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Original Article

Anti-thrombotic Effects of Modified *Jeho-tang* using a $FeCl_3$-induced Carotid Arterial Thrombosis Model

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Objectives: The aim of this study was to examine the antithrombotic effects of the four herbal ingredients (Mume Fructus, MF; Santali Albi Lignum, SAL; Amomi Tsao-Ko Fructus, ATF; and Amomi Fructus, AF) of modified *Jeho-tang* (MJHT) in a ferric chloride ($FeCl_3$)-induced carotid arterial thrombosis model.
Methods: Thirty minutes prior to a 35% $FeCl_3$ application, Sprague-Dawley (SD) rats were injected with saline, MF, SAL, ATF or AF (100 mg/kg, intraperitoneal injection), respectively. The effect of the MJHT ingredients was examined for time to occlusion (TTO) and thrombus weight (TW) in a $FeCl_3$-induced thrombosis model. Histological analysis was performed to examine the effect of the MJHT ingredients on collagen fiber damage using hematoxylin & eosin and Masson's trichrome staining.
Results: Compared with vehicle treatment, MF, SAL and ATF treatment delayed TTO (vehicle, 8.11 ± 0.60 min; MF, 16.67 ± 1.03 min; SAL, 17.50 ± 1.52 min and ATF, 13.33 ± 1.21 min; $P < 0.001$) and inhibited thrombus formation (vehicle, 0.79 ± 0.03 mg/mm; MF, 0.61 ± 0.07 mg/mm; SAL, 0.57 ± 0.03 mg/mm and ATF, 0.72 ± 0.02 mg/mm; $P < 0.001$). In addition, each herbal ingredient of MJHT except for AF prevented the collagen fiber damage induced by a 35% $FeCl_3$ application. These results indicate that the MJHT ingredients MF ≧ SAL 〉 ATF 〉 AF possess antithrombotic activity in a $FeCl_3$-induced carotid arterial thrombosis.
Conclusions: Altogether, these results are the first evidence that the MJHT ingredients MF, SAL and ATF have the ability to prevent vascular damage and thrombus formation in $FeCl_3$-induced carotid arterial thrombosis.

Key Words : modified *Jeho-tang*, $FeCl_3$-induced carotid arterial rat model, thrombosis

Introduction

Thrombosis is the formation of a thrombus inside a blood vessel, obstructing the blood flow through the circulatory system[1]. Thrombus formation in blood vessels contributes to the development of cardiovascular diseases such as atherosclerosis, stroke and hypertension[2]. Therefore, the inhibition of thrombus formation is important to prevent cardiovascular disease[3]. Animal models of thrombosis that mimic human diseases have been widely implemented in antithrombotic drug development[4]. Among the animal models, $FeCl_3$-induced arterial thrombosis is a model generally used in mice, rats and rabbits[5,6]. The exposure of the vessel to $FeCl_3$ leads to vessel injury

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and occlusion, which then stops blood flow[5]. This thrombosis model has been used to study antithrombotic pharmacological interventions; hence, the primary endpoint has been the capacity of various drugs to reduce thrombus size, to delay the time to occlusion or to prevent occlusion[7-11]. Especially, previous study has reported that factor Xa inhibitor YM466 and GPIIb/IIIa antagonist YM128 inhibited thrombotic occlusion and neointima formation[8]. However, these drugs possess side effects such as systematic bleeding and short half-life, and long-term use is difficult. For this reason, there are multiple ongoing investigations utilizing various herbal medicines to identify safer and inexpensive thrombus preventive agents to overcome such disadvantages[12,13]. This model has been used to demonstrate drug availability in the circulatory system and antithrombotic effects[4,5,7,10,14]. *Jeho-tang* (JHT), a traditional Korean herbal medicine, has been used for many years in Korea to treat dehydration[15] and is effective in quenching thirst, strengthening the stomach, relieving diarrhea, and regulating intestinal function[16-18]. In addition, JHT stimulates the growth of intestinal bacteria and immune cells[19]. However, no study had been performed regarding the protective effect of JHT on thrombus formation. Hence, this study investigated the potential beneficial properties of modified *Jeho-tang* (MJHT) in the $FeCl_3$-induced carotid arterial thrombosis model. MJHT consists of four herbal medicines (Mume Fructus, MF; Santali Albi Lignum, SAL; Amomi Tsao-Ko Fructus, ATF; and Amomi Fructus, AF) from JHT, but lacks honey, and is a Korean herbal medicine with antithrombotic properties[20]. Recently, we found that MJHT has potent antithrombotic effects through the inhibition of platelet aggregation and adhesion in vitro[20] and effectively inhibits thrombosis and anti-peripheral blood flow in the $FeCl_3$-induced carotid arterial thrombosis model[21]. Thus, to investigate the differences of antithrombotic activity effects between each herbal ingredient of MJHT, we used a topical $FeCl_3$-induced carotid arterial thrombosis rat model.

Materials and methods

1. Extract preparation

MJHT is composed of the medicinal herbs shown in Table 1. The herbs used in the present study were purchased from a commercial supplier in Daejeon, Korea. All herbs were identified by the Herbal Quality Control Team and deposited at the herbarium of the department of Herbal Medicine Research Division, Korea Institute of Oriental Medicine (KIOM). The four herbal ingredients were incubated at 80 °C for 12 hours in a water bath and were refluxed with distilled water at 100 °C for 2 hours. All extracts were concentrated under a vacuum using a rotary evaporator after filtration and were freeze-dried to yield extract powder.

2. The $FeCl_3$ injury thrombosis model

All experimental procedures were approved by the Animal Care and Use Committee of the Korea Institute of Oriental Medicine (Approval No. 12-033). Male Sprague-Dawley (SD) rats (220-250 g, Orient Bio, Korea) were used in this study, and $FeCl_3$-induced arterial thrombosis was instigated according to the previously described method, with minor modifications[5,21,22,24,25]. Briefly, after exposure to 3% isoflurane, the rats were anesthetized with 1.5% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen using facemask anesthesia, which was continued throughout the surgical procedure. An incision in the skin was created directly on top of the region of the left common carotid artery. Carotid blood flow was measured with a miniature laser Doppler flowmeter (LDF; BFL21, Transonic Instrument, USA). Thirty minutes prior to the $FeCl_3$

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application, the rats were injected with saline, MF, SAL, ATF or AF (100 mg/kg, intraperitoneal injection). A small piece of filter paper (2 x 2 mm) soaked in $FeCl_3$ solution (35%, w/v) was then applied topically to the carotid artery of the SD rats. To measure the occlusion time in the carotid artery, carotid blood flow was continuously monitored for 30 min after $FeCl_3$ application.

3. Data analysis – time to occlusion

The time to occlusion was determined by measuring the time (in minutes) from removal of $FeCl_3$-saturated filter paper until the blood flow was 0 mL min-1. Occlusion was defined as blood flow reduced to 0 mL min-1 for more than 5 min. If occlusion did not occur after 30 min, the occlusion time was reported as 30 min, even though no occlusion occurred during the observation period. Time to occlusion (TTO) was recorded using Transonic TS420 and Chart4 software (ADI, Australia). A chamber temperature of 37℃ was maintained for the duration of the experiment.

4. Immunohistochemistry

The rat carotid artery were dissected and fixed in 4% paraformaldehyde, transferred to 70% ethanol, dehydrated and embedded in paraffin. The blood vessels were then cut into 4-μm sections and stained with hematoxylin and eosin (H&E) and Masson's trichrome. The stained sections were examined using light microscopy (BX51, Olympus, Japan).

5. Statistical analysis

The arterial thrombosis data were analyzed using one-way ANOVA followed by the Tukey's post-hoc test. All results are presented as the mean ± SD. Values of $P < 0.05$ were considered to be significant. All analyses were performed using the Statistical Package for Social Science (SPSS, version 12.0, USA) and R package version 3.0.0 for Windows.

Results

1. The antithrombotic effects of MJHT ingredients in a rat arterial model of thrombosis

Table 1 shows that the effect of each MJHT ingredient on $FeCl_3$-induced thrombus formation was examined using a Doppler flow probe system. The TTO of the carotid artery was 8.11 ± 0.60 min in the saline-treated group of the $FeCl_3$-induced carotid arterial thrombosis model

Table 1. Effects of Herbal Ingredients on the 35% $FeCl_3$-Induced Carotid Artery Thrombosis Model

Herbal medicine	Yield rate (%)	Treatment dose (mg/kg)	n	TTO (min)	TTO reduction (ratio)	TW (mg/mm)	TW inhibition (%)
Vehicle	-	Saline	9	8.11±0.60c	-	0.79±0.03a	-
Mume Fructus	16.22	100	6	16.67±1.03a***	2.08±0.13	0.61±0.07c***	21.28±9.10
Santali Albi Lignum	1.51	100	6	17.50±1.52a***	2.19±0.19	0.57±0.03c***	26.58±4.78
Amomi Tsao-Ko Fructus	11.28	100	6	13.33±1.21b***	1.68±0.22	0.72±0.02b***	10.25±4.57
Amomi Fructus	11.26	100	4	8.25±0.96c	0.97±0.12	0.79±0.01^{a,b}	-0.47±2.10

Results are mean ± SD (n=4–9). Means of letters recorded as a, b and c within a column indicate the same level of antithrombotic effects within the values determined by one–way ANOVA, Tukey's post–hoc test. $^{***}P<0.001$ indicate significant differences between each group and Vehicle. TTO: time to occlusion (minute), TTO: time to occlusion (minute), TW: thrombus weight.

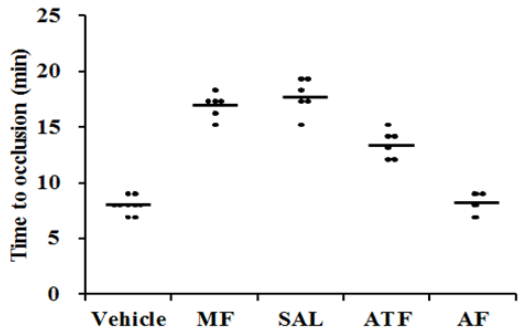

Fig. 1. The effect of the MJHT ingredients on time to occlusion in $FeCl_3$-treated rats. Thirty minutes prior to $FeCl_3$ application, the rats were injected with saline, MF, SAL, ATF and AF (100 mg/kg, intraperitoneal injection). The common carotid arteries were exposed, and a filter paper saturated with 35% $FeCl_3$ was placed on top of the exposed vessel for 3 min. The data are presented as the mean ± SD (n = 4-9). Vehicle, Saline pretreated $FeCl_3$ rat group; Mume Fructus, MF; Santali Albi Lignum, SAL; Amomi Tsao-Ko Fructus, ATF; and Amomi Fructus, AF.

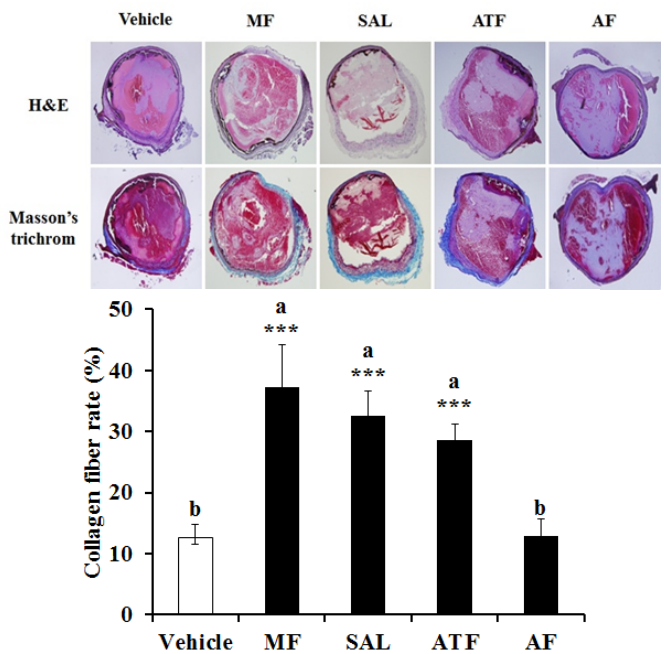

Fig. 2. The effects of the MJHT ingredients on collagen fiber rate in $FeCl_3$-treated rats. Immunohistochemical analysis was performed for $FeCl_3$-induced injury of the vessels in the saline, MF, SAL, ATF and AF-treated rats. The four herbal medicines were given intraperitoneally 30 min prior to $FeCl_3$ application. All sections were stained using H&E and Masson's trichrome. The data are presented as the mean ± SD in each group (n = 4-9). Statistical presentation of the quantitative data of the Masson's trichrome stain. Means of letters recorded as a and b within a column indicate the same level of antithrombotic effects within the values determined by one-way ANOVA, Tukey's post-hoc test. ***P<0.001 indicate significant differences between each group and Vehicle. Vehicle, Saline pretreated $FeCl_3$ rat group; Mume Fructus, MF; Santali Albi Lignum, SAL; Amomi Tsao-Ko Fructus, ATF; and Amomi Fructus, AF.

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(Fig. 1). The TTO of the MF and SAL-treated groups (16.67 ± 1.03 and 17.50 ± 1.52 min, respectively; P < 0.001) was delayed more than 2 times compared with that of the saline-treated group. In addition, the TTO of ATF-treated group was 13.33 ± 1.21 min (P < 0.001), and that of AF-treated group was similar to that of saline-treated group (8.25 ± 0.96 min; P = 0.376).

Also, compared with the vehicle group (0.79 ± 0.03 mg/mm), the MF, SAL and ATF groups (0.61 ± 0.07, 0.57 ± 0.03, and 0.72 ± 0.02 mg/mm, respectively; P < 0.001) showed a significant reduction in thrombus weight (TW); however, AF treatment (10.79 ± 0.01 mg/mm, P = 0.948) did not affect $FeCl_3$-induced thrombus weight (Table 1).

2. The effects of MJHT ingredients on histological changes and collagen fiber damage in the rat carotid arterial model of thrombosis

Tissue sections were stained with H&E for histology staining and with Masson's trichrome for collagen fiber staining. In this study, the histological changes were observed in the carotid arteries of SD rats after $FeCl_3$-induced thrombosis (Fig. 2). In the detection of type I collagen in the vessels, the collagen fiber stained blue, the nuclei stained black and the background stained red. The collagen fiber damage in the vessels induced by $FeCl_3$ was prevented by MF, SAL and ATF treatments (vehicle, 12.59 ± 2.24%; MF, 37.33 ± 6.80%; SAL, 32.61 ± 4.03% and ATF, 28.61 ± 2.53; P < 0.001; Fig. 2). However, AF treatment did not prevent collagen fiber damage (12.93 ± 2.73%, P = 0.816). In addition, the collagen fiber rate of the MF and SAL-treated groups was approximately 3 times higher than the saline-treated group, indicating that MF and SAL provided an excellent recovery of collagen fiber damage in $FeCl_3$-induced vessel injury.

Discussion

Worldwide, the incidence of vascular disease has increased together with extended lifespans[24]. Many studies have reported new drugs for the treatment and prevention of vascular disease. Recently, buckwheat seed, red ginseng, *Sokmyeung-tang* and *Ginkgo biloba* extracts showed antithrombotic effects in $FeCl_3$-induced thrombosis[12,13,23,24]. In addition, Wuslsan is known to have antithrombotic activity through the inhibition of platelet aggregation and an increased blood flow rate[26]. Although animal thrombosis models are only loosely linked to the human disorder of medical interest, they may provide insight into the mechanism of action involved in thrombus formation. $FeCl_3$-induced vascular injury is a widely used model in thrombosis research because it allows variable levels of injury in different vascular beds and can be monitored by microscopic visualization or blood flow measurement[27]. We have previously reported that 35% $FeCl_3$ induces the experimental thrombus formation, and treatment with a 100 mg/kg concentration of the MJHT ingredients to SD rats thirty minutes before $FeCl_3$ application does not induce toxicity[21]. In addition, the LD50 value of MJHT in ICR mice was evaluated more than 5000 mg/kg[28].

Therefore, this indicates that treatment of MJHT ingredients to rats after $FeCl_3$ application is stable and suitable for experimental thrombosis study. The TTO and carotid blood flow in the carotid artery were measured with continuous monitoring for 30 min after $FeCl_3$ application. The TTO of MF and SAL-treated groups was delayed more than 2-fold compared with that of the saline-treated group in $FeCl_3$-treated vessels. TW also was decreased in the MF, SAL and

ATF-treated groups compared with the saline-treated group. Collagen exposure has been reported to induce thrombus formation in mice lacking coagulation factor XII[29]. Collagen mediates platelet adhesion when subendothelial extracellular matrix proteins are exposed to blood and may also play a crucial role in atherothrombosis[14]. We showed that MF, SAL and ATF prevented collagen fiber damage in injured vessels more than 3-fold compared with the vehicle group.

From the above results, we found that MF, approximately equal to SAL, showed antithrombotic activity more effectively than ATF in $FeCl_3$-treated rats, and AF had no effect compared to the vehicle.

In conclusion, the present study established an in vivo animal model system of thrombosis to assess the efficacy of each herbal ingredients of MJHT and showed that MF, SAL and ATF may have an antithrombotic effect through delaying TTO and reducing TW and collagen fiber damage induced by the $FeCl_3$-induced carotid arterial thrombosis model.

Further studies are required to define the potential effectiveness of MJHT. Further study is required to determine the mechanisms of action of MJHT and its ingredients to aid in the discovery of new drugs for the prevention and treatment of the arterial thrombosis.

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