# Antiplatelet and antithrombotic activities of purpurogallin *in vitro* and *in vivo*

*Sae-Kwang Ku[1] & Jong-Sup Bae[2,]\**

[1]Department of Anatomy and Histology, College of Korean Medicine, Daegu Haany University, Gyeongsan 712-715, [2]College of Pharmacy, CMRI, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 702-701, Korea

**Enzymatic oxidation of pyrogallol was efficiently transformed to an oxidative product, purpurogallin (PPG). Here, the anticoagulant activities of PPG were examined by monitoring activated partial thromboplastin time (aPTT), prothrombin time (PT), and the activities of thrombin and activated factor X (FXa). And, the effects of PPG on expression of plasminogen activator inhibitor type 1 (PAI-1) and tissue-type plasminogen activator (t-PA) were evaluated in tumor necrosis factor (TNF)-α activated human umbilical vein endothelial cells (HUVECs). Treatment with PPG resulted in prolonged aPTT and PT and inhibition of the activities of thrombin and FXa, as well as inhibited production of thrombin and FXa in HUVECs. In addition, PPG inhibited thrombin-catalyzed fibrin polymerization and platelet aggregation. PPG also elicited anticoagulant effects in mice. In addition, treatment with PPG resulted in significant reduction of the PAI-1 to t-PA ratio. Collectively, PPG possesses antithrombotic activities and offers a basis for development of a novel anticoagulant. [BMB Reports 2014; 47(7): 376-381]**

## INTRODUCTION

Primary haemostatic events are triggered in response to damage of the vascular wall by exposure of the subendothelial extracellular matrix to blood (1, 2). Thrombin is the key effector enzyme of the coagulation system, with many biologically important functions, including activation of platelets, conversion of fibrinogen to a fibrin network, and feedback amplification of coagulation (2, 3). The precise and balanced generation of thrombin at sites of vascular injury is the result of an ordered series of reactions collectively referred to as blood coagulation (2, 3). Clots are eventually broken down by plasmin, which is

activated by tissue-type plasminogen activator (t-PA) from plasminogen. Thrombin is also an activator of inflammation and an inhibitor of fibrinolysis (4). The hemostatic plug that forms within blood vessels, often within the veins or arteries of the heart, in pathological conditions associated with arterial disease, referred to as a thrombus (4), is a major cause of morbidity and death. Clotting time assays measure the time required for generation of thrombin (5) and activated partial thromboplastin time (aPTT) measures the efficacy of the contact activation and common coagulation pathways (5). In addition, the aPTT or prothrombin time (PT) mainly serves to aid in diagnosis of deficiencies in certain factors (6).

Purpurogallin (Fig. 1A), a benzotropolone containing natural product, occurs in the nut gall of *Quercus spp.* (7). Purpurogallin has been reported to inhibit glutathione S-transferase, xanthine oxidase, and catechol O-methyltransferase (8-10). It is also effective in the cytoprotection of hepatocytes (11), kidney cells (9), and cardiac cells (12). In addition, purpurogallin exhibited antibacterial activity against gram-positive bacteria and phytotoxicity against all plants tested (13). In our recent study, we reported on the barrier protective and anti-inflammatory effects of PPG in human endothelial cells (14). Inflammation and coagulation play pivotal roles in the pathogenesis of vascular disease (15). Increasing evidence points to extensive cross-talk between these two systems, whereby inflammation leads not only to activation of coagulation, but coagulation also has a considerable effect on inflammatory activity (15). Activation of coagulation and fibrin deposition as a consequence of inflammation is known to occur and can be viewed as an essential part of the body's host defense against infectious agents (15). Together with the previous report, which demonstrated the potential anti-inflammatory effects of PPG (14), we hypothesized that PPG may have anti-coagulant activities. Furthermore, no studies on the anticoagulant activities of PPG have been reported. Therefore, in the current study, we examined the anticoagulant activities of PPG in production of FXa and thrombin, and their effects on PT and aPTT and on fibrinolytic activity.

## RESULTS AND DISCUSSION

Purpurogallin (PPG, Fig. 1A) has been reported to have anti-in-

flammatory and cytoprotective activities (9, 11, 12, 14). In this study, we examined the anticoagulant effects of PPG for the first time and attempted to identify the mechanisms respon-



**Fig. 1.** Chemical structures of PPG and effects of PPG on fibrin polymerization in human plasma and cytotoxicity. (A) Structure of PPG. (B) Thrombin-catalyzed fibrin polymerization at the indicated concentrations of PPG was monitored using a catalytic assay, as described in the "Materials and Methods" section. The results are Vmax values expressed as percentages versus controls. (C) Effect of PPG on mouse platelet aggregation induced by 3 U/ml thrombin. Data represent the mean ± SEM of three independent experiments performed in triplicate. (D) Effect of PPG on cellular viability was measured by MTT assay. **P < 0.01 vs. Th alone.

sible for these effects.

### Effects of PPG on aPTT and PT
Incubation with PPG resulted in changes in the coagulation properties of human plasma. The anticoagulant properties of PPG in human plasma were tested using aPTT and PT assays; a summary of the results is shown in Table 1. Although the anticoagulant activities of PPG were weaker than those of heparin, aPTT and PT were significantly prolonged by treatment with PPG at concentrations greater than 20 μg/ml. The result showing prolongation of aPTT suggests inhibition of the intrinsic and/or the common pathway, whereas prolongation of PT indicates that PPG could also inhibit the extrinsic coagulation pathway. To confirm these data *in vivo*, PPG was administered to mice via intravenous injection. As shown in Table 1, treatment with PPG resulted in significantly prolonged tail bleeding times. Because the average blood volume of a mouse is 2 ml, the amounts of PPG used were 40 or 100 μg per mouse.

### Effects of PPG on thrombin-catalyzed platelet aggregation and fibrin polymerization and cellular viability
The effects of PPG on thrombin-catalyzed fibrin polymerization in human plasma were monitored as changes in absorbance at 360 nm, as described in the Materials and Methods section. The results, shown in Fig. 1B, demonstrate that incubation of human plasma with PPG resulted in a significant decrease in the maximal rate of fibrin polymerization. To eliminate the effect of sample pH, all dilutions were performed using 50 mM TBS (pH 7.4). We also evaluated the ef-
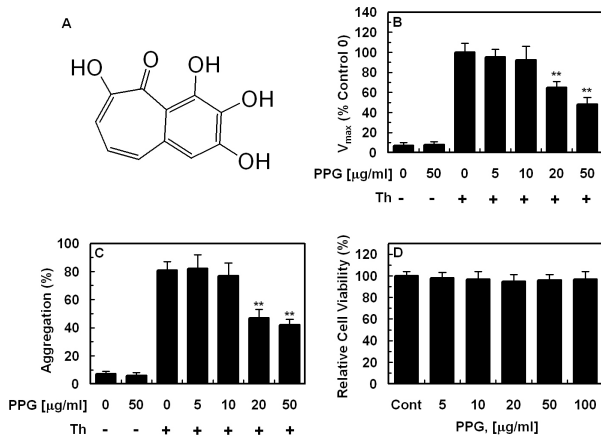
**Table 1.** Anticoagulant activity of PPG[a]

| | | *In vitro* coagulant assay | | |
|---|---|---|---|---|
| Sample | Dose | aPTT (s) | PT (s) | PT (INR) |
| Control | Saline | 31.4 ± 0.4 | 14.4 ± 0.4 | 1.00 |
| PPG | 1 μg/ml | 31.6 ± 0.6 | 14.8 ± 0.6 | 1.06 |
| | 2 μg/ml | 31.4 ± 0.2 | 15.2 ± 0.6 | 1.13 |
| | 5 μg/ml | 32.2 ± 0.4 | 15.8 ± 0.8 | 1.23 |
| | 10 μg/ml | 33.4 ± 1.4 | 15.6 ± 0.4 | 1.19 |
| | 20 μg/ml | 44.4 ± 0.6** | 22.8 ± 0.6 | 2.75** |
| | 50 μg/ml | 53.6 ± 0.8** | 25.6 ± 0.2 | 3.55** |
| Heparin | 5 μg/ml | | 5 μg/ml | 5.18** |
| | 132.4 ± 1.2** | 30.4 ± 0.8** | | |

| | | *In vivo* bleeding time | |
|---|---|---|---|
| Sample | Dose | Tail Bleeding time (s) | n |
| Control | Saline | 43.8 ± 1.8 | 10 |
| PPG | 40 μg/mouse | 67.8 ± 1.2** | 10 |
| | 100 μg/mouse | 82.4 ± 0.8** | 10 |
| Heparin | 1 mg/mouse | 124.3 ± 1.4** | 10 |

[a]Each value represents the means ± SEM (n = 10).
**P < 0.01 as compared to control.

fect of the same volume of DMSO on human plasma; however, coagulation properties were unaffected. To confirm the anticoagulant activities of PPG, thrombin-catalyzed platelet aggregation assay was performed. As shown in Fig. 1C, treatment with PPG resulted in significantly inhibited mouse platelet aggregation induced by thrombin (final concentration: 3 U/ml) in a concentration dependent manner. In order to exclude the possibility that the decrease of polymerization could be due to a direct effect on thrombin leading to a decrease in fibrin production rather than polymerization of fibrin formed, reptilase-catalyzed polymerization assay was performed. Results showed that PPG induced a significant decrease in reptilase-catalyzed polymerization (data not shown). To determine the cellular viability of PPG, cellular viability assay (MTT assay) was performed in HUVECs treated with PPG for 24 h. PPG did not affect cell viability at concentrations up to 100 µg/ml (Fig. 1D).

### Effects of PPG on the activities of thrombin and FXa

In order to elucidate the mechanism responsible for inhibition of coagulation by PPG, the inhibitory effects of PPG on the activities of thrombin and FXa were measured using chromo-
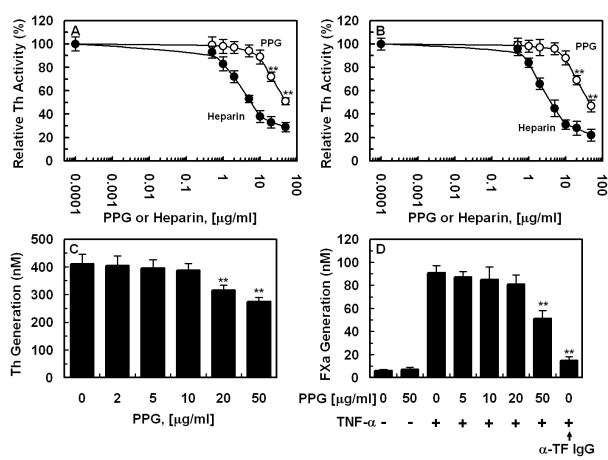
genic substrates. Results are shown in Fig. 2A; treatment with PPG resulted in dose-dependent inhibition of the amidolytic activity of thrombin, indicating direct inhibition of thrombin activity by the anticoagulant. In addition, we also investigated the effects of PPG on activity of FXa. PPG inhibited the activity of FXa (Fig. 2B). These results are consistent with those of our antithrombin assay, and therefore suggest that the antithrombotic mechanisms of PPG are due to inhibition of fibrin polymerization and/or the intrinsic/extrinsic pathway.

### Effects of PPG on production of thrombin and FXa

In a previous study, Sugo et al. reported that endothelial cells are able to support prothrombin activation by FXa (16). In the current study, pre-incubation of HUVECs with FVa and FXa in the presence of $CaCl_2$ prior to addition of prothrombin resulted in production of thrombin (Fig. 2C). In addition, treatment with PPG resulted in dose-dependent inhibition of production of thrombin from prothrombin (Fig. 2C). According to findings reported by Rao et al., the endothelium provides the functional equivalent of pro-coagulant phospholipids and supports activation of FX (17), and, in TNF-α stimulated HUVECs, activation of FX by FVIIa occurred in a TF expression-dependent manner (18). Thus, we investigated the effects of PPG on activation of FX by FVIIa. HUVECs were stimulated with TNF-α for induction of TF expression, and, as shown in Fig. 2D, the rate of FX activation by FVIIa was 16-fold higher in stimulated HUVECs (91.3 $\pm$ 6.4 nM) than in non-stimulated HUVECs



**Fig. 2.** Effects of PPG on inactivation and production of thrombin and factor Xa. (A) Inhibition of thrombin (Th) by PPG was measured using a chromogenic assay, as described in the "Materials and Methods" section. (B) Inhibition of factor Xa (FXa) by PPG was monitored using a chromogenic assay, as described in the "Materials and Methods" section. Heparin (●) was used as a positive control. (C) HUVEC monolayers were pre-incubated with FVa (100 pM) and FXa (1 nM) for 10 min with the indicated concentrations of PPG. Prothrombin was added to a final concentration of 1 µM and prothrombin activation was determined 30 min later, as described in the "Materials and Methods" section. (D) HUVECs were pre-incubated with the indicated concentrations of PPG for 10 min. TNF-α (10 ng/ml for 6 h) stimulated HUVECs were incubated with FVIIa (10 nM) and FX (175 nM) in the absence or presence of anti-TF IgG (25 µg/ml) and FXa production was determined as described in the "Materials and Methods" section. **P < 0.01 vs. 0 (A, B, C) or TNF-α alone (D).
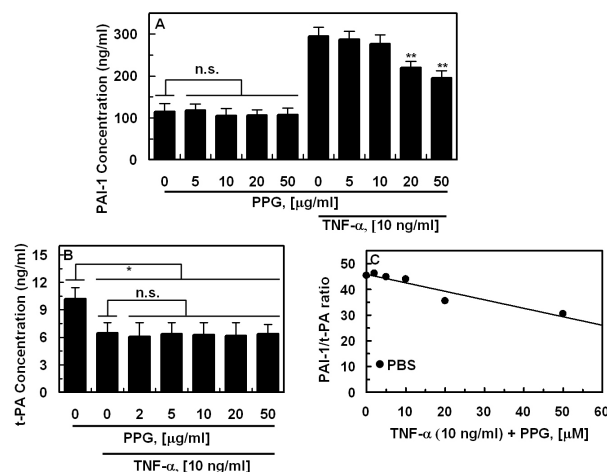


**Fig. 3.** Effects of PPG on secretion of PAI-1 and tPA. (A) HUVECs were cultured with PPG in the absence or presence of TNF-α (10 ng/ml) for 18 h and PAI-1 concentrations in culture media were determined as described in the "Materials and Methods" section. (B) HUVECs were cultured with PPG in the absence or presence of TNF-α (10 ng/ml) for 18 h and t-PA concentrations in culture media were determined as described in the "Materials and Methods" section. (C) PAI-1/t-PA ratio in TNF-α activated HUVECs from (A) and (B). *P < 0.05 or **P < 0.01 vs. TNF-α alone or 0; n.s., not significant.

(5.5 ± 1.4 nM), and this increase in activation was abrogated by anti-TF IgG (11.8 ± 1.8 nM). In addition, pre-incubation with PPG resulted in dose-dependent inhibition of FX activation by FVIIa (Fig. 2D). Therefore, these results suggest that PPG can inhibit production of thrombin and FXa.

### Effects of PPG on secretion of PAI-1 or t-PA protein

TNF-α is known to inhibit the fibrinolytic system in HUVECs by inducing production of PAI-I, and altering the balance between t-PA and PAI-1 is known to lead to modulation of coagulation and fibrinolysis (19, 20). In order to determine the direct effects of PPG on TNF-α-stimulated secretion of PAI-1, HUVECs were cultured in media with or without PPG in the absence or presence of TNF-α for 18 h. As shown in Fig. 3A, treatment with PPG resulted in dose-dependent inhibition of TNF-α-induced secretion of PAI-1 from HUVECs, and these decreases became significant at a PPG dose of 20 μg/ml.

TNF-α does not have a significant effect on t-PA production (21) and the balance between plasminogen activators and their inhibitors reflects net plasminogen-activating capacity (2, 3, 5); therefore, we investigated the effect of TNF-α with PPG on secretion of t-PA from HUVECs. The results obtained were consistent with those of a previous study reporting a modest decrease in production of t-PA by TNF-α in HUVECs (22). This decrease was not significantly altered by treatment with PPG (Fig. 3B). Therefore, collectively, these results indicate that the PAI-1/t-PA ratio was increased by TNF-α and that PPG prevented this increase (Fig. 3C).

In a recent study, we reported that the active compound, PPG, an important component of nut gall of *Quercus spp*, exhibited anti-inflammatory responses in lipopolysaccharide (LPS) treated human endothelial cells (14). We showed that LPS mediated inflammatory responses, such as enhanced vascular permeability, expression of cell adhesion molecules and adhesion and migration of leukocytes, and pretreatment of human endothelial cells with PPG resulted in suppression of LPS-mediated pro-inflammatory responses. There is ample evidence indicating that inflammation and coagulation are intricately related processes that may have a considerable effect on each other (15, 23). This cross-talk occurs at the levels of platelet activation, fibrin formation, and resolution, as well as physiological anticoagulant pathways (15, 23). Based on our previous and current experimental studies, it can be hypothesized that inhibitory modulation of both coagulation and inflammation by PPG could give promising anti-coagulant and anti-inflammatory mediators.

In conclusion, results of this study demonstrate that PPG inhibited the extrinsic and intrinsic blood coagulation pathways through inhibition of FXa and thrombin production in HUVECs, and that PPG inhibited TNF-α-induced secretion of PAI-1. These results add to previous work on the topic, and should be of interest to those designing pharmacological strategies for treatment or prevention of vascular diseases.

## MATERIALS AND METHODS

### Reagents
Purpurogallin (PPG, Fig. 1) was purchased from Sigma (St. Louis, MO, USA). TNF-α was purchased from Abnova (Taiwan). Anti-tissue factor antibody was purchased from Santa Cruz Biologics (Santa Cruz, CA). Factor V, VII, VIIa, FX, FXa, antithrombin III (AT III), prothrombin, and thrombin were obtained from Haematologic Technologies (Essex Junction, VT, USA). aPTT assay reagent and PT reagents were purchased from Fisher Diagnostics (Middletown, Virginia, USA), and the chromogenic substrates, S-2222 and S-2238, were purchased from Chromogenix AB (Sweden). PAI-1 and t-PA ELISA kits were purchased from American Diagnostica Inc. (Stamford, CT, USA). Other reagents were of the highest commercially available grade.

### Isolation of plasma
Blood samples were taken in the morning from 10 healthy volunteers in fasting status (aged between 24 and 28 years, four males and six females) without cardiovascular disorders, allergy and lipid or carbohydrate metabolism disorders, and untreated with drugs. All subjects gave written informed consent before participation. Healthy subjects did not use addictive substances or antioxidant supplementation, and their diet was balanced (meat and vegetables). Human blood was collected into sodium citrate (0.32% final concentration) and immediately centrifuged (2,000 × 15 min) in order to obtain plasma.

### Anticoagulation assay
aPTT and PT were determined using a Thrombotimer (Behnk Elektronik, Germany), according to the manufacturer's instructions, as described previously (24). In brief, citrated normal human plasma (90 μl) was mixed with 10 μl of PPG and incubated for 1 min at 37°C. aPTT assay reagent (100 μl) was added and incubated for 1 min at 37°C, followed by addition of 20 mM CaCl₂ (100 μl). Clotting times were recorded. For PT assays, citrated normal human plasma (90 μl) was mixed with 10 μl of PPG stock and incubated for 1 min at 37°C. PT assay reagent (200 μl), which had been pre-incubated for 10 min at 37°C, was then added and clotting time was recorded. PT results are expressed in seconds and as International Normalized Ratios (INR), and aPTT results are expressed in seconds. INR = (PT sample/PT control)$^{ISI}$. ISI = international sensitivity index.

### Platelet aggregation assay
Mouse platelets from platelet-rich plasma (PRP) were washed once with HEPES buffer (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH₂PO₄, 2 mM MgCl₂, 5.6 mM glucose, 0.1% BSA (w/v), pH to 7.45). The platelet aggregation study was performed according to a previously reported method (25). Washed platelets were incubated with the indicated concentration of PPG for 3 min, and followed by stimulation with thrombin (3U/ml, Sigma) in 0.9% saline solution at 37°C for 5

min. Platelet aggregation was recorded using a aggregometer (Chronolog, Havertown, PA, USA).

## Thrombin-catalyzed fibrin polymerization

Thrombin-catalyzed polymerization was determined every 6 s for 20 min by monitoring turbidity at 360 nm using a spectrophotometer (TECAN, Switzerland) at ambient temperature. Control plasma and plasma incubated with PPG were diluted three times in TBS (50 mM Tris-buffered physiological saline solution pH 7.4) and clotted with thrombin (final concentration - 0.5 U/ml). The maximum polymerization rate (Vmax, ΔmOD/min) of each absorbance curve was recorded (26). All experiments were performed three times.

## Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and were maintained using a previously described method (27, 28). Briefly, cells were cultured until confluent at $37^{o}C$ at 5% $CO_2$ in EBM-2 basal media supplemented with growth supplements (Cambrex Bio Science).

## Cell viability assay.

MTT was used as an indicator of cell viability. Cells were grown in 96-well plates at a density of $5 \times 10^3$/well. After 24 h, cells were washed with fresh medium, followed by treatment with PPG. After a 48-h incubation period, cells were washed, and 100 μl of 1 mg/ml MTT was added, followed by incubation for 4 h. Finally, 150-μl DMSO was added in order to solubilize the formazan salt formed, the amount of which was determined by measuring the absorbance at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). Data were expressed as mean ± SD of at least three independent experiments.

## Factor Xa production on the surfaces of HUVECs

TNF-α (10 ng/ml for 6 h in serum-free medium) stimulated confluent monolayers of HUVECs (preincubated with the indicated concentrations of PPG for 10 min) in a 96-well culture plate were incubated with FVIIa (10 nM) in buffer B (buffer A supplemented with 5 mg/ml bovine serum albumin [BSA] and 5 mM $CaCl_2$) for 5 min at $37^{o}C$ in the presence or absence of anti-TF IgG (25 μg/ml). FX (175 nM) was then added to the cells (final reaction mixture volume, 100 μl) and incubated for 15 min. The reaction was stopped by addition of buffer A (10 mM HEPES, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) containing 10 mM EDTA and the amounts of FXa generated were measured using a chromogenic substrate. Changes in absorbance at 405 nm over 2 min were monitored using a microplate reader. Initial rates of color development were converted into FXa concentrations using a standard curve prepared with known dilutions of purified human FXa.

## Thrombin production on the surfaces of HUVECs

Measurement of thrombin production by HUVECs was quanti-

tated as previously described (24, 28). Briefly, HUVECs were pre-incubated in 300 μl containing PPG in 50 mM Tris-HCl buffer, 100 pM FVa, and 1 nM FXa for 10 min, followed by addition of prothrombin to a final concentration of 1 μM. After 10 min, duplicate samples (10 μl each) were transferred to a 96-well plate containing 40 μl of 0.5 M EDTA in Tris-buffered saline per well in order to terminate prothrombin activation. Activated prothrombin was determined by measuring the rate of hydrolysis of S2238 at 405 nm. Standard curves were prepared using amounts of purified thrombin.

## Thrombin activity assay

PPG in 50 mM Tris-HCl buffer (pH 7.4) containing 7.5 mM EDTA and 150 mM NaCl was mixed in the absence or presence of 150 μl of AT III (200 nM). Heparins with AT III (200 nM) were dissolved in physiological saline and placed in the sample wells. Following incubation at $37^{o}C$ for 2 min, thrombin solution (150 μl; 10 U/ml) was added, followed by incubation at $37^{o}C$ for 1 min. S-2238 (a thrombin substrate; 150 μl; 1.5 mM) solution was then added and absorbance at 405 nm was monitored for 120 s using a spectrophotometer (TECAN, Switzerland).

## Factor Xa (FXa) activity assay

These assays were performed in the same manner as the thrombin activity assay, but using factor Xa (1 U ml/1) and S-2222 as substrates.

## *In vivo* bleeding time

Tail bleeding times were measured using the method described by Dejana *et al*. (24, 29). Briefly, ICR mice were fasted overnight before experiments. One h after intravenous administration of PPG, tails of mice were transected at 2 mm from their tips. Bleeding time was defined as the time elapsed until bleeding stopped. When the bleeding time exceeded 15 min, bleeding time was recorded as 15 min for the analysis. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Kyungpook National University.

## ELISA for PAI-1 and t-PA

The concentrations of PAI-1 and t-PA in HUVEC cultured supernatants were determined using ELISA kits (American Diagnostica Inc. CT, USA).

## Statistical Analysis

Data are expressed as mean ± SEM (standard error of the mean) of at least three independent experiments. Statistical significance between two groups was determined using the Student's *t*-test. Statistical significance was accepted for P values < 0.05.

## Conflict of interest statement

The authors have no conflict of interest to declare.

## ACKNOWLEDGEMENTS

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