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Original Article

A Trend of *Yin*-tonifying Formulas Compared with *Yang*-tonifying Formulas on Anti-platelet and Anti-thrombotic Activity

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Objectives: Formulas for treatment of *yin* or *yang* deficiency conditions have been commonly used in traditional Korean medicine. The aim of this study is to examine the possible inhibitory effects of *yin*- or *yang*-tonifying formulas on *in vivo* anti-platelet activity and *in vivo* anti-thrombotic activity.
Methods: We tested the effects of 26 types of *yin*- or *yang*-tonifying formulas on platelet aggregation induced by collagen in human whole blood using the impedance method of aggregometry and accessed a biomarker of platelet activation using thromboxane B_2 immunoassay. We also tested the anti-thrombotic effects of effective candidates on experimental models of thrombosis in mice.
Results: 3 types of *yin*-tonifying formulas and 3 types of *yin-yang*-tonifying formulas were selected to be the most effective candidates ($p<0.01$). Also, through *in vivo* study, the antithrombotic activities of *Igyeong-tang*, *Gamisipjeondaebo-tang*, and *Gamisoyo-san*-treated groups, with recovery rate of 60, 50, and 45.45%, respectively, were observed to be higher than those of the control group (saline, 36.8%) in mouse acute thrombosis.
Conclusion: These results show that *yin*-tonifying formulas are more effective in anti-platelet and anti-thrombotic activity than *yang*-tonifying formulas.

Key Words : formulas, tonifying, *yin*, *yang*, platelet aggregation, antithrombotic.

Introduction

Traditional Korean medicine (TKM) now plays an important role in medical treatment in Korea. TKM is often used for the treatment of various diseases including *yin-yang* deficiency conditions, menopausal disorders, autonomic imbalances, cold syndromes, etc. These diseases are still difficult to treat using Western medicine; however, by correcting the imbalance of the whole body, TKM are relatively effective treatments[1-3]. TKM uses eight treatment methods which are tonification, diaphoresis, emesis, purgation, mediation, warming, clearing and resolution. All these are for the harmonization of *yin* and *yang*[4-6].

In terms of TKM, thrombosis is a type of blood indigestion caused by circulation problems in the blood veins. Blood stasis is defined as blood indigestion of the internal body. It is a pathological effect and a factor that induces blood circulation disorders related to pathological processes. It also

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means hemorrheological disorder, peripheral circulatory disturbance and hemodynamic disorder. Thrombus is the main hemorrheological element involved in these[7,8]. Platelet aggregation plays an important role in both physiological hemostatic and pathological thrombotic processes. Once vascular injury occurs, platelets will be activated by endogenous agonists such as ADP, collagen, and thrombin and adhere to the site of injury. The formation and release of thromboxane (TBX) A_2 is a central component in the platelet response to a variety of agonists[9]. The modulation of platelet activity by specific pharmacological agents has proven to be a successful strategy for preventing thrombosis[10]. Platelets have multiple activation signaling mechanisms. Therefore, agents with anti-platelet and anti-thrombotic effects may have wide therapeutic potential for circulatory diseases[11-13].

In this context, in the search of another prospective of *yin*- and *yang*-tonifying, we selected 26 types of *yin*- or *yang*-tonifying formulas of every functional category based on *Donguibogam*[14] and evaluated the potential effect of these formulae on the blood circulation and blood stasis using *in vitro* whole blood aggregation assay and mouse acute thrombosis model.

Materials and Methods

1. Materials

Collagen and epinephrine were obtained from Chrono-Log Co. (USA). Thromboxane B_2 immunoassay kit was obtained from R&D systems (USA). 68 species of medicinal herbs used in the study were purchased from a commercial supplier in Seoul, Korea, in 2003. All herbs were identified by and deposited at the herbarium of the Herbal Quality Control Center, Korea Institute of Oriental Medicine (KIOM) in Daejeon, Korea. All of the herbs were ground using a food mixer. These ground powders were prepared to formulas according to *Donguibogam*[14]. Each mixture of formula was refluxed with distilled water (10 g/100 mL) for 30 min below 100℃. The extracted solutions were filtered and concentrated respectively under vacuum using a rotary evaporator and then freeze-dried to obtain dry extract powders.

2. Volunteers

A total of 8 healthy volunteers (females, aged range 20-28 years) took part in the study. For this study, blood was collected in Vacutainer tubes (Becton Dickinson, USA) containing 3.8% sodium citrate with a draw volume of 2.7 mL using standard phlebotomy techniques. All subjects had no history of bleeding disorders or cardiovascular disease, and refrained from any pharmacological therapy for at least 2 weeks before enrollment. None of the subjects smoked, and none exhibited hypertension, diabetes or abnormal hematocrit (HCT). All subjects gave written informed consent before participation. This study was approved by the Ethics Committee of the Korea Institute of Oriental Medicine in Daejeon, Korea.

3. Platelet-cell cytotoxicity

We examined platelet-cell cytotoxicity by formula extracts with a Coulter counter. The complete blood count (CBC) was analyzed using a Coulter JT automatic blood cell analyzer (Becton Dickinson, USA). Briefly, platelet-cell damage was studied in whole blood adjusted to a count of 4×10^8 platelet cells/mL with 0.9% saline in plastic cuvettes (Chrono-Log, USA) incubated at 37℃ for 10 min. 50-μL of formula extracts or saline (vehicle control) was added and incubated at 37℃ for 5 min and were counted using a blood cell analyzer.

4. Whole-blood platelet aggregation

We investigated inhibition of platelet aggregation from selected formulas by non-cytotoxicity results. For the whole blood studies, blood was drawn intravenously from healthy volunteers into vacuum

tubes to give 3.2% sodium citrate. Platelet aggregation was studied in whole blood preparation according the platelet-cell cytotoxicity process but containing a magnetic stir bar (Chrono-Log, USA). Whole blood aggregation was monitored for 10 min after addition of collagen (final concentration of 2μg/mL); during aggregation testing, samples were continuously stirred at 200μg in 37℃ with a 500VS Chrono-Log aggregometer (Chrono-Log, USA) using the impedance method[15,16], which allows the quantification of aggregation by electrical resistance between two electrodes immersed in whole blood.

5. Thromboxane B_2 immunoassay

For the TXB_2 immunoassay, whole blood samples after aggregation assay were centrifuged at 2,000 g for 15 min. Supernatant aliquots were immediately frozen and stored at -20℃ until assay. The concentration of TXB_2, a stable metabolite of thromboxane A_2 (TXA_2), was quantified using an ELISA kit (R&D systems, Inc. USA) according the manufacturer's instructions. The absorbance was measured at 405 nm using a SpectraMax 340 reader (Molecular Devices, USA). The concentration of TXB_2 calculated corresponded to the mean absorbance from the standard curve.

6. Acute pulmonary thromboembolism model

A modification of the method reported by DiMinno and Silver was used[17]. ICR mice, aged 3 weeks, were purchased from Orient Co. (Korea). The most effective extracts were suspended in saline and administered orally to ICR mice (4 weeks) once a day at a dose of 100 mg/kg. All experiments were performed 1 h after the final administration. A thrombotic challenge was induced by rapid intravenous injection of a mixture of collagen (1,200 μg/kg) and epinephrine (120 μg/kg) into the mouse tail. Each mouse was carefully watched for 15 min to determine whether the mouse was paralyzed, died or recovered from paralysis caused by the acute thrombotic challenge. Animal experiments were approved by the committee on animal care at our institute and were carried out according to institutional guidelines.

7. Statistical analysis

The results are presented as mean±standard deviation (S.D.). Groups were compared with a paired t-test and a x^2 test using SPSS 11.0 for Windows (SPSS, Chicago, IL, USA). Values of $p<0.05$ were considered to be statistically significant.

Results

1. Influence on platelet-cell cytotoxicity

For the evaluation on platelet damage, we examined 26 types of formula on platelet-cell cytotoxicity because platelets are a most important cell in whole blood aggregation[18,19]. These results showed whether the inhibitory influence of extracts on platelet aggregation do or do not cause platelet-cell damage. Using a Coulter counter, the formula extracts were examined for platelet-cell cytotoxicity effects. As shown in Table 1, platelet-cell cytotoxicity was observed in the presence of formula extracts. Among them, 4 types of *yin*-tonifying formulas, 1 type of *yang*-tonifying formula, and 5 types of *yin-yang* tonifying formulas did not cause any damage on platelet counts and histograms. Finally, we selected the extract of 10 types of formulas as candidates for screening for anti-platelet activity.

2. Inhibition on platelet aggregation in human whole blood

Control aggregation in saline induced by collagen was systematically carried out at the beginning of each experiment to verify the physiological status of the platelets. Using the impedance method of aggregometry, we tested the *in vitro* effect of 10 types of formula on the platelet aggregation that is

Table 1. Cytotoxicity on Platelet in Human Whole Blood Treated Tonifying Formulas

Classification	Formulae	Platelet	
		Count (x 10^9/L)	Histogram
Control	Saline	6.4 ± 1.1	Normal
Yin-tonifying	*Boeum-san*	6.5 ± 0.7	Normal
	Iui-hwan	8.8 ± 1.5†	Normal
	Boeum-hwan	8.0 ± 0.0†	Normal
	Daeboeum-hwan	6.0 ± 0.0	Normal
	Sayangboeum-tang	8.3 ± 0.5†	Normal
	Gamisoyong-san	6.5 ± 0.7	Normal
	Igyeong-tang	7.5 ± 0.7	Normal
Yang-tonifying	*Osaeng-hwan*	8.5 ± 0.7*	Normal
	Gyebu-tang	n.d.	Abnormal
	Samgigeonjung-tang	6.3 ± 3.1	Normal
	Doksam-tang	8.5 ± 0.7*	Normal
	Insamhwanggi-tang	8.0 ± 0.0†	Normal
	Hwanggi-tang	9.5 ± 0.6‡	Normal
	Samhyang-san	8.0 ± 0.0†	Normal
	Jeonggiboheo-tang	n.d.	Abnormal
	Jeungsonyangnyeongtang	7.5 ± 0.6*	Normal
Yin-yang-tonifying	*Ssanghwa-tang*	6.5 ± 0.7	Normal
	Palmul-tang	5.75 ± 0.5	Abnormal
	Sipjeondaebo-tang	6.3 ± 0.6	Abnormal
	Gamisipjeondaebo-tang	6.0 ± 0.0	Normal
	Hwanggisipbo-tang	5.25 ± 0.5*	Abnormal
	Gojineumja	6.5 ± 0.7	Normal
	Sijessangbo-hwan	6.0 ± 0.0	Normal
	Insamnyangyeong-tang	6.3 ± 0.5	Abnormal
	Boigyagyeong-tang	6.0 ± 0.0	Normal
	Jaeumdaebo-hwan	8.0 ± 0.0†	Normal

Results are mean ± S.D.
* $p<0.05$ represents significant differences between the experimental and control values.
† $p<0.01$ represents significant differences between the experimental and control values.
‡ $p<0.001$ represents significant differences between the experimental and control values.

Table 2. Effects on Platelet Aggregation in Human Whole Blood Induced by Collagen

Classification	Formulae	Aggregation	
		Ohm (Ω)	Inhibition (%)
Control	Saline	12.9 ± 2.7	
Yin-tonifying	*Boeum-san*	6.0 ± 4.2†	63.5 ± 12.3
	Daeboeum-hwan	8.7 ± 2.1	29.6 ± 9.4
	Gamisoyong-san	1.3 ± 0.6‡	90.4 ± 1.8
	Igyeong-tang	3.0 ± 2.0‡	78.2 ± 12.3
Yang-tonifying	*Samgigeonjung-tang*	9.0 ± 5.3	45.6 ± 21.9
Yin-yang-tonifying	*Ssanghwa-tang*	8.0 ± 1	38.8 ± 14.5
	Gamisipjeondaebo-tang	4.7 ± 3.1‡	68.3 ± 12.3
	Gojineumja	2.7 ± 1.2‡	82.4 ± 7.8
	Sijessangbo-hwan	10.3 ± 3.2	31.9 ± 22.2
	Boigyagyeong-tang	6.3±5.0‡	55.7 ± 34.9

Results are mean ± S.D.
* $p<0.05$ represents significant differences between the experimental and control values.
† $p<0.01$ represents significant differences between the experimental and control values.
‡ $p<0.001$ represents significant differences between the experimental and control values.

Table 3. Effects on Thromboxane B2 Production in Human Whole Blood Induced by Collagen

Classification	Herbal formulas	Thromboxane B_2 (pg/mL)
Control	Saline	494.7 ± 28.0
Yin-tonifying	*Boeum-san*	419.5 ± 7.1
	Gamisoyong-san	484.8 ± 9.1
	Igyeong-tang	393.7 ± 5.2 (p=0.117)
Yin-yang-tonifying	*Gamisipjeondaebo-tang*	474.2 ± 29.6
	Gojineumja	370.7 ± 3.0 (p=0.099)
	Boigyagyeong-tang	391.1 ± 11.3

induced by collagen in human whole blood. Table 2 lists the inhibitory effects of 10 types of formula selected from the 26 types of formula on human platelet aggregation. Among them, 3 types of *yin*-tonifying extracts and 3 types of *yin-yang*-tonifying extracts showed significant inhibiting effect on whole blood aggregation (p<0.001). The inhibition percentages for *Boeum-san*, *Gamisoyo-san*, and *Igyeong-tang* among *yin*-tonifying formulas were 63.5 ± 12.3, 90.4 ± 1.8, and 78.2 ± 12.3%, while the values for *Gamisipjeondaebo-tang*, Gojineumja, and Boigyagyeong-tang among *yin-yang*-tonifying formulas were 63.3 ± 12.3, 82.4 ± 7.8, and 55.7 ± 34.9%, respectively. These results indicate that *yin*- or *yin-yang*-tonifying formulas were the potentially effective extracts in inhibition of platelet aggregation induced by collagen. In contrast, *yang*-tonifying formulas displayed only mild anti-platelet effects. In addition, we accessed biomarkers of platelet activation using thromboxane B_2 by ELISA assay. Low decrease in thromboxane B_2 production was seen in the presence of 6 types of effective extracts (Table 3).

3. Protection of mice from thrombotic challenge by oral administration

Intravenous injection of a mixture of collagen (1,200 μg/kg) and epinephrine (120 μg/kg) into mouse tail induces massive thrombus formation in the pulmonary artery, causing acute paralysis that can lead to sudden death. Most of the mice were paralyzed within 3 min and approximately 80% of them died within 15 min. Only approximately 33.3% of the mice recovered from the paralysis within 15 min as shown with the control groups in Table 4. Oral administration

Table 4. Effects on Antithrombotic Activity

	Herbal formulas (100 mg/kg)	No. killed or paralyzed / No. tested	Recovery (%)
Control	Saline	12/18	33.3
Yin-tonic	*Boeum-san*	8/10	20.0
	Gamisoyong-san	6/11	45.5
	Igyeong-tang	4/10	60.0 (p=0.172)
Yin and *Yang*-tonic	*Gamisipjeondaebo-tang*	5/10	50.0
	Gojineumja	9/13	30.7
	Boigyagyeong-tang	8/9	11.1

of the 3 types of *yin*-tonifying formula extracts and the 3 types of *yin-yang*-tonifying formula extracts once a day increased the recovery rates from the acute thrombotic challenge. The recovery rates of 60.0 (p=0.172), 50.0, 45.5% respectively in the *Igyeong-tang*, *Gamisipjeondaebo-tang*, and *Gamisoyo-san*-treated (100 mg/kg) groups were higher than the rate in the control group. The recovery rates were not enhanced in mice treated with Gojineumja, *Boeum-san* or Boigyagyeong-tang (Table 4).

Discussion

Recent research has been geared towards the scientific evaluation of traditional medicine theory including *yin-yang* theory. For instance, oxidant and antioxidant phenomenon are related to *yin-yang*. This is partially supported by the fact that the *yin*-tonifying traditional Chinese herbs have more antioxidant activity and polyphenolic content than the *yang*-tonifying herbs[20-23]. Another study investigated the pharmacological basis of '*yang*-invigorating' action, where correlations with myocardial ATP-generation capacity were found in mice, but noted that none of the 'blood-enriching' herbs produced any detectable changes[5,24].

In the present study, 26 types of *yin*- or *yang*-tonifying formulas on the inhibition of whole blood aggregation were investigated. The results showed 6 types of formula to be the most promising candidates within the *in vitro* assay. From these six, the most effective candidates within the *in vivo* assay were chosen. These formulae are *Igyeong-tang*, *Gamisipj-eondaebo-tang*, and *Gamisoyo-san*, and the indication cited in *Donguibogam* follows. *Igyeong-tang* treats flusteredness and fearful throbbing caused by heart blood deficiency owing to excessive thinking; *Gamisoyo-san* treats hemoptysis; *Gamisipjeondaebo-tang* treats consumptive disease formed by dual deficiency of qi and blood and gradual formation of phthisis[14]. We discuss the anti-platelet activities of candidates by evaluating its *in vitro* effects on the whole blood aggregation and *in vivo* effects on the acute thrombosis model. According to the investigation *Igyeong-tang* and *Gamisoyo-san* among *yin*-tonifying formulas significantly inhibited collage-induced platelet aggregation as well as thromboxane production, a biomarker of platelet activation. In addition, results of oral administration on mouse acute pulmonary thromboembolism model also showed their effect of reducing formation of thrombus. From these results extracts of *Igyeong-tang* and *Gamisoyo-san* were considered to have antithrombotic activity and the decreasing rate of producing thrombus suggested that they help promote blood circulation by inhibiting platelet aggregation. However, to determine detailed mechanisms of anti-thrombus and platelet aggregation inhibition more research is necessary.

In conclusion, the high effectiveness shown in *yin*-tonifying formulas meant that such formulas promote blood circulation effects with high bioactivity and a correlation was found in the efficacy of *yin*-tonifying formulas in promoting blood circulation. In this study, we found that *Igyeong-tang* was the most effective formula showing anti-platelet and anti-thrombotic activity. Particularly, our study reported here reveals that such a correlation is supported, at least, by the trend of anti-platelet activity and anti-thrombotic activity of *yin*-tonifying formulas in TKM.

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