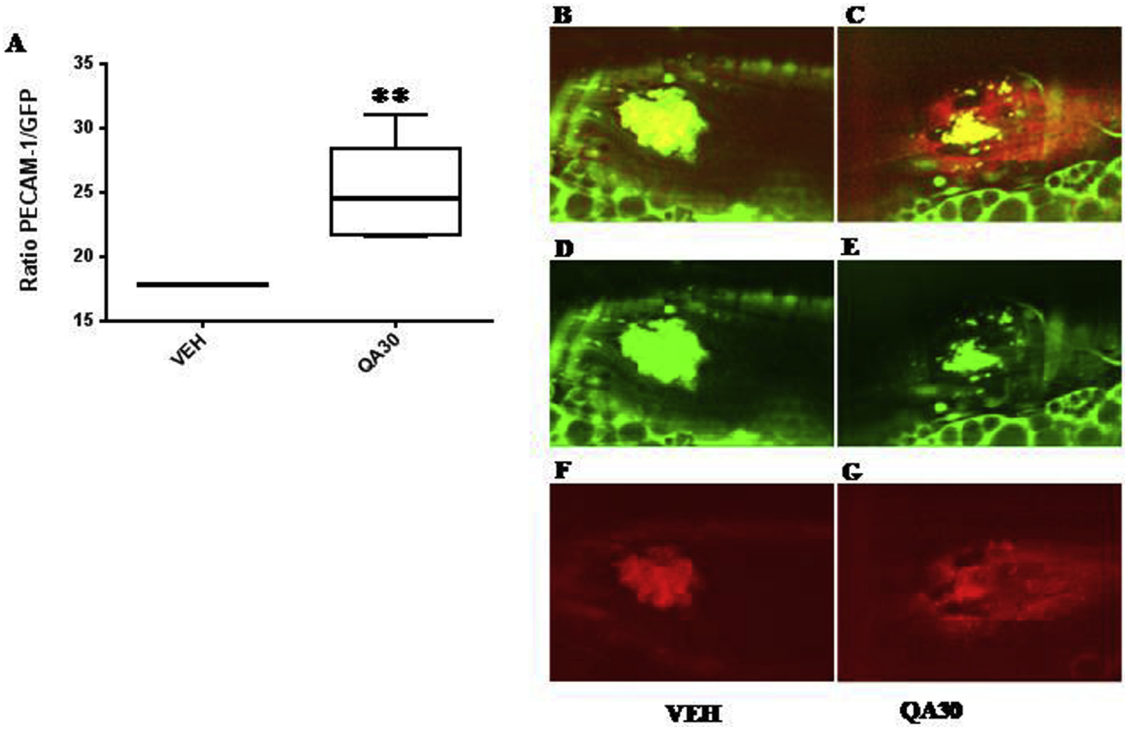

Fig. 5. Effect of intravenous QA administration on [A] the ratio of PECAM-1 (red) to GFP (green) fluorescence of platelet-rich thrombus formed at the site of laser-injured mesenteric vein in mice. Intensity of fluorescence was recorded 10 min after QA administration for 5 min. [B–C] representative images of the GFP platelet-rich thrombus expressing: merged PECAM-1 and GFP fluorescence in [B] VEH group and [C] QA treated group; GFP fluorescence in [D] VEH group and [E] QA treated group; and PECAM-1 fluorescence in [F] VEH group and [G] QA treated group. VEH – control group; QA30 – dose of quinolinic acid (mg/kg); $p < 0.01$** compared with VEH. Water immersion, x200.

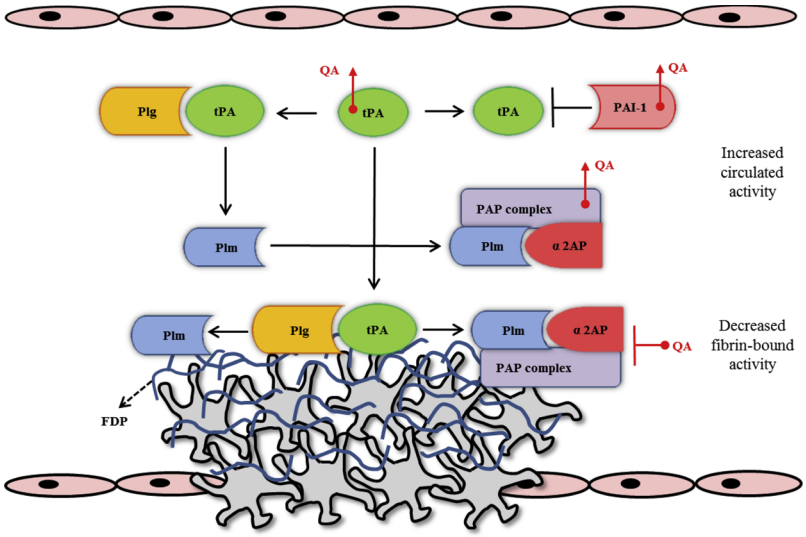

Fig. 6. Schematic representation of interaction between fibrinolysis and QA.
Endothelial injury leads to platelet aggregation and coagulation activation, resulting in the formation of the platelet and fibrin hemostatic plug. Tissue injury also activates fibrinolytic activators and inhibitors, of which tPA and PAI-1 play an important role in the arterial lumen. Released after tissue injury, tPA, if is not inhibited by PAI-1, can convert plasminogen (Plg) to plasmin (Plm) in the circulation and/or at the fibrin site. As a result, both circulated and fibrin-bound plasmin are activated, which then degrade fibrin into fibrin degradation products (FDP) or undergo inactivation by α2AP to form PAP complexes. The red arrows indicate the increased activity and concentration of tPA, PAI-1, and PAP complexes in the circulation, respectively, as an effect of 14 days of QA administration in thrombotic rats. The red line indicates the probable inhibitory effect of QA on fibrin-bound tPA or PAP complex activity.

at a lower dose, but it was inhibited by increased PAI-1 activity at the highest doses. Particularly, tPA activity and plasmin activation were observed at all examined doses due to an increased concentration of PAP complexes in rat plasma. Furthermore, it was previously reported that tPA is able to activate circulating plasminogen [35] as well as clot-bound plasminogen, thereby releasing plasmin [36]. Similarly, plasmin can be bound and inhibited by α2AP by the formation of PAP complexes present in the circulation or α2AP can be crosslinked to the fibrin surface and thereby inhibit plasmin by the formation of fibrin-bound PAP complexes [37].

In the light of the above, it seems that QA could inhibit plasmin activation and deactivation at the fibrin site through the attenuation of tPA affinity to clot-bound plasminogen as well as the attenuation of α2AP crosslinking to the fibrin surface and/or formation of fibrin-bound plasmin-α2AP(PAP) complexes(see Fig. 6), which in turn did not result in clot lysis or clot progression or resistance to lysis, respectively. These opposite effects can also explain the lack of changes in thrombus weight measured in rats. Undoubtedly, our experimental data of 14 days of QA administration in drinking water on hemostatic profile in rats revealed its new and till now unknown properties, nevertheless further studies evaluating the binding capacity of labeled tPA and antiplasmin to the clot structure in the presence of QA must be undertaken.

Taking into account that we observed the effects of QA on hemostasis that occurred during approximately 45 min after electric injury of the endothelium, we decided to analyze events that occur within the vein over a shorter period of time, expecting to observe if QA would affect platelet activity. Therefore, we involved the intravital model for the evaluation of acute thrombosis developing at the site of laser-induced injured endothelium in the mesenteric vein of transgenic GFP mice using a highly sensitive technique of intravital microscopy [38]. Laser injury of the endothelium was described to result in rapid platelet-rich thrombus formation [39,40].

Firstly, we examined if 14-day QA (30 mg/kg) administration in drinking water would affect thrombus formation in mice. As it turned out, QA exerted a prothrombotic effect, as it increased relative fluorescence intensity of platelet-rich thrombus, developing during 5 min after laser injury that also corresponded with increased fluorescence intensity of platelets accumulated at the site of the injured endothelium calculated as AUC. Secondly, we examined if single-dose IV administration of QA (30 mg/kg) would exert similar effects on hemostasis in GFP mice. Laser injury to induce thrombosis was done10 min after intravenous administration of QA and was recorded for 5 min. To our surprise, QA decreased the area of platelet-rich thrombus, displaying its antithrombotic activity.

Trying to find the involvement of QA in the regulation of thrombus formation, we decided to determine whether QA is able to regulate platelet activity through the mechanism involving platelet endothelial cell adhesion molecule, PECAM-1. Recent studies have demonstrated that the activation of PECAM-1 prior to the stimulation of platelets results in the inhibition of platelet aggregation and activation [41,42] by a range of receptors, including the glycoprotein VI (GPVI), GPIb-V-IX complex, and FcγRIIA receptor or G-protein–coupled receptor agonists such as thrombin [43]. The contribution of endothelial PECAM-1in the regulation of thrombus formation indicate a more complex relationship and still is the subject of ongoing investigations. According to the current state of knowledge, endothelial PECAM-1 mediates homophilic interactions between PECAM-1 molecules supporting the integrity of endothelial cell-cell junctions, whereas platelet PECAM-1, as it was mentioned above, is involved in the negative regulation of platelet aggregation and activation via its heterophylic interactions [44].

The complexity of these interactions is consistent with the research carried out by Falati et al. [43]. The authors proved that platelet PECAM-1 is involved in the regulation of thrombus growth, but also suggested that endothelial PECAM-1 may be involved at different phases in thrombus formation (at a later phase), according to observation in PECAM-1 knockout mice that received transplants of wild type (WT) bone marrow. This study indicated a more complex relationship between platelet and endothelial PECAM-1in thrombosis.

In our experiment, laser-induced endothelial injury did not target platelet PECAM-1 selectively, thus we measured platelet PECAM-1 expression as a ratio between the area of PECAM-1 fluorescence and the area of GFP fluorescence of platelet-rich thrombus. For that purpose, GFP mice received both PECAM-1 fluorescent antibody and QA 30 mg/kg intravenously. The effect of QA on laser-induced thrombosis was observed 10 min after QA administration. As it was found, the antithrombotic effect of QA was statistically significantly related to an increase in the relative fluorescence intensity of PECAM-1 in the area of platelet-rich thrombus in GFP mouse mesenteric vein. These data provide evidence for the involvement of QA-mediated PECAM-1 upregulation in the inhibition of thrombus formation, which may contribute to the inhibition of platelet adhesion and aggregation at a site of injured endothelium at an early phase of thrombosis.

5. Conclusions

Summing up, we demonstrate here - to our knowledge for the first time- the effect of QA on hemostatic profile in thrombotic rats and mice. Fourteen days of QA administration in rats resulted in divergent, depending on dose, increases in the concentration of active form of tPA

and PAI-1, total PAI-1 concentration as well as PAP complexes concentration in circulation observed at a later phase of thrombosis induction. In light of the results, none of the fibrinolytic markers influenced thrombus weight, thus it is highly probable that QA could have inhibited plasmin activation and deactivation at the fibrin site through the attenuation of tPA affinity to fibrin-bound plasminogen as well as attenuation of α2AP crosslinking to fibrin and/or formation of plasmin-α2AP (PAP) complexes at fibrin site. In turn, 14 days of QA administration in GFP mice revealed its prothrombotic activity, increasing platelet aggregation while single-dose IV administration revealed its antithrombotic activity, through the up-regulation of PECAM-1 expression observed at an early phase of thrombosis induction. Our data suggest that the amount of QA in circulation may regulate the balance between the inhibition and the activation of hemostasis. This may be important to determine the threshold level for hypercoagulability and hyperfibrinolysis in CKD patients. Our findings of QA biological activity *in vivo* may have potentially important physiological implications; thus, further studies on the effects of QA on the hemostatic profile and its direct mechanism on hemostasis must be undertaken.

Conflict of interests

The authors declare no conflict of interests.

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The author contribution

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Data Collection: Agnieszka Leszczyńska, Tomasz Misztal, Natalia Marcińczyk.

Statistical Analysis: Agnieszka Leszczyńska, Tomasz Kamiński.

Data Interpretation: Agnieszka Leszczyńska, Dariusz Pawlak, Ewa Chabielska.

Manuscript Preparation: Agnieszka Leszczyńska, Dariusz Pawlak.

Literature Search: Agnieszka Leszczyńska, Karol Kramkowski.

Funds Collection: Agnieszka Leszczyńska, Dariusz Pawlak.

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