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Suppression of plasminogen activator inhibitor-1 (PAI-1) activity by crocin ameliorates lipopolysaccharide-induced thrombosis in rats☆

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ABSTRACT

The imbalance between clot formation and fibrinolysis is mainly attributed to increased levels of plasminogen activator inhibitor type 1 (PAI-1), an inhibitor of fibrinolysis closely involved in inflammatory responses such as septic shock. This increase is mediated by many factors, including reactive oxygen species (ROS). The present study was designed to evaluate the prophylactic effect of crocin, a potent natural antioxidant, on PAI-1 in the rat model of endotoxic shock. Lipopolysaccharide-infused rats (500 μg/kg) showed significant changes in thrombosis-related haematological parameters such as decrease of platelet blood counts and increase (7 fold) of PAI-1 concentration in blood plasma. No effect on t-PA activity was observed. Crocin administration in two different doses (10 mg/kg and 100 mg/kg) 30 min prior to the injection of LPS, inhibited the reduction of platelet counts and ameliorated the concentration of PAI-1 in the liver and the brain. Moreover, crocin inhibited the deposition of fibrin in the renal glomeruli. No significant changes were recorded in the healthy groups of crocin (10 mg/kg and 100 mg/kg) compared to the control group. These data demonstrate the potential of crocin to prevent LPS-induced organ injury and suggest it is worthwhile to investigate the use of antioxidants for the treatment of septicemia.

1. Introduction

Crocin, crocetin and safranal, the chemical constituents of saffron (*Crocus sativus* L.) have several pharmacological effects, including antithrombotic effects mainly by inhibition of the factor X activity, as shown by both *in vitro* and *in silico* studies (Ditsa et al., 2009; Sinakos and Geromichalos, 2016). Preventive administration of crocetin in rabbits significantly improved the haematological parameters and inhibited kidney fibrosis due to sepsis, the most common cause of disseminated intravascular coagulation (DIC), established after infusion of endotoxin (Tsantarliotou et al., 2013).

Platelets are key modulators of coagulation and when activated they constitute the main source of blood plasminogen activator inhibitor-1 (PAI-1), thus playing a major role in thrombotic diseases; their activation is modulated by ROS (Stief, 2004; Thushara et al., 2013). PAI-1 is a 50 kDa glycoprotein that is the main physiological inhibitor of tissue-type and urokinase-type plasminogen activators (t-PA and u-PA, respectively). These two activators convert inactive plasminogen into its fibrin-degrading form, plasmin. Normally, plasma and tissue concentrations of PAI-1 remain low and increase only under pathologic conditions. This increase is mediated by many factors, including ROS (Stief, 2004; Vulin and Stanley, 2004). Elevated levels of PAI-1 have been identified in several inflammatory diseases, such as septic shock, acute lung inflammation (Schwartz and King, 2011), thrombotic diseases or tissue fibrosis (Declerck et al. 1994; van Mejer and Pannekoek 1995; Ha et al. 2009). Thus, PAI-1 has been correlated in a positive manner with diseases where inflammatory processes induce ROS production and therefore PAI-1 is suggested as a prognostic marker of the severity of sepsis (Pralong et al., 1989; Declerck et al.,1994; Tipoe et al., 2018). These inflammations are characterized by high levels of thrombin activity, which lead to fibrin deposition and formation of microthrombi throughout the microcirculation.

On the other hand, crocin and crocetin protect cells and tissues against injury mediated by reactive oxygen species (ROS). For example, crocetin attenuated LPS-induced acute lung injury in mice and increased LPS-impaired superoxide dismutase activity in the lung (Yang

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et al., 2012). Superoxide dismutase (SOD) is an enzyme responsible for the production of H_2O_2 or O_2 by the dismutation of superoxide radicals. H_2O_2, which can easily cross cell membranes and exert cytotoxic effects is converted to H_2O by catalase (CAT), glutathione peroxidase and peroxiredoxin activities, collectively called H_2O_2 decomposing activity. The above enzymes are antioxidant molecules and serve as redox biomarkers (Veskoukis et al., 2016).

The actions of inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), cause production of large amounts of tissue factor (TF) from monocytes and endothelial cells, leading to marked activation of coagulation. Moreover, LPS and cytokines inhibit the expression of thrombomodulin (TM), an anti-clotting protein in the vascular endothelium, thus increasing coagulation activation. According to Okada et al. (1998), PAI is over-expressed in the vascular endothelium due to the action of LPS and cytokines. Therefore, whether or not there are changes in t-PA or u-PA activities, fibrinolysis is eventually suppressed. In particular, in rat cardiac microvascular endothelial cells IL-1 may inhibit fibrinolysis and contribute to vascular injury by inducing PAI-1 and type 1 collagen expression in a ROS-dependent manner. Hence, PAI-1 constitutes an important target for therapy in patients with sepsis and antioxidants acquire an additional physiological role; that is the antithrombotic role (Schwartz and King, 2011).

As for the animal model, systematic analysis revealed that the rat is by far the preferred species amongst animal models and LPS is the preferred inducer of sepsis and DIC (Berthelsen et al., 2011). Thus, our present study was designed to evaluate the preventive effect of the carotenoid crocin in a rat experimental LPS-induced sepsis model and to determine the potential of crocin to impede or diminish the deleterious effects of endotoxin.

2. Methods

2.1. Animals and treatments

In the present study, 29 adult female Wistar rats weighing 200 ± 22 g were used. As far as we know there are no differences in reactive oxygen metabolites and blood antioxidant capacity between sexes in rats (Brunelli et al., 2014). The rats were isolated from the other animals hosted in the animal care facility to minimize noise stress, in a well-ventilated room with a 12-h:12-h light/dark cycle at 22 °C and 50% humidity. The animals were fed with commercial laboratory rat chow and water ad libitum. The experimental protocol was approved by the Committee for Animal Welfare, Medicine and Application of Central Macedonian Region, Greece (permission code 449209/4837-22/1/2015).

The animals were anaesthetized with an intraperitoneal (ip) injection of a combination of 5 mg/kg diazepam (Adelco, Piraeus, Greece) and 40 mg/kg of ketamine hydrochloride (Merial, Lyon, France), followed by ip supplements in order to maintain the anesthesia throughout the experiment. The rats were divided into six groups (Table 1): control (C): normal saline (n = 4), endotoxin (E): 500 μg LPS/kg (n = 5), Crocin 10 (CR_{10}): 10 mg crocin/kg (n = 4), Crocin 100 (CR_{100}): 100 mg crocin/kg (n = 4) and ECR_{10}, ECR_{100}: endotoxin with prior administration of crocin 10 mg/kg (n = 6) and 100 mg/kg (n = 6), respectively. The LPS solution (2 mg/ml N.S. + 5% BSA) was administered in the groups E, ECR_{10}, ECR_{100} by a single ip injection (LPS from *Escherichia coli*, serotype O127:B8, Sigma, Poole, UK). The C, CR_{10}, and CR_{100} groups were injected with a normal saline solution. Two solutions of crocin ($C_{44}H_{64}O_{24}$, Sigma-Aldrich GMBH, Germany) were freshly prepared (10 mg/ml and 100 mg/ml) in NS right before use. In all groups, the ip administration of crocin or NS was carried out 30 min before the ip injection of either endotoxin or N.S. The specific doses of LPS and crocin were chosen based on published data (Ngo and Declerck, 1998; Alavizadeh and Hosseinzadeh, 2014) and preliminary data from our group. Three hours after endotoxin administration all animals were euthanatized by anesthesia and cervical dislocation. Two blood samples were collected from each animal by cardiac puncture. Samples collected in tubes containing EDTA were used for whole blood analysis and complete blood count (CBC), whereas samples collected in tubes with 3.8% sodium citrate were used to determine plasma t-PA and PAI-1 activities.

Table 1
Experimental groups and administration regime of all compounds. The LPS solution (2 mg/ml N.S. + 5% BSA) was administered in the groups E, ECR_{10}, ECR_{100} by a single ip injection (LPS from *Escherichia coli*, serotype O127:B8, Sigma, Poole, UK). The C, CR_{10}, and CR_{100} groups were injected with a normal saline solution.

	i.p. Administration		
Group	t = 0 min	t = 30 min	t = 210 min
Control (C)	N.S.	N.S.	Euthanasia
CR_{10}	Crocin (10 mg/kg)	N.S.	Euthanasia
CR_{100}	Crocin (100 mg/kg)	N.S.	Euthanasia
Endo (E)	N.S.	LPS (500 μg/kg)	Euthanasia
ECR_{10}	Crocin (10 mg/kg)	LPS (500 μg/kg)	Euthanasia
ECr_{100}	Crocin (100 mg/kg)	LPS (500 μg/kg)	Euthanasia

Brain and liver were removed from each animal immediately after euthanasia, washed in NS, frozen and stored at −70 °C. The whole organs were used for the assays. Both kidneys were cut dorsally in the middle in two parts; one half of each kidney was fixed in 10% neutral-buffered formalin for the histopathological assay and the other halve was frozen and stored at −70 °C.

Tissue specimens were homogenized in 0.1 M Tris-HCl buffer, pH 7.4, in a motor driven Kinematica homogenizer in a ratio of 100 mg tissue in 1 ml of buffer. The buffer contained 1% Triton X-100. The homogenate was centrifuged (4000 rpm, 4 °C, 20 min) and the supernatant was passed through a Millipore filter (pore size 1.2 μm) and the flow-through was used for the assays.

2.2. Haematological and biochemical methods

CBC was counted in an ADVIA 120 Haematology analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Optical microscopy of the peripheral blood smear after staining with May-Grünwald-Giemsa was used to confirm the results from the analyzer.

2.3. Functionally active t-PA activity assay

Activity of functionally active (PAI-1-free) rat t-PA was determined using the t-PA activity ELISA kit (Molecular Innovations, Inc. USA) according to manufacturer's instructions. Briefly, 0.1 ml of blood plasma or tissue homogenate were added in each PAI-1-coated well and unbound proteins were washed away after appropriate washing steps. PAI-1-bound t-PA was captured by polyclonal anti-rat t-PA primary antibody. Excess antibody was washed away and bound antibody was then reacted with horseradish peroxidase (HRP)-conjugated secondary antibody. Following an additional washing step, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate was used for color development at 450 nm. The amount of color is directly proportional to the concentration of active t-PA in the sample.

2.4. Functionally active PAI-1 activity assay

Activity of functionally active (PA-free) rat PAI-1 was determined using the PAI-1 activity ELISA kit (Molecular Innovations, Inc. USA) according to manufacturer's instructions. Briefly, 0.1 ml blood plasma or tissue homogenate were added in each t-PA-coated well and unbound proteins were washed away. The t-PA-bound PAI-1 was captured by anti-rat PAI-1 primary antibody. Following washes, the bound

antibody was detected by an HRP-conjugated secondary antibody. Following an additional washing step, TMB substrate was used for color development at 450 nm. The amount of color is directly proportional to the concentration of active PAI-1 in the sample.

2.5. Superoxide dismutase (SOD) activity

The assessment of SOD activity in the liver specimens was based on the xanthine oxidase-cytochrome c method according to McCord and Fridovich (McCord and Fridovich, 1969) modified by Veskoukis et al. (2016). The system of xanthine oxidase and its substrate, xanthine, is used to generate O_2^- which reduces cytochrome *c*. The determination of SOD activity is based on the inhibition of cytochrome *c* reduction monitored at 550 nm.

In particular, 740 μL of 50 mM potassium phosphate buffer (25 mM KH_2PO_4, 25 mM K_2HPO4, 0.1 mM EDTA, pH 7.8), 100 μL of 0.5 mM xanthine (Sigma-Aldrich, St. Louis, Missouri, U.S.A.), 100 μL of 0.1 mM cytochrome *c* solution and 10 μL of the homogenate were added in a plastic test tube. The mixture was vortexed, transferred to a cuvette and 50 μL of bovine xanthine oxidase (diluted 1/50 from a 25-Unit stock) were added before the cuvette was rapidly inverted 3 times and the absorbance was read at 550 nm for 1 min. SOD activity was determined with the use of the molar extinction coefficient of cytochrome *c* (19.2 L/mol/cm) as follows:

$$\text{SOD (U/mg protein)} = (\Delta Abs_{control} \text{ per min} - \Delta Abs_{sample} \text{ per min}/19.2) \times 1000 \times \text{dilution factor/Protein concentration.}$$

2.6. H_2O_2 decomposing activity

H_2O_2 decomposing activity was determined based on the method of Aebi (1984) modified by Veskoukis et al. (2016). For the hepatic H_2O_2 decomposing activity, 5 μL of liver homogenate was diluted 112 times in 0.01M PBS solution. From the diluted homogenates, 40 μL were transferred to plastic test tubes and 2955 μL of 67 mM Phosphate Buffer (67 mM KH_2PO_4 and 67 mM Na_2HPO_4; pH 7.4) were added. The samples were vortexed and incubated at 37 °C for 10 min. The content was transferred to quartz cuvette and 5 μL of 30% hydrogen peroxide (H_2O_2) was added; the sample was then inverted three times after a thin layer of parafilm was placed and the absorbance was read at 240 nm for 1.5 min H_2O_2 decomposing activity was calculated using the molar extinction coefficient of H_2O_2 (40 L/mol/cm), as follows:

$$\text{CAT (U/mg protein)} = (\Delta Abs_{sample} \text{ per min}/40) \times \text{dilution factor/Protein concentration.}$$

2.7. Histopathological methods

Kidney midsections were fixed in 10% neutral-buffered formalin, embedded in paraffin and stained with haematoxylin-eosin or the Martius–Scarlet–Blue (MSB) histochemical stain for fibrin. Neutrophils and fibrin deposition were quantified in haematoxylin-eosin and MSB-stained kidney sections, respectively. For each assessment, thirty 40× high power field images of the affected glomeruli were captured per treatment group and 10 images from each group were randomly selected for counts. Images were analyzed with the Image J image processing and analysis program (NIH, Bethesda, Maryland, USA). Neutrophils in glomeruli were counted manually using the cell counter plug-in, whereas automatic calculation of the numbers of red pixels corresponding to fibrin in images appeared in a histogram after processing each image with the threshold color plug-in. Morphometric counts were recorded as the number of neutrophils or red-labelled pixels per image.

2.8. Statistical analysis

Whenever normal distribution of data was noticed, statistical analysis was processed with one way analysis of variance (ANOVA) and comparisons between pairs were performed with Tukey's test. Data either with a non normal distribution or with a Levene criterion result smaller than 0.05 ($p < 0.05$) were analyzed with the Kruskal-Wallis test and pairwise comparisons were performed with the Mann-Whitney *U* test. Statistical significance level was set at $p < 0.05$. All experimental data were analyzed with the SPSS version 24.0 (IBM Corp., Armonk, New York, U.S.A.) and are presented as mean ± SEM.

3. Results-discussion

The endotoxin rat model for sepsis is an established, widely-used model for the study of the pathophysiology of the disease and the potential intervention with different chemical and natural compounds to improve the clinical outcome of the disease (Puig et al., 2016; Brooks et al., 2017). In the present study, adult female rats were ip injected with LPS (500 μg/kg) to induce a septic shock. Within three hours (3 h) endotoxin induced a 26% reduction in platelet count ($p = 0.014$) compared to control animals that were injected with NS (Fig. 1A and B). This indicates that endotoxin induced the coagulation process in the circulation, a fact also ascertained by the extensive deposition of fibrin in the glomeruli of the endotoxin-treated animals, as revealed by the

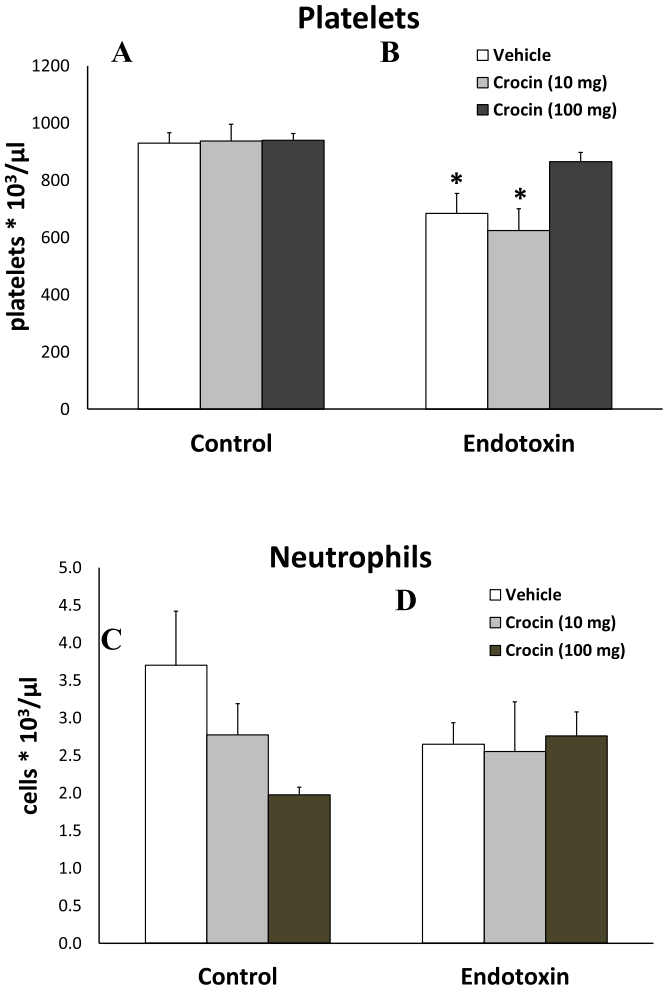


Fig. 1. Concentration of platelets **(A, B)** and neutrophils **(C, D)** in the blood. Whole blood was collected in EDTA-treated vials and platelet and neutrophil numbers were determined by an automated cell counter. The results are presented as number of corpuscles per μl of total blood.

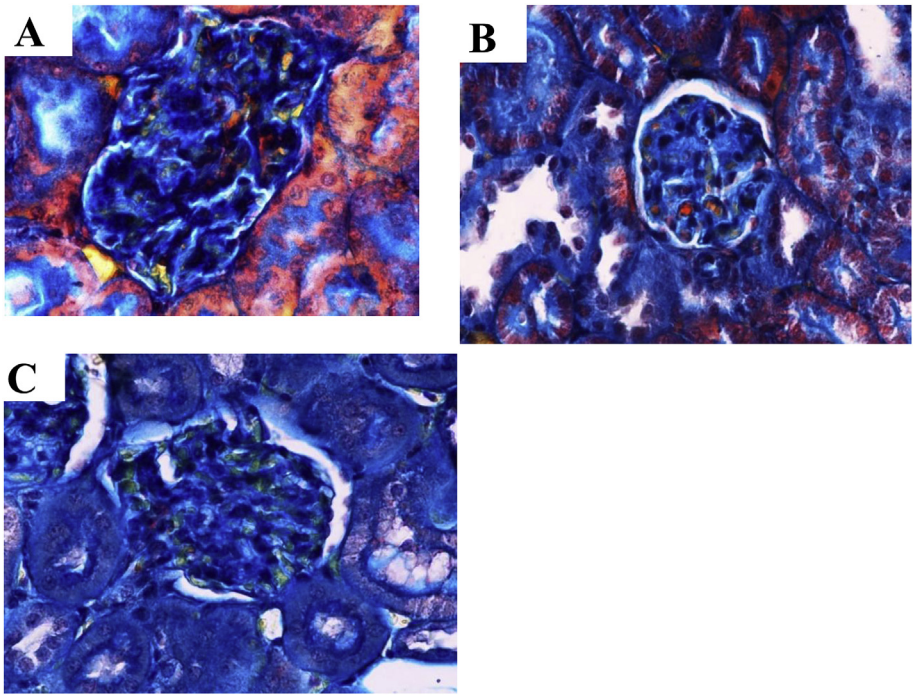


Fig. 2. Histopathological analysis of kidneys. **A.** Endotoxin-treated animal that received only NS as a pre-treatment. Fibrin deposits are red-colored **B, C.** Endotoxin-treated animals that received CR_{10} and CR_{100}, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

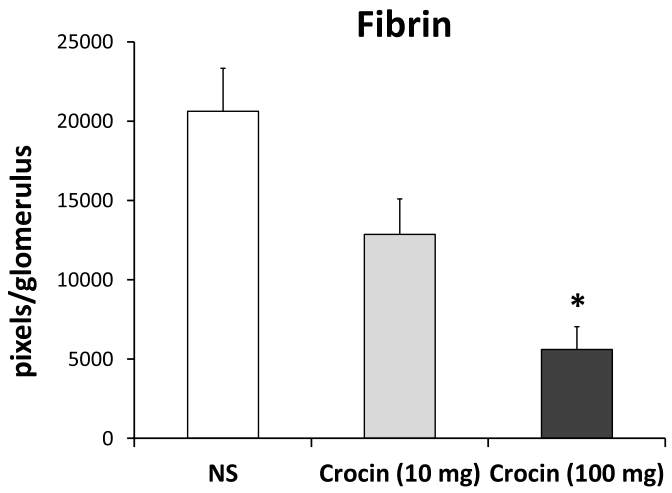


Fig. 3. Relative quantitation of the fibrin deposits in the kidneys of the LPS-treated animals. The fibrin deposition is expressed as pixels/glomerulus in the kidneys of animals that were pre-treated with NS (vehicle), 10 mg or 100 mg crocin/kg bw 30 min before the administration of LPS. The asterisk indicates $p \leq 0.005$.

Table 2
Leukocyte (WBC) and neutrophil concentration in the blood.

Group	WBC (Mean*10^3 cells/μl ± SEM)	Neutrophils (Mean*10^3 cells/μl ± SEM)
C	4.05 ± 0.58	3.7 ± 0.72
E	3.25 ± 0.29	2.65 ± 0.41
ECR_{10}	4.15 ± 0.45	2.55 ± 0.66
ECR_{100}	3.84 ± 0.35	4.27 ± 0.32
CR_{10}	4.33 ± 0.33	2.78 ± 0.41
CR_{100}	3.85 ± 0.35	1.98 ± 0.28

histological analysis (Figs. 2A and 3). The concentration of leukocytes in the blood of treated animals was not affected (Table 2), despite a slight reduction in the concentration of neutrophils (Fig. 1C and D). Conflicting data have been reported in the literature regarding the effect of endotoxin on leukocytes. However, it appears that in the rat one of the immediate responses to LPS is neutropenia. This effect of LPS was diminished in rats that have been rendered tolerant to LPS (Barroso-Aranda et al., 1991).

Oxidative stress plays a key role in the manifestation of endotoxin-induced sepsis (Sakaguchi and Furusawa, 2006). Taking this under consideration, we anticipated that reinforcing the antioxidant status of an organism would fortify its defense and resistance against the toxic insult by endotoxin. For this reason, we investigated the potential protective role of crocin, a potent antioxidant, in our experimental system. Pre-treatment of the animals with 100 mg crocin effectively inhibited the reduction of platelets ($p = 0.01$), which was evoked by endotoxin in the LPS group (Fig. 1B). Thus, animals that received the high concentration of crocin before they were inoculated with LPS retained platelet concentration in their blood to values that are similar to those of the control group (Fig. 1A and B, dark grey bars). However, when administered at lower concentration (10 mg/kg), crocin did not prevent the reduction of platelets by LPS (Fig. 1A and B light grey bars). Crocin had no effect on platelet concentration in the control animals (Fig. 1A).

The animals that were injected with LPS exhibited a slight reduction in the concentration of neutrophils that was not inhibited by prior administration of crocin (Fig. 1D). This may reflect recruitment of neutrophils in the microthrombi and the sites of inflammation. Crocin did not alleviate this effect of LPS, probably because it does not affect the formation of thrombi but rather facilitates their destruction by inducing the thrombolytic mechanism. However, when administered in control animals, crocin reduced the concentration of neutrophils in the circulation in a dose-dependent manner (Fig. 1C). This is in the line with previous data which demonstrate an approximately 10% reduction in neutrophil count in rats that received two doses of crocin (96 mg/kg) within 12 h (Jayalakshmi, 2015).

The histopathological analysis of the kidneys revealed that LPS induced the deposition of fibrin in the glomeruli of treated animals (Fig. 2A). This was anticipated, since septicemia induces extensive coagulation in the microcirculation, a characteristic of DIC. Crocin effectively inhibited the deposition of fibrin in the glomeruli of endotoxin-treated animals in a dose-dependent manner (Figs. 2 and 3). Specifically, when administered at a concentration of 100 mg/kg

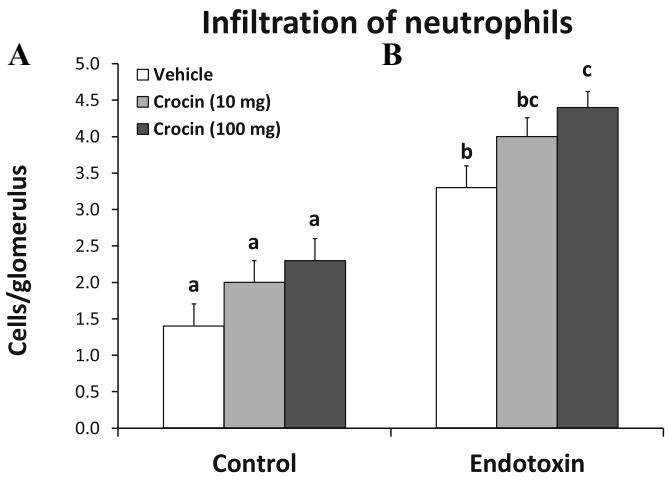


Fig. 4. Number of neutrophils that have infiltrated the glomeruli. **A.** Control, animals that were injected with NS, **B.** Endotoxin, animals that were injected with LPS. Different letters indicate statistically different values ($p \leq 0.05$).

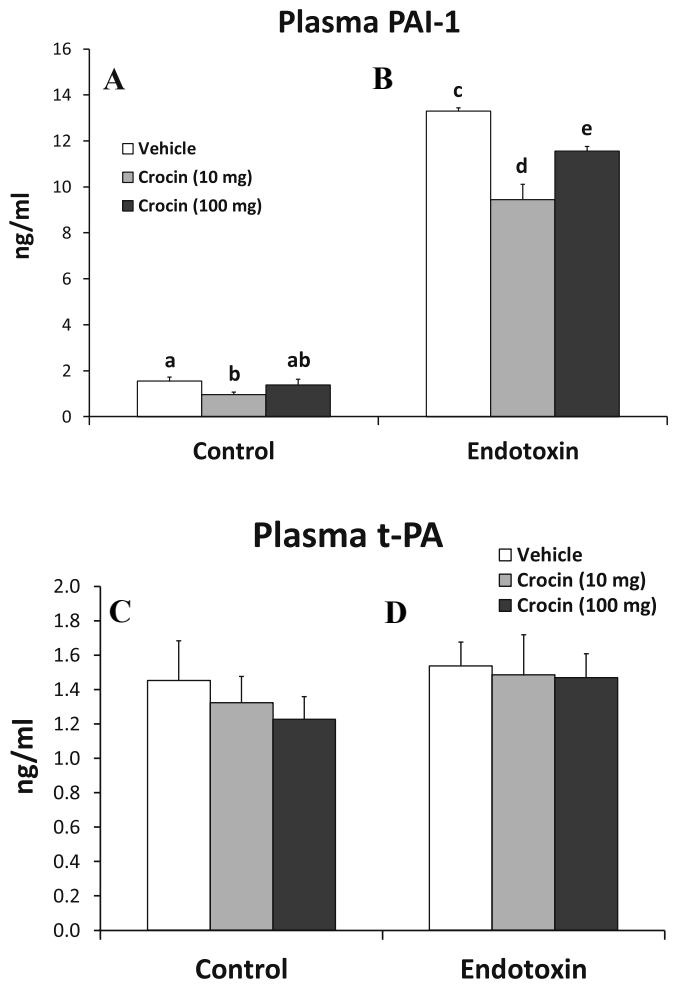


Fig. 5. Active PAI-1 (**A, B**) and active t-PA (**C, D**) concentrations were determined in the blood plasma by ELISA. **A, C** control (C), animals that were injected with NS, **B, D** Endotoxin (E), animals that were injected with LPS. Different letters correspond to statistically significant differences from the control values ($p \leq 0.05$).

(Fig. 3, dark grey bar) crocin diminished the deposition of fibrin in the glomeruli by 73% ($p < 0.001$). Moreover, the number of neutrophils that infiltrated the glomeruli of LPS-treated animals increased two-fold ($p = 0.013$) compared to the number of cells that were found in the glomeruli of control animals (Fig. 4, white bars). This is in accordance with the reduction in neutrophil counts in the blood of these animals (Fig. 1C and D) and reinforces previous data from our research group that indicate a protective role of crocetin, another carotenoid constituent of the stigmata of crocus (saffron), in the rabbit model of endotoxin shock (Tsantarliotou et al., 2013). Surprisingly, just as crocin slightly decreased neutrophil counts in the blood of control animals, it also slightly enhanced infiltration by neutrophils in a dose-dependent manner.

Researchers and reviewers of the molecular mechanisms implicated in sepsis and DIC have been challenged by the paradox imposed by data indicating the activation of both coagulative and fibrinolytic mechanisms in animal models. This has been attributed to the disruption of the balance between coagulation and fibrinolysis, where the fibrinolytic parameter PAI-1 appears to be a key factor (Asakura, 2014). PAI-1 is a key factor implicated in the pathogenesis of septic shock (Pralong et al., 1989) and levels of PAI-1 have been correlated with the outcome and the severity of sepsis (Tipoe et al., 2018; Hoshino et al., 2017; Koyama and Nunomiya, 2017). We investigated the potential of crocin to modulate the t-PA/PAI-1 system in the endotoxin shock rat model. We used two ELISA systems that quantitate the activities of (PAI-1-free) t-PA and (t-PA-free) PAI-1, each. Therefore, we quantitated the active but not the inactive or latent forms of these proteins. As it was anticipated, 3 h after the administration of LPS, the concentration of active PAI-1 levels in the plasma was increased approximately 8.5-fold ($p = 0.014$) compared to those of the control animals (Fig. 5A and B white bars).

Interestingly, animals that received crocin 30 min prior to the injection of LPS displayed lower levels of active PAI-1 than those that received only LPS ($p = 0.006$). The inhibitory effect of crocin on endotoxin-mediated induction of PAI-1 was very effective, despite the fact that it did not completely block the induction of PAI-1. The animals that received only crocin displayed a 38% reduction in plasma PAI-1 levels compared to those of the control animals (Fig. 5A) (Orbe et al., 2001; Liu, 2008). Neither endotoxin nor crocin had any effect on active t-PA levels in the plasma (Fig. 5C and D).

To sum up, the endotoxic shock mediated by inoculation of rats with LPS disrupted the fibrinolytic balance by increasing PAI-1, thus inhibiting the fibrinolytic activity. Therefore, microthrombi appear throughout the circulation, as demonstrated by the fibrin deposition in the glomeruli (Fig. 2). Probably, the release of t-PA-free PAI-1 from activated platelets (Chandler, 2012) or from endothelial cells (Asakura, 2014) can be accounted for this increase. The newly released PAI-1 is acutely active and mediates its antifibrinolytic effect either by interacting with t-PA or by other indirect ways (Handt et al., 1996). Endotoxin induces coagulation, and the formed thrombi induce the tPA/plasmin fibrinolytic activity. This may be the result of increased secretion of t-PA by the endothelial cells. At the same time, the activated platelets that are recruited in the thrombi release the PAI-1 that they carry. Part of this PAI-1 may interact with the t-PA that has been induced by LPS, and thus render it inactive. This explains the relatively stable levels of active t-PA 3 h after the administration of LPS (Fig. 5D). In any case, the inhibition of the fibrinolytic activity would contribute to the increased coagulation and the formation of microthrombi in the circulation.

Crocin inhibited the LPS-mediated induction of both PAI-1 and coagulation. This effect could be attributed to the inhibition of platelet aggregation by crocin, which is the result of its antioxidant properties, as previously demonstrated (Thushara et al., 2013). Therefore, in our model, the fortification of the antioxidant potential of the organism with crocin may have resulted in inhibition of platelet aggregation that was induced by LPS. As a result, crocin hindered the elevation of PAI-1, which is otherwise induced by LPS, and thus more t-PA was active to induce fibrinolysis. Therefore, fibrin deposition was dramatically decreased in the glomeruli of animals that were preconditioned with

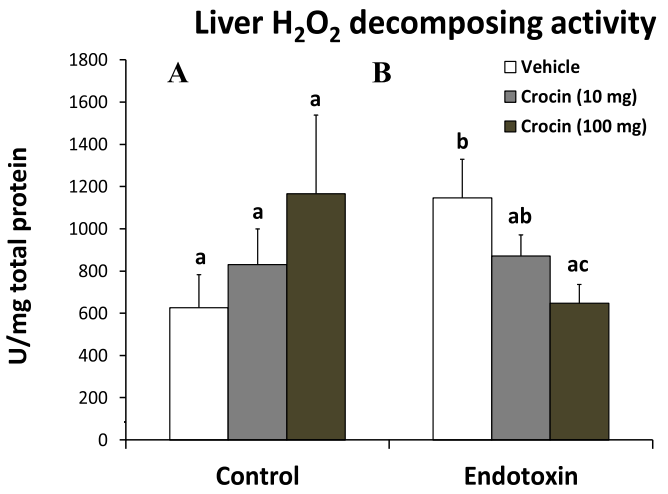


Fig. 6. H_2O_2 decomposing activity in the liver was determined by a biochemical colorimetric assay, and is expressed as units (U) of H_2O_2 decomposing activity per mg of total protein. **A,** control (C), animals that were injected with NS, **B,** Endotoxin (E), animals that were injected with LPS. Bars with different letters have statistically significant different values ($p \leq 0.05$).

crocin prior to the injection of LPS (Fig. 2B and C).

PAI-1 has a key role in diverse physiopathological processes in different tissues and organs, including the liver and brain. Our research group, amongst others, has shown that the t-PA/PAI-1 system is modulated by oxidative stress and antioxidants can play an important role in inhibiting these effects (Liu, 2008; Tsantarliotou et al., 2008; Zervos et al., 2011; Lavrentiadou et al., 2013). Therefore, we determined how LPS affects the levels of PAI-1 and t-PA in the liver and brain. Furthermore, we investigated the antioxidant role of crocin in these two organs.

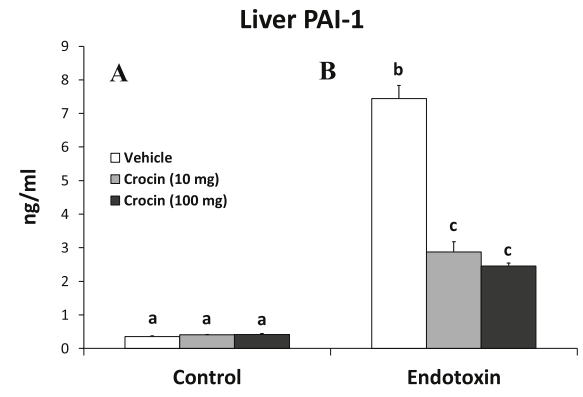
The endotoxin shock induced the H_2O_2 decomposing activity in the liver (Fig. 6) but had no effect on hepatic SOD activity (Table 3). H_2O_2 decomposing induction by LPS could be attributed to the stimulation of the liver by the increase in ROS during the endotoxic shock. The hepatic cells counteract the elevated ROS levels by increase their antioxidant armor. However, pretreatment of animals with crocin (100 mg/kg) effectively inhibited this effect ($p = 0.045$), reducing the H_2O_2 decomposing activity in the liver of these animals to levels similar to those of control animals (Fig. 6B). On the other hand crocin, when administered at high concentration, induces H_2O_2 decomposing activity in the liver. However, this increase was not statistically significant (Fig. 6A).

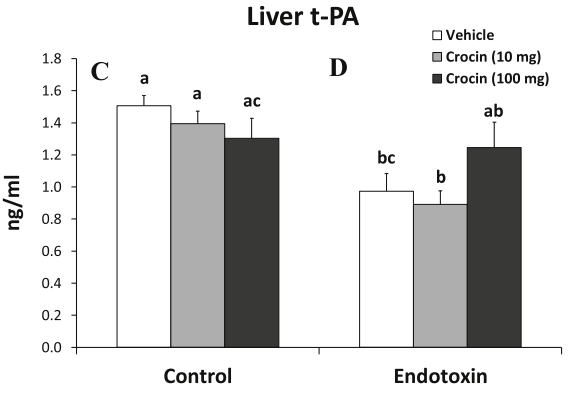
Interestingly, LPS induced active PAI-1 levels in both the liver (Fig. 7A and B) and the brain (Fig. 8A,B) and decreased active t-PA levels in the liver (Fig. 7C and D), but had no effect on active t-PA levels in the brain (Fig. 8C and D). In the liver, PAI-1 levels exhibited a 21-fold increase ($p = 0.014$), whereas active t-PA was decreased by 40% ($p = 0.019$). This dual effect of endotoxin resulted in a dramatic decrease in the ratio of active t-PA over active PAI-1 activity by 97% compared to the ratio determined in control animals ($p = 0.014$) (Fig. 7E and F).

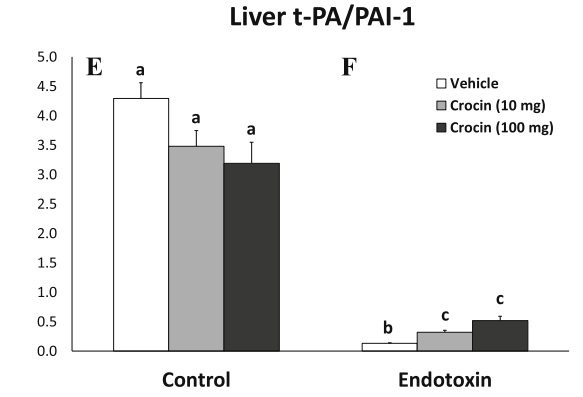
As anticipated, LPS disturbed the fibrinolytic balance by increasing

Table 3
Superoxide Dismutase (SOD) activity (U/mg protein) in the liver and brain.

Group	Liver	Brain
C	75.9 ± 8.3	70.8 ± 9.3
E	104.7 ± 12.2	61.3 ± 13.6
ECR_{10}	87.3 ± 4.2	62.4 ± 4.5
ECR_{100}	78.4 ± 5.4	51.1 ± 7
CR_{10}	83.8 ± 12.4	58.2 ± 8.5
CR_{100}	118.5 ± 27.5	57.8 ± 12.8







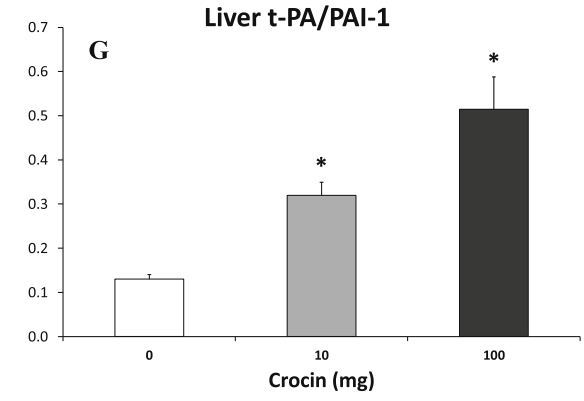


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Fig. 7. Active PAI-1 (**A, B**) and active t-PA (**C, D**) concentrations were determined in the liver of the animals and are expressed as ng/ml of tissue homogenate. The ratio of t-PA/PAI-1 was calculated (**E, F**) and the ratios that correspond to the endotoxin-treated animals are shown in a larger scale in **G**. **A-F:** Different letters on the bars represent statistically different values ($p \leq 0.05$). **G:** The asterisks indicate values that are statistically different compared to the endotoxin (E) group ($p = 0.006$).

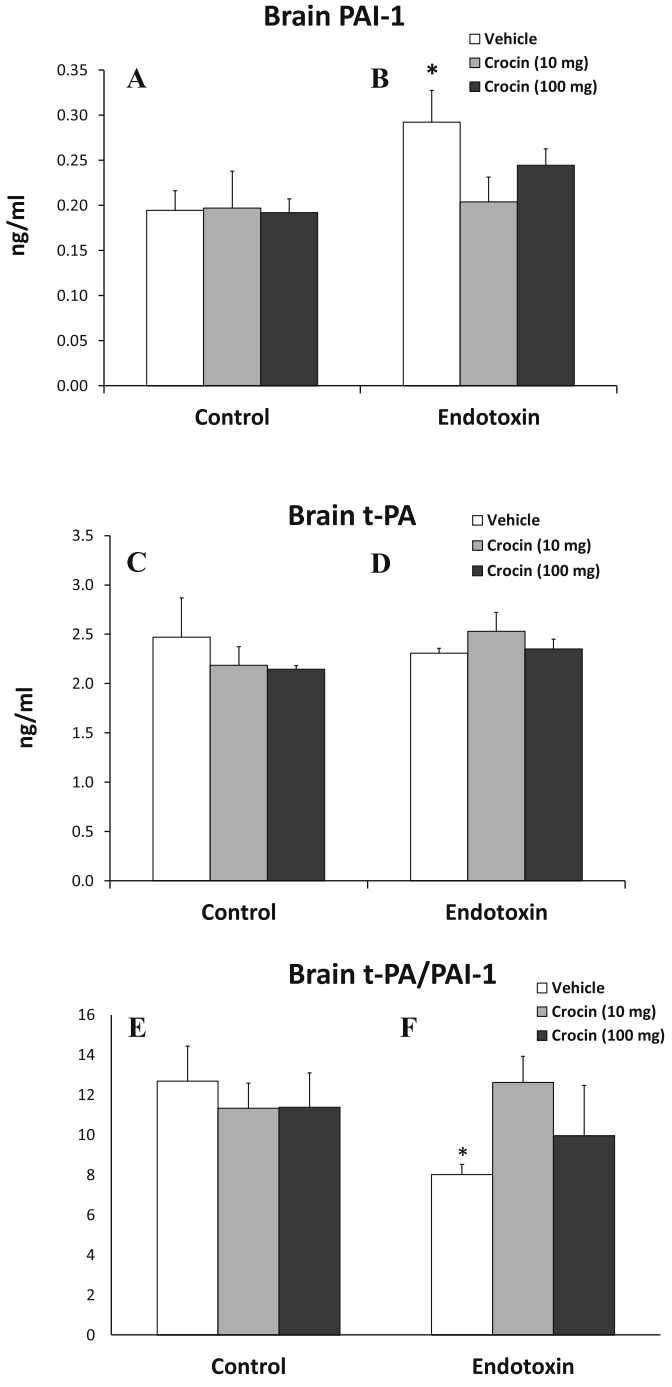

Fig. 8. Active PAI-1 (**A, B**) and active t-PA (**C, D**) concentrations in the brain of the animals expressed as ng/ml of tissue homogenate. The ratio t-PA/PAI-1 was calculated and is presented in **E, F. A, C, E:** control (C) animals, **B, D, F:** LPS-treated (E) animals. Asterisks correspond to statistically different values ($p \leq 0.05$).

PAI-1 and decreasing t-PA levels, thus dramatically reducing the t-PA/PAI-1 ratio. The t-PA/PAI-1 ratio was also significantly ($P = 0.027$) reduced in the brain of LPS-treated rats (Fig. 8E and F). The observed increase of PAI-1 levels in the liver may be due to the activation of PAI-1 gene transcription in both endothelial cells and hepatocytes (Fearns and Loskutoff, 1997) and can be the result of oxidative stress (Vulin and Stanley, 2004). Crocin efficiently alleviated the effects of LPS in both the liver and the brain. In particular, both concentrations of crocin reduced PAI-1 activity in the LPS-treated animals by almost 60% in the liver (Fig. 7B) and by almost 30% in the brain (Fig. 8B).

When crocin was administered at high concentration (100 mg/kg) it moderately inhibited the reduction of t-PA by LPS in the liver (Fig. 7D, dark grey bar). Consequently, crocin reversed the inhibitory effect of LPS on t-PA/PAI-1 ratios (Figs. 7G and 8F). In the liver, this ratio was increased ($p = 0.006$) but was not restored to control values (Fig. 7E and F). It appears therefore that the antioxidant properties of crocin are effective in protecting the liver and the brain from oxidative damage by modulating the levels of active PAI-1. A similar effect of other antioxidants in the liver and other organs has been described previously (Vulin and Stanley, 2004; Tsantarliotou et al., 2008; Zervos et al., 2011). However, the molecular events implicated in the modulation of PAI-1 and t-PA by ROS and the molecular targets of crocin have yet to be elucidated.

4. Conclusion

This work demonstrates the protective role of crocin, a constituent of the crocus, against endotoxic shock in female rats. Crocin administration 30 min prior the LPS insult effectively maintains low levels of active PAI-1 in blood plasma, liver and brain. Therefore, crocin alleviates the inhibition of the fibrinolytic mechanism, thus improving the clinical status of the animals that are challenged by sepsis.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.01.001.

References

Aebi, H., 1984. Catalase *in vitro*. Methods Enzymol. 105, 121–126.
Alavizadeh, S.H., Hosseinzadeh, H., 2014. Bioactivity assessment and toxicity of crocin: a comprehensive review. Food Chem. Toxicol. 64, 65–80.
Asakura, H., 2014. Classifying types of disseminated intravascular coagulation: clinical and animal models. J. Intensive Care 2, 1–7.
Barroso-Aranda, J., Schmid-Schönbein, G.W., Zweifach Benjamin, W., Mathison, J.C., 1991. Polymorphonuclear neutrophil contribution to induced tolerance to bacterial lipopolysaccharide. Circ. Res. 69, 1196–1206.
Berthelsen, L.O., Kristensen, A.T., Tranholm, M., 2011. Animal models of DIC and their relevance to human DIC: a systematic review. Thromb. Res. 128, 103–116.
Brooks, M.B., Turk, J.R., Guerrero, A., Narayanan, P.K., Nolan, J.P., Besteman, E.G., et al., 2017. Non-lethal endotoxin injection: a rat model of hypercoagulability. PLoS One 12 (1), e0169976. https://doi.org/10.1371/journal.pone.0169976.
Brunelli, E., Domanico, F., La Russa, D., Pellegrino, D., 2014. Sex differences in oxidative stress biomarkers. Curr. Drug Targets 15, 1–5.
Chandler, W., 2012. Diagnosis of fibrinolytic disorders. In: Kottke-Marchant, K., Davis, B. (Eds.), Laboratory Hematology Practice. Blackwell Publishing Ltd., West Sussex, UK, pp. 460–467.
Declerck, P.J., Juhan-Vague, I., Felez, J., Wiman, B., 1994. Pathophysiology of fibrinolysis. J. Intern. Med. 236, 425–432.
Ditsa, M., Geromichalou, E., Markala, D., Geromichalos, G., Dalezis, P., Trafalis, D.T.P., Papageorgiou, A., Sinakos, Z., 2009. In vitro and in silico study of the effect of crocin and safranal on human plasma coagulation. In: Proceedings of 3rd International Symposium on Saffron, pp. 60 Kozani, Greece.
Fearns, C., Loskutoff, D.J., 1997. Induction of plasminogenactivator inhibitor 1 gene expression in murine liver by lipopolysaccharide. Cellular localization and role of endogenous tumor necrosis factor-α. Am. J. Pathol. 150 (2), 579–590.
Ha, H., Oh, E.Y., Lee, H.B., 2009. The role of plasminogen activator inhibitor 1 in renal and cardiovascular diseases. Nat. Rev. Nephrol. 5, 203–211.
Handt, S., Jerome, W.G., Tietze, L., Hantgan, R.R., 1996. Plasminogen activator inhibitor-1 secretion of endothelial cells increases fibrinolytic resistance of an in vitro fibrin clot: evidence for a key role of endothelial cells in thrombolytic resistance. Blood 87, 4204–4213.

Hoshino, K., Kitamura, T., Nakamura, Y., Irie, Y., Matsumoto, N., Kawano, Y., Ishikura, H., 2017. Usefulness of plasminogen activator inhibitor-1 as a predictive marker of mortality in sepsis. J. Intensive Care 5, 42. https://doi.org/10.1186/s40560-017-0238-8.

Jayalakshmi, K., 2015. Haematological effect of crocin on male albino rat. Life Sci. Arch. (LSA) 1 (3), 175–181.

Koyama, K., Nunomiya, S., 2017. Single measurement of plasminogen activator inhibitor-1 in sepsis: is it useful for evaluating the severity or prognosis of sepsis? J. Intensive Care 5, 63–64.

Lavrentiadou, S.N., Tsantarliotou, M.P., Zervos, I.A., Nikolaidis, E., Georgiadis, M.P., Taitzoglou, I.A., 2013. CCl_4 induces tissue-type plasminogen activator in rat brain; protective effects of oregano, rosemary or vitamin E. Food Chem. Toxicol. 61, 196–202.

Liu, R.-M., 2008. Oxidative stress, plasminogen activator inhibitor 1, and lung fibrosis. Antioxidants Redox Signal. 10, 303–319. https://doi.org/10.1089/ars.2007.1903.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.

Ngo, T.H., Declerck, P.J., 1998. Suppression of plasminogen activator inhibitor 1 (PAI-1) activity in rats by monoclonal antibodies. Fibrinolysis Proteolysis 12, 335–339.

Okada, H., Woodcock-Mitchell, J., Mitchell, J., Sakamoto, T., Marutsuka, K., Sobel, B.E., Fujii, S., 1998. Induction of plasminogen activator inhibitor type 1 and type 1 collagen expression in rat cardiac microvascular endothelial cells by interleukin-1 and its dependence on oxygen-centered free radicals. Circulation 97, 2175–2182.

Orbe, J., Rodriguez, J., Calvo, A., Grau, A., Belzunce, M., Martinez-Caro, D., Páramo, J., 2001. Vitamins C and E attenuate plasminogen activator inhibitor-1 (PAI-1) expression in a hypercholesterolemic porcine model of angioplasty. Cardiovasc. Res. 49, 484–492. https://doi.org/10.1016/S0008-6363(00)00260-1.

Pralong, G., Calandra, T., Glauser, M.P., Schellekens, J., Verhoef, J., Bachmann, F., Kruithof, E.K., 1989. Plasminogen activator inhibitor 1: a new prognostic marker in septic shock. Thromb. Haemostasis 61, 459–462.

Puig, F., Herrero, R., Guillamat-Prats, R., Gómez, M.N., Tijero, J., Chimenti, L., et al., 2016. A new experimental model of acid- and endotoxin-induced acute lung injury in rats. Am. J. Physiol. Lung Cell Mol. Physiol. 311, L229–L237.

Sakaguchi, S., Furusawa, S., 2006. Oxidative stress and septic shock: metabolic aspects of oxygen-derived free radicals generated in the liver during endotoxemia. FEMS Immunol. Med. Microbiol. 47 (2), 167–177.

Schwartz, M., King, T., 2011. Coagulation and fibrinolysis in lung injury and repair. In: Interstitial Lung Disease, fifth ed. PMPH, USA.

Sinakos, Z., Geromichalos, G., 2016. The effect of saffron (crocus sativus) carotenoids on hemostasis and atherosclerosis. Next Generat. Sequence Appl. 3, 1–3. https://doi.org/10.4172/2469-9853.1000e102.

Stief, T.W., 2004. Regulation of hemostasis by singlet-oxygen (1Delta O_2^*). Curr. Vasc. Pharmacol. 2, 357–362.

Thushara, R.M., Hemshekhar, M., Santhosh, M.S., Jnaneshwari, S., Nayaka, S.C., Naveen, S., et al., 2013. Crocin, a dietary additive protects platelets from oxidative stress-induced apoptosis and inhibits platelet aggregation. Mol. Cell. Biochem. 73 (1–2), 73–83.

Tipoe, T.L., Wu, W.K.K., Chung, L., Gong, M., Dong, M., Liu, T., et al., 2018. Plasminogen activator inhibitor 1 for predicting sepsis severity and mortality outcomes: a systematic review and meta-analysis. Front. Immunol. 9, 1218. https://doi.org/10.3389/fimmu.2018.01218.

Tsantarliotou, M., Zervos, I., Lavrentiadou, S., Kokoli, A., Taitzoglou, I., 2008. Diet supplementation with oregano or rosemary increases plasminogen activator activity in healthy rat brain. In: Proceedings of XIIXth International Congress on Fibrinolysis and Proteolysis, pp. 159 Vienna, Austria.

Tsantarliotou, M.P., Markala, D., Kazakos, G., Sapanidou, V., Lavrentiadou, S., Zervos, I., Taitzoglou, I., Sinakos, Z., 2013. Crocetin administration ameliorates endotoxin-induced disseminated intravascular coagulation in rabbits. Blood Coagul. Fibrinolysis 24, 305–310.

van Mejer, M., Pannekoek, H., 1995. Structure of plasminogen activator inhibitor 1 (PAI-1) and its function in fibrinolysis: an update. Fibrinolysis 9, 263–276.

Veskoukis, A., Kyparos, A., Paschalis, V., Nikolaidis, M.G., 2016. Spectrophotometric assays for measuring redox biomarkers in blood. Biomarkers 21 (3), 208–217. https://doi.org/10.3109/1354750X.2015.1126648.

Vulin, A.I., Stanley, F.M., 2004. Oxidative stress activates the plasminogen activator inhibitor type 1 (PAI-1) promoter through an AP-1 response element and cooperates with insulin for additive effects on PAI-1 transcription. J. Biol. Chem. 279 (24), 25172–25178.

Yang, R.Y., Yang, L., Shen, X., Cheng, W., Zhao, B., Ali, K.H., et al., 2012. Suppression of NF-kB pathway powered by crocetin contributes to attenuation of lipopolysaccharide-induced acute lung injury in mice. Eur. J. Pharmacol. 674, 391–396.

Zervos, I.A., Nikolaidis, E.A., Lavrentiadou, S.N., Tsantarliotou, M.P., Eleftheriadou, E.K., Papapanagiotou, E.P., et al., 2011. Endosulfan-induced lipid peroxidation in rat brain and its effect on t-PA and PAI-1: ameliorating effect of vitamins C and E. J. Toxicol. Sci. 36 (4), 423–433.