

# Inhibition effect of cardiotonic pills on venous thrombosis induced in rat mesentery by photochemical reaction

Fang Wang<sup>a</sup>, Yu-Ying Liu<sup>a,\*</sup>, Lian-Yi Liu<sup>a</sup>, Jun Guo<sup>a</sup>, Kai Sun<sup>a</sup>, Chuan-She Wang<sup>a,b</sup>, Jing-Yu Fan<sup>a</sup> and Jing-Yan Han<sup>a,\*</sup>

<sup>a</sup> *Tasly Microcirculation Research Center, Peking University Health Science Center, Haidian, Beijing 100083, China*

<sup>b</sup> *Department of Integrated Traditional Chinese and Western Medicine, Peking University Health Science Center, Haidian, Beijing 100083, China*

**Abstract.** This paper was aimed to explore the inhibition effect of cardiotonic pills (CP) on venous thrombosis induced in rat mesentery by photochemical stimulation (PS). Male Sprague-Dawley rats were anesthetized with urethane. Thrombosis was induced in the mesenteric venule by PS with photosensitizer hematoporphyrin and an ultraviolet beam. The rats were divided into three groups: control ( $n = 6$ ), PS ( $n = 6$ ) and PS + CP group ( $n = 6$ ) where CP solution (0.4 g/kg.b) was administrated orally 60 min before PS. Microcirculatory disturbances in the mesentery were observed under an inverted microscope with a color video-camera. Based on the recorded images, the development of thrombosis was evaluated in term of time of thrombosis appearance ( $T_a$ ) and area ratio of thrombus/vessel (AR). The expression of adhesion molecule (CD31) of platelet was examined in blood taken from the abdominal aorta, using flow cytometry. The  $T_a$  was approximately 10 seconds after PS in PS group, but it was approximately 20 seconds in PS + CP group. The AR in PS + CP group was significantly reduced, compared to that in PS group, during the period of the observation. The CD31 expression was not changed in both groups, while positive cells were significantly increased in the number. It is suggested that CP might suppress thrombus development under the interaction of platelet with endothelium.

Keywords: Cardiotonic pills, venous thrombosis, adhesion molecules, platelet, videomicroscopy

## 1. Introduction

Cardiotonic pills (CP) consist of *Salvia miltiorrhiza*, *Panax notoginseng* and Borneol, which have been widely used for treatment of coronary heart disease, brain thrombosis, venous thrombosis and thrombus-related diseases [1]. In previous papers, we studied several effects of CP in microcirculatory disturbances induced by ischemia/reperfusion (I/R) and chronic alcohol. CP could inhibit the leukocyte adhesion to venules, oxidative stress, albumin leakage and mast cell degranulation under ischemia/reperfusion [2, 3] and also could diminish the leukocyte adhesion to liver sinus in chronic alcoholic [4]. According to Tang et al. [5], *Salvia miltiorrhiza* (major effective component of CP), and dihydroxyphenyl lactic acid (LDA) (major water soluble component of *Salvia miltiorrhiza*) have an inhibitory effect on platelet aggregation. This indicates that CP may inhibit thrombosis, but this possibility has not been proved *in vivo*.

\*Corresponding authors. E-mail: kan@chuigaku.co.jp (J.-Y.H.); liuyyliu@sohu.com (Y.-Y.L.).

Photochemical reaction has been used for induction of thrombosis *in vivo* [6–10]. Sato et al. [7,8] induced thrombosis in rat mesentery by injecting photosensitizer hematoporphyrin with an ultraviolet beam on venules. The mesenteric venular thrombosis model is most available for *in vivo* studies of venous thrombosis. In the present study, we used this technique to induce thrombosis in rat mesentery. By using videomicroscopy, we investigated inhibition effects of CP on the venous thrombosis. Furthermore, we also measured the expression of CD31 of platelet *in vitro*, using flow cytometry.

## 2. Materials and methods

### 2.1. Preparation of CP solution

CP was provided by Tianjin Tasly group (Tianjin, China). The CP solution was prepared by dissolving in saline solution at the concentration of 0.1 g/ml.

### 2.2. Animal preparation

Male Sprague-Dawley (SD) rats (220–250 g bw) were provided by the Animal Center of Peking University Health Science Center. The rats were fasted with normal drink during 12 hours before the experiment, and anesthetized by intramuscular injection of urethane (1 ml/100 g.bw).

The rats ( $n = 18$ ) were divided into three groups: control ( $n = 6$ ), photochemical stimulation (PS;  $n = 6$ ) and photochemical stimulation + CP group (PS + CP;  $n = 6$ ).

### 2.3. Microscopic observation of microcirculatory disturbances

In each rat, the abdomen was opened via a midline (incision 20–30 mm long). The ileocecal portion of the mesentery (10–15 cm caudal) was gently drawn out, exteriorized, and mounted on a transparent plastic stage. The mesentery was kept warm and moist by continuous superfusion with saline solution at 37°C.

Microcirculation in the mesentery were observed under an inverted microscope (DM-IRB Leica, Germany) with a transilluminator. As an objective lens,  $\times 20$  was used. The microscopic images were projected on a monitor by a color video camera (Toshiba, 3CCD camera, Japan) mounted on the microscope, which were recorded with a DVD videocassette recorder (DVR-R25, Malata, China). A video timer (For.a, VTG-55B, Japan) was used to monitor the time and stopwatch function on the monitor.

#### 2.3.1. Induction of thrombosis

Photosensitizer hematoporphyrin (PHP; 0.25 mg/kg.bw) (Pharmaceutical Industry Institute, China) was injected through the caudal vein [5]. Then, 10 minutes later, an ultraviolet beam (455 nm wavelength) was irradiated from a mercury burner (100 W) to the fluorescence microscope [11]. It was passed through an objective lens to be focused on a single vessel. Its size was approximately 250  $\mu\text{m}$  in diameter. In Fig. 1 is shown schematic drawings of the beam and vessel with thrombosis.

#### 2.3.2. Experimental protocol

In control group, after anesthesia, rat mesentery was exposed and observed under the videomicroscope for 30 minutes. In PS group, anesthetized rats were injected with PHP and irradiated with an ultraviolet beam. In PS + CP group, CP solution was administrated orally in dose of 0.4 g/kg bw into rats at 30 minute before PHP injection.

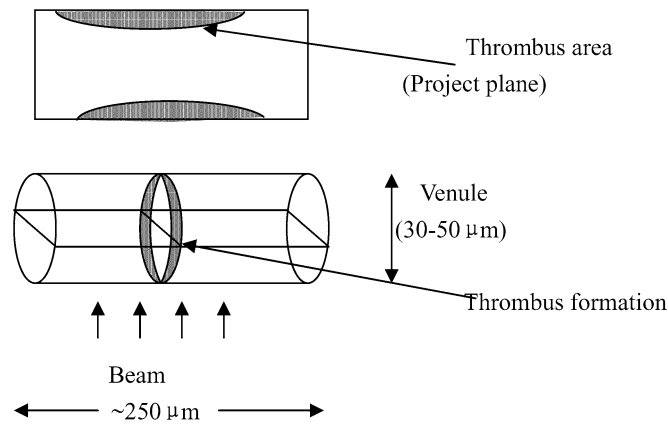


Fig. 1. Configuration of a venule (30–50  $\mu\text{m}$  in diameter) and an ultraviolet beam ( $\sim 250 \mu\text{m}$  in diameter). The area of thrombus induced is shown by shadow on the 2D-projected plane.

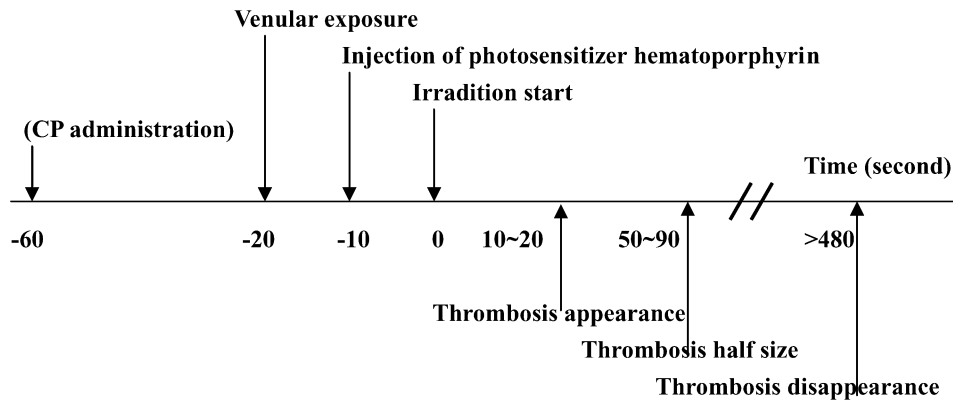


Fig. 2. Protocol (time course) of experiment in PS and PS + CP group.

By selecting a single and unbranched venule (30–50  $\mu\text{m}$  in diameter; 250  $\mu\text{m}$  in length) in each rat, we irradiated to induce thrombosis. The irradiation was made until the thrombus approached to a half size of the venular diameter. The microscopic observation was continued during 30 minutes (Fig. 2).

### 2.3.3. Evaluation of microcirculatory disturbances

Based on the recorded image, we examined the microcirculatory disturbances in terms of the following parameters.

(a) *Thrombus appearance time ( $T_a$ )*. This was defined by the time duration from the start of irradiation to the appearance of thrombus on the image. The thrombus appearance was determined on basis of the platelet adhesion to the venular wall.

(b) *Area ratio of thrombus/venule (AR)*. The thrombosis formation and development was evaluated within 250  $\mu\text{m}$  length of venule on the projected image (see Fig. 3). The area was measured every 10 (or 15) seconds during 5 minutes of irradiation, using Image-Pro-Plus software (Media Cybernetics Inc, USA). The area ratio (AR) of thrombus/venule was defined by the ratio derived from thrombus area divided by the venular area.

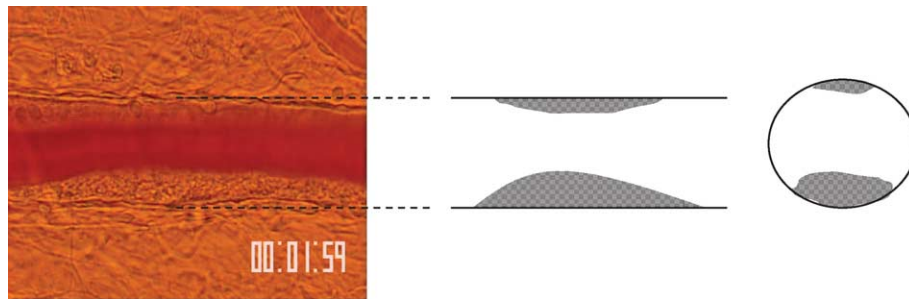


Fig. 3. An example of video image at a time-stage of thrombosis from the initiation to flow downstream on the projected plane of thrombus and the cross-sectional thrombus in PS + CP group.

(c) *Venular diameter (D)*. The diameters were measured at a proximal site to the area subject to the ultraviolet beam before and 10, 20, and 30 min after the irradiation, using Image-Pro-Plus software [12].

#### 2.4. Expression of adhesion molecules (CD31)

Thirty minutes after PS, 3 ml blood (anti-coagulated with EDTA) was collected from the mesenteric vein in 6 rats of each group (control, PS and PS + CP). The platelets were separated using a centrifuger, and 5000 platelets were examined using flow cytometry (FACS). The expression of adhesion molecule CD31 of platelets was depicted in terms of fluorescence intensity (mean) of phycoerythrin (PE)-labeled CD31 antibody positive cells [13].

#### 2.5. Statistic analysis

The quantitative data were expressed as mean  $\pm$  SEM ( $n = 6$ ). One-way ANOVA was performed using SPSS 11.0 statistic analysis software. Statistical significance was assigned by  $P < 0.05$ .

### 3. Results

Table 1 shows the diameter ( $D$ ), the time ( $T_a$ ) of thrombus appearance, and the time ( $T_h$ ) for thrombosis to grow to a half size to the vessel in PS and PS + CP groups. In the control group, no thrombus formation was observed within the period of observation times. In PS group, thrombi appeared at approximately 10 seconds after irradiation, while it appeared at approximately 21 seconds in PS + CP group. The venular diameter showed a vasodilatory tendency by PS in both PS and PS + CP groups, but these differences were not significant during the period of observation.

#### 3.1. Development of thrombosis on the projected image

The thrombosis appeared at one side of venule at a short period (10–20 seconds), which developed up to a comparable size to the venular diameter until it flowed downstream. Figure 3 shows an example of videoimage together with the cross-section (illustrated) in PS + CP group.

Figure 4 shows time-changes of the thrombus area measured on the projected plane following the irradiation of ultraviolet beam in a rat of PS and PS + CP groups. In PS group, the thrombus area started approximately 10 seconds after the irradiation, increasing up to approximately  $3500 \mu\text{m}^2$  30 seconds

Table 1

The mean  $\pm$  SEM ( $n = 6$ ) of venular diameter ( $D$ ), the time ( $T_a$ ) of thrombus appearance, and the time ( $T_h$ ) for thrombosis to grow to a half size to the vessel in PS ( $n = 6$ ) and PS + CP group ( $n = 6$ )

	Vessel diameter $D$ ( $\mu\text{m}$ )		Time of thrombosis appearance $T_a$ (sec)	Time of thrombosis half size $T_h$ (sec)
	0 sec	30 min		
PS group	37.5 $\pm$ 3.4	38.3 $\pm$ 3.4	9.8 $\pm$ 1.0	48.0 $\pm$ 8.9
Mean $\pm$ SEM				
PS + CP group	37.5 $\pm$ 2.8	38.7 $\pm$ 3.2	20.8 $\pm$ 2.6 <sup>#</sup>	90.2 $\pm$ 7.1 <sup>#</sup>
Mean $\pm$ SEM				

<sup>#</sup> $p < 0.05$  vs PS.

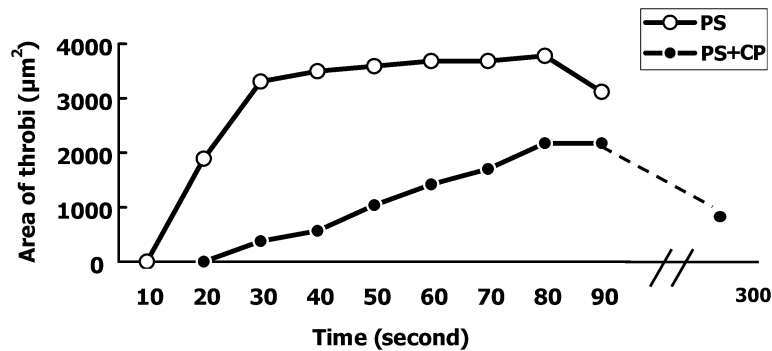


Fig. 4. Time-changes of the thrombus area measured on the projected plane in a rat of PS and PS + CP groups.

after, which lasted during 50 seconds. In PS + CP group, the thrombus area started at approximately 20 seconds, increasing up to approximately 2000  $\mu\text{m}^2$  80 seconds after, which lasted during 10 seconds. Apparently, the onset and development of thrombus area were suppressed under the effect of CP.

### 3.2. The area ratio of thrombus/venule

Figure 5 shows time-changes of the area ratio (mean  $\pm$  SEM) of thrombus/venule induced in the mesentery by PS in 6 rats. In PS group, it increased from 0.2  $\pm$  0.06 (15 seconds after PS) to 0.4  $\pm$  0.08 (30 seconds after PS), which lasted until 75 seconds after the irradiation. In PS + CP group, it was 0.07  $\pm$  0.04, 0.15  $\pm$  0.05, 0.30  $\pm$  0.06, respectively, 15, 30, 75 seconds after irradiation, respectively. These indicate that pre-treatment with CP significantly suppressed the increase in the area ratio of thrombus/venule following PS.

### 3.3. The expression of adhesion molecules CD31 of platelets

Figure 6 shows fluorescence intensities of adhesion molecule CD31 of platelets and number of the positive cells in control, PS and PS + CP groups. There appeared no significant difference in the expression of adhesion molecule CD31 of platelets, but positive cells were significantly different in the number between control and PS groups.

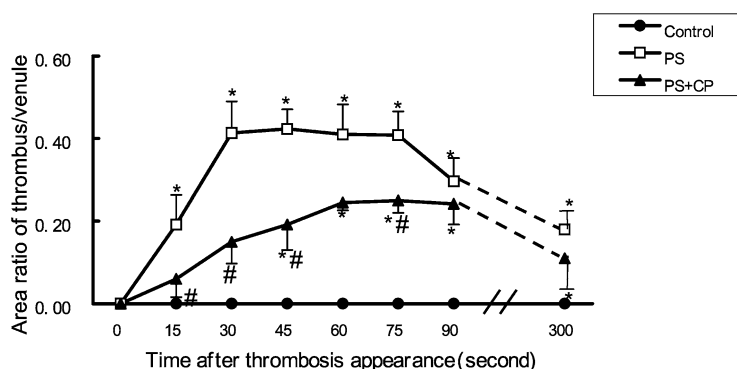


Fig. 5. The time course of the area ratio of thrombus/venule in 6 rats of PS and PS + CP groups. \* $p < 0.05$  vs control, # $p < 0.05$  vs PS.

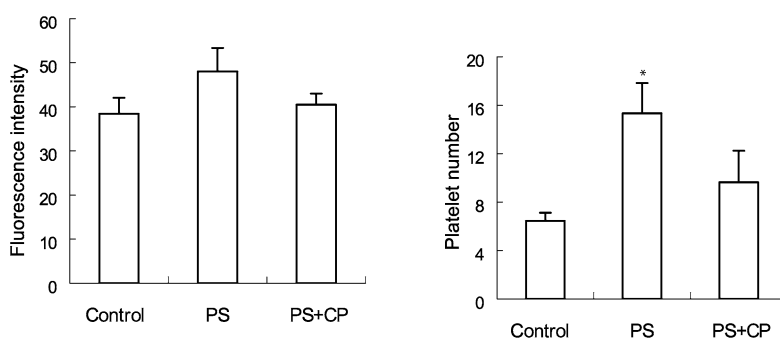


Fig. 6. Fluorescence intensities of adhesion molecule CD31 of platelets (left) and number of the positive cells (right) in three groups (control, PS and PS + CP). \* $p < 0.05$  vs control.

#### 4. Discussion

Using a videomicroscopic technique in a rat mesenteric model of venous thrombosis, we have evaluated the appearance time and the area of thrombosis induced by PS after the irradiation of ultraviolet beam. The areas of thrombus were summed by those measured on the both sides of vascular wall on the two-dimensional projected plane. In the present experiment, the ultraviolet beam might be irradiated sufficiently over the vessel, so that the endothelial cells might be damaged effectively along the circumference of vessel. It is, therefore, reasonable to suppose that the area of thrombosis measured on the projected plane should be related to the volume of three-dimensional thrombus.

The present experiment has demonstrated that the CP could delay the thrombus appearance (Table 1 and Fig. 4) and also suppressed the increase in the thrombus area (Fig. 5). The present *in vitro* result using flow cytometry has demonstrated that the expression of CD31 of platelet induced by PS was significantly enhanced by PS, while administration of CP did not influence its expression but the number of positive cells. Injection of photosensitizer hematoporphyrin followed by irradiation with ultraviolet may elicit the focal production of single oxygen and/or superoxide radical, which results in harming vessel endothelium and activation of platelet [14,15]. However, it has not yet been reported whether PS may interfere with the expression of CD31 of platelets or not.

The platelets have various adhesion molecules on their surface, including CD31, CD61, CD62p, and their expression can give rise to adhesion of platelets with vessel endothelium, which play an important

role in the thrombosis [16,17]. However, no increase was observed in expression of CD31 of platelets by PS, and pre-treatment with CP has not affect on expression of CD31 of platelets by PS in this study. It remains unclear whether CP may influence the expression of these thrombosis-related adhesion molecules.

It has been proved *in vivo* that pre-treatment with CP could inhibit venous thrombosis induced in rat mesentery by PS. It is suggested that CP may be useful for clinical treatment of thrombus-related diseases [18–21].

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