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Technical Note

The synergistic effects of Korean Red Ginseng and *Cervi Parvum Cornu* ameliorating $FeCl_3$-induced arterial thrombosis by downregulating ICAM-1 and VCAM-1

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ABSTRACT

In this study, we compared antithrombotic activities of Korean Red Ginseng (KRG) and *Cervi Parvum Cornu* (CPC) on rats with induced thrombosis. Results indicate that KRG and CPC suppressed the arterial occlusion and the combination of KRG and CPC (KRG + CPC) treatment exhibited a synergistic effect with maximum reduction in thrombosis.

Thrombosis is the thrombus formation in cardiovascular system due to cohesion of platelets and blood components [1]. The activation of blood coagulation factors leads to the development of arterial thrombus [2]. Also, abnormal cohesion of blood cells such as leukocytes to endothelium of vascular wall, caused by action of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), results in initiating the formation of atherosclerotic deposits [3]. Earlier, many natural herbal medicines have been developed to improve the therapy of thrombogenesis such as arteriosclerotic and vascular disorders [4]. Korean Red Ginseng (KRG) is known for its anti-thrombotic and anti-platelet effects and has shown potential as a therapeutic agent in platelet aggregation, thrombosis and inflammation [5]. Also, KRG had demonstrated synergistic effects along with other herbal medicine in managing various disease [6]. *Cervi Parvum Cornu* (CPC), a traditional medicine derived from slices of deer antlers, is used for improving heart function and lung efficiency, and also as a major blood-tonifying agent [7]. In previous studies, CPC was reported to contain sugar molecules, sialic acid, minerals, free amino acids, and proteins [7,8]. Among them, sialic acid is one of the active ingredients which acts as a hemorrheologic agent and for restoring cardiac function [9]. Previous studies suggest reduced sialic acid levels can induce thrombosis and erythrocyte aggregation [10]. Recently, studies have been reported on the potential anti-inflammatory effects of CPC and synergistic effect of CPC with KRG [11,12]. However, the synergistic effects of KRG with CPC on therapeutic potential on thrombosis are not yet explored. This study aims to evaluate the antithrombotic effects of KRG or CPC and investigate the synergistic ability of KRG + CPC on suppressing thrombosis formation in a rat model.

KRG and CPC were kindly supplied by Korea Ginseng Corporation (KGC; Daejeon, Korea). Ginsenosides were separated using HPLC system (Waters, USA) with photo diode array detector at 203 nm. The system is equipped with ACQUITY BEH C18 column (1.7 μm, 2.1 × 100mm) and mobile phase consisted of distilled water (A) and acetonitrile (B). Gradient elution was performed with 0–0.5 min (15 % B), 14.5 min (30 % B), 15.5 min (32 % B), 18.5 min (38 % B), 24.0 min (43 % B), 27.0 min (55 % B), 27.0–31.0 min (55 % B), 33.0 min (90 % B), 33.0–38.0 min (90 % B), 38.1 min (15 % B), and 38.1–43.0 min (15 % B). The flow rate for elution was set at 0.6 mL/min with 2.0 μL injection volume. HPLC analysis for KGC revealed the amount of ginsenosides as follows: Rg1 0.68 mg/g, Re 0.88 mg/g, Rf 1.26 mg/g, Rh_1 1.52 mg/g, Rg_2(s) 1.03 mg/g, Rb_1 1.5 mg/g, Rc 5.62 mg/g, Rb_2 2.13 mg/g, Rd 2.06 mg/g, Rg_3(s) 0.97 mg/g, and Rg_3(r) 3.16 mg/g by evaporative light scattering detector. Likewise, sialic acid content was 1.48 mg/g in CPC and 0.81 mg/g in the combination KRG + CPC (1:1). Ferric chloride ($FeCl_3$) were obtained from Sigma-Aldrich (St. Louis, MO, USA), and all other reagents were of reagent or biological grade from Sigma. 42 male wistar

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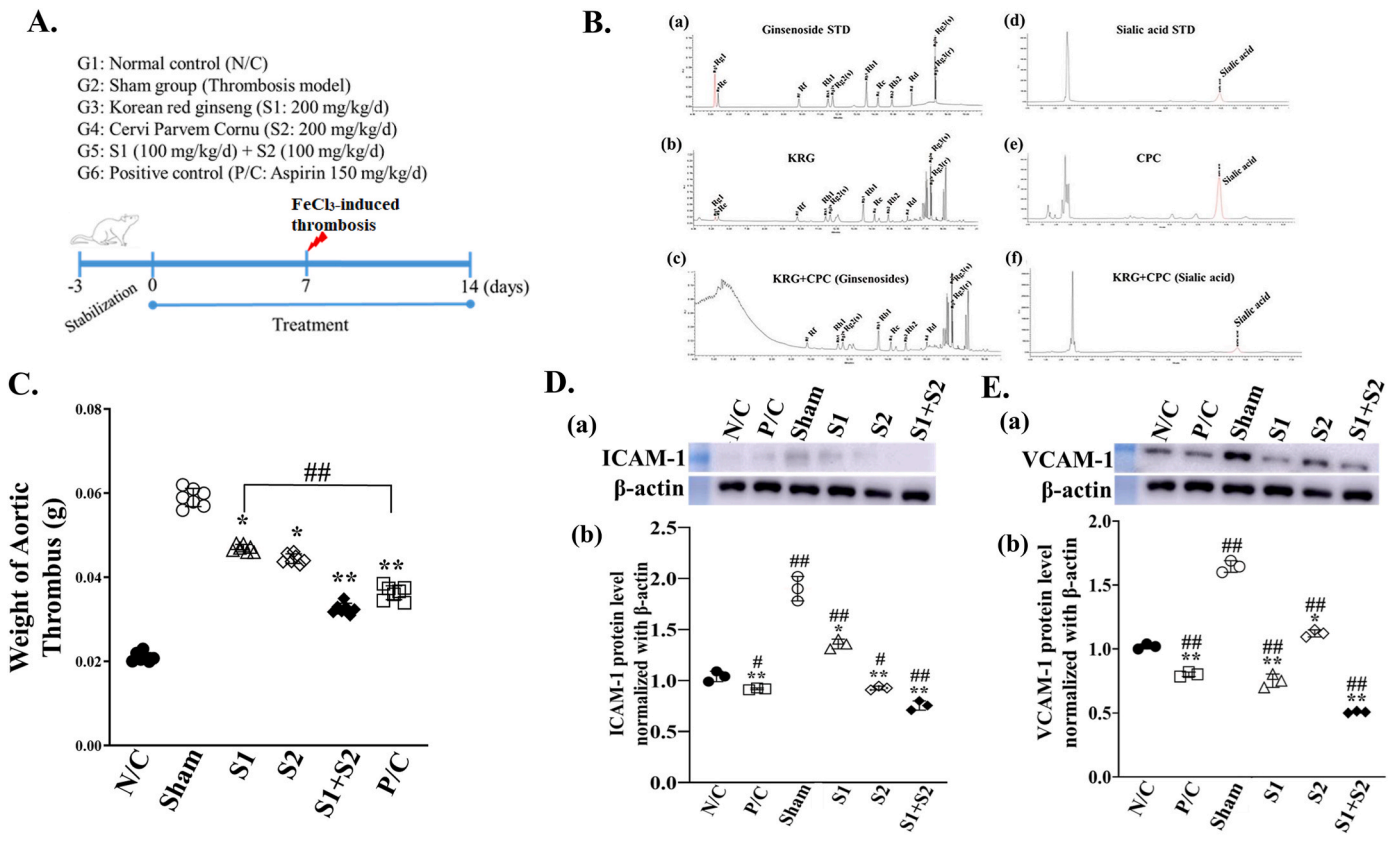

Fig. 1. Anti-thrombotic effect of KRG(S1) and CPC(S2) in rat arterial thrombosis model. (A) Experimental protocol; (B) HPLC chromatograms of (a) ginsenoside standard (Ginsenoside STD), (b) KRG, (c) amount of ginsenosides in KRG + CPC, (d) sialic acid standard (Sialic acid STD), (e) CPC, and (f) amount of sialic acid in KRG + CPC; (C) average weight of aortic thrombus (n = 7); (D) ICAM-1 expression, (a) Western blot analysis and (b) protein level normalized with β-actin; (E) VCAM-1 expression, (a) Western blot analysis and (b) protein level normalized with β-actin. Data are value of triplicates with means ± SD. *p < 0.05 and **p < 0.01 when treatment groups are compared with sham (thrombosis) group, and #p < 0.05 and ##p < 0.01 when treatment groups are compared with normal control group.

rats (Samtako-Seoul, Korea) weighing 250 ± 20 g were used, the experiment was performed in the room conditions at 12 h light/dark cycle, with 22 °C ± 2 °C, 50 % ± 5 % relative humidity. All in vivo studies followed in accordance with international guidelines for animal experiments and principles of laboratory animal care of Jeonbuk National University (IACUC No. CBNU 2020–037, Jeonju, Korea). Animals (n = 7) were randomly allocated into normal control (N/C), sham (thrombosis-induced), KRG-treated (S1; 200 mg/kg/d), CPC-treated (S2; 200 mg/kg/d), KRG + CPC-treated (100 mg/kg/d of each S1+S2), and positive control (aspirin 150 mg/kg/d) groups. Animals were stabilized for 3 days, and treatment of KRG, CPC, and KRG + CPC initiated for the respective groups up to 7 days. After 7 days of treatment, thrombosis was induced on the right carotid artery by exposing the artery using 40 % $FeCl_3$ saturated filter paper 1 cm × 0.5 cm for 1 h [13]. After removing the filter paper, the exposed artery was sutured, and the animal were placed on a heating pad for 1 h before transferring back to its cage. After, respective treatment of KRG, CPC, and KRG + CPC were continued for 7 days to the animals and then it was sacrificed. Weight of thrombosis induced artery were measured after surgically removing on a filter paper. For SEM and hematoxylin-eosin (HE) stain, 5 μm thick sections of artery previously fixed in 10 % formalin for 48 h and staining of the sections were performed as per the standard protocol [14,15]. Anti-thrombotic effect is evaluated by measuring levels of endothelial adhesion molecules such as ICAM-1 and VCAM-1 (Primer sets were received from Bioneer, Daejeon, Korea). Confocal microscopic images were also analyzed through immunohistochemistry (IHC) [16], fluorescence intensity measurements were calculated using FOBI (NEOimage for FOBI, Korea). All data were analyzed using statistics of variance (ANOVA) followed by Tukey's multiple comparison study. Values are expressed as mean standard deviation and probability of $p < 0.05$ is considered as statistically significant. In results, the weight of thrombosis-induced artery were reduced in KRG(**S1**) or CPC(**S2**) treatment group compared to the sham group. Especially, the weight of artery in KRG + CPC(**S1+S2**) group were lower than KRG or CPC groups, displaying synergistic effect of KRG with CPC (Fig. 1C). ICAM-1 and VCAM-1 are inflammatory markers at intracellular and vascular levels, directly related to thrombotic complications[17]. Western blot analysis also revealed that the synergistic effects of S1 with S2 by suppressing ICAM-1 expression compared to the sham, S1, and S2 groups, which means the combination of S1 with S2 show even more suppression of ICAM-1. (Fig. 1D). Likewise, VCAM-1 level were significantly decreased in the combination of S1 with S2 compared to S1 or S2 group, suggesting synergistic effects of S1 with S2 by suppressing VCAM-1 expression (Fig. 1E). HE staining showed S1+S2 group had drastically reduced the occlusion in lungs and carotid artery compared to S1 or S2 group (Fig. 2A). Similarly, masson trichome stain indicates robust reduction of occlusion in lungs and carotid artery for S1+S2 group (Fig. 2B). Confocal images indicates higher expression of ICAM-1 than VCAM-1 in S1, S2, and S1+S2 groups (Fig. 2C). The fluorescence intensity levels of ICAM-1 and VCAM-1 were only marginally low in S1+S2 group than S1 or S2 group. Whereas, the expression of ICAM-1 and VCAM-1 in S1 or S2 group showed slightly higher than S1+S2 group (Figs. 2C–1 and C-2), which means the combination of KRG and CPC more effectively inhibits the expression of ICAM-1 and VCAM-1. In this regard, we would like to present three possibilities. The first possibility is that in S1 or S2 group, the dosage is 200 mg/kg, whereas in the S1+S2 group, the dosage is only 100 mg/kg for each. Therefore, it can be expected that the expression of ICAM-1 and VCAM-1 appeared differently depending on the difference

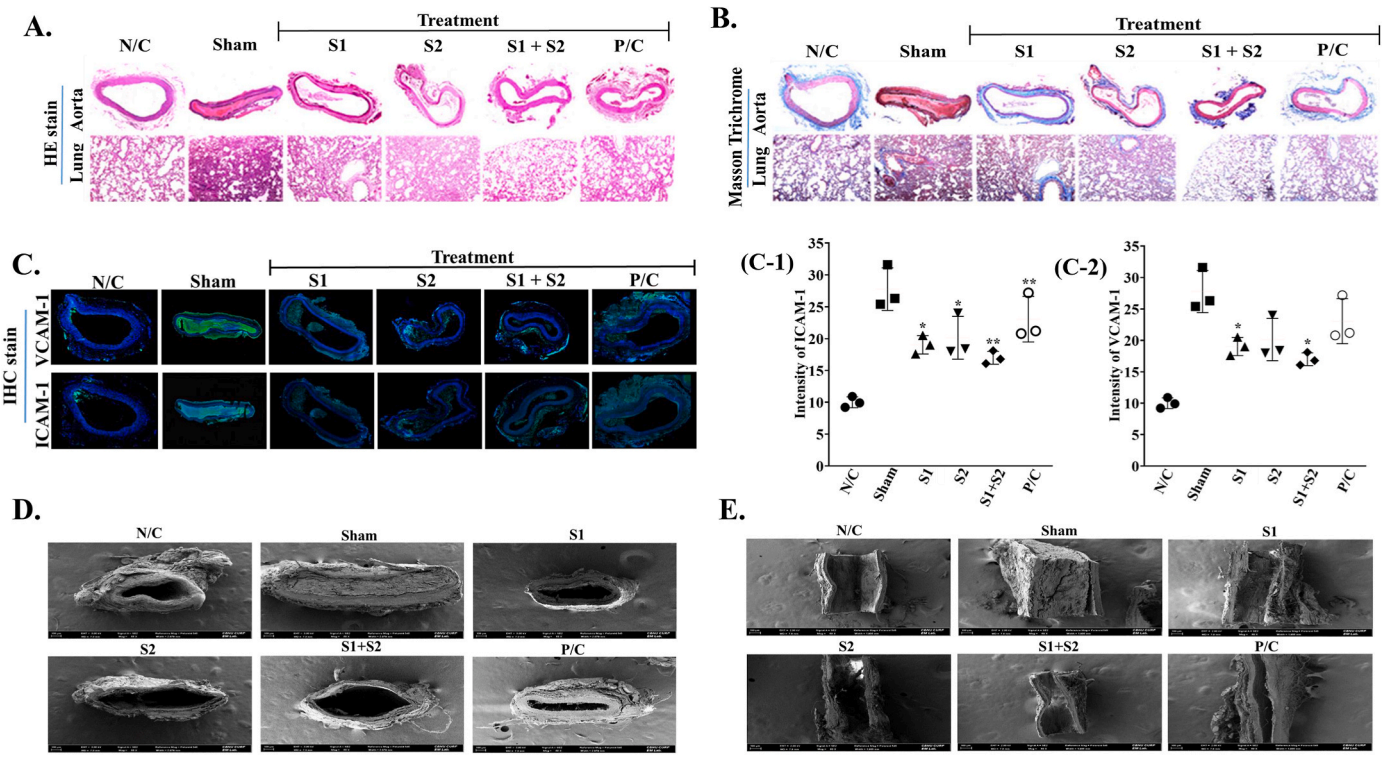

Fig. 2. Anti-thrombotic effect of KRG(S1) and CPC(S2) on aorta and lung tissue. Sections observed at a magnification of ×100, were stained with (A) HE stain; (B) masson's trichrome; (C) immunohistochemistry staining, (C-1) relative histogram for ICAM-1 and (C-2) relative histogram for VCAM-1, respectively; (D) SEM images from cross-section of artery; and (E) longitudinal section of artery were shown. Data are value of triplicates with means ± SD. *p < 0.05 and **p < 0.01 when treatment groups are compared with sham group, and #p < 0.05 and ##p < 0.01 when treatment groups are compared with normal control group.

in concentration between KRG and CPC, and the second possibility is that the expression of ICAM-1 and VCAM-1 was affected by the mutual agonism on specific components of KRG and CPC upon mixtures of KRG and CPC. It could be predicted that the mutual restrict relationship between KRG and CPC components might be affected due to the differences in characteristics of natural components. The third possibility can be anticipated due to the interaction of transcription factors related to the ICAM-1 and VCAM-1 in mixtures of KRG and CPC. This may be the result of components of KRG or CPC acting agonistically to each other. However, further study is required to identify such interaction for KRG and CPC. On the other hand, SEM analysis reveals a significant thrombus formation in the sham group compared to S1, S2, and S1+S2 groups, demonstrating severe thrombus on aorta walls. However, S1 or S2 group indicates a reduction in thrombus and the combined treatment of S1 and S2 exhibited even less thrombus formation (Fig. 2D and E) than sham group, S1, or S2 group. Limitations to our finding is that both KRG and CPC are not completely water soluble, which might restrict on estimating the exact active ingredients involved in this therapeutic effect. Second limitation is that, the physicochemical nature and stability of the extracts in combination needs to be investigated in detail in order to validate its stability. However, based on the above results, it is evident that treatment of KRG + CPC as well as KRG or CPC, respectively, could inhibit thrombus formation by controlling inflammation in inducing downregulation of ICAM-1 and VCAM-1. Our data suggests that the combination of KRG and CPC might exhibit synergistic activity in ameliorating thrombosis. Nevertheless, the bioactive components of KRG and CPC need to be thoroughly investigated to elucidate the mechanism behind this antithrombotic effect.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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