Experimental Study on Promoting Blood Circulation to Remove Blood Stasis of EM-X

Ke Bin [a)], Zhengxian Zhong [b)], Teruo Higa [c)]

[a)]*EM Research Organization, Okinawa, Japan*
[b)]*Guangxi Institute of Traditional Medical and Pharmalceutical Sciences, Nanning, China*
[c)]*College of Agriculture, University of the Ryukyus, Japan*

Abstract

The test in animal demonstrated that EM-X both significantly prolonged the coagulation time of mice by oral administration, and prolonged the calcium-recovering tiems plasma, but no influence of prothrombin time and fibrinolysin activity in rabbits and mice using administration of EM-X *in vitro* and *in vivo*. It shortened the time of acute pulmony embolism induced by ADP and lowered TG contents in serum thrombus formation *in vivo*, and had definite improvement on blood viscosity and ethrocye electrophoretic time of acute stasis syndrome rats. All these suggest that EM-X has the definite actions of promoting blood circulation to remove blood stasis on many strain animal models.

EM-X is a beverage with antioxidant effects prepared by Okinawa Tropical Plant Research Institute in Japan. To study the pharmacological action of EM-X on promoting blood circulation to remove blood stasis, we studied the actions of anticoagulant, anti-acute pulmonary embolism, lipid-lowering, anti-platelet aggregation and blood rheology etc of EM-X using animal experiment.

Materials and Methods

1.1 Drugs and Reagent

EM-X, 500ml/bottle, was proved by EM Research Organization in Japan. Aspirin was purchased from pharmaceutical Factory of Hunang (Changsha, China), fenofibrate from Yimin Pharmaceutical Factory of (Beijing, China), danshen injection (DSI) from Longhua Pharmaceutical Factory of Guangzhou military region (Guangzhou, China), frozen-dried coagulase from RongShen Biol technology Co. (Shanghai, China); both the kits of serum total cholesterol (TC), triglyceride (TC), high density lipoprotein cholesterol (HDL-C) from Dongou Biol Engin Co of Wnzhou (Wenzhou, China).

1.2 Animals

Both NIH strain mice (18-22g) and SD strain rat (250-300g), including both sexes, were obtained form Animal Department of Guangxi Institute of Traditional Medical and Pharmaceutical Sciences (Guangxi, China); New Zealand strain white rabbit from Guangxi Grass Estate Center of China. All animals were sex-separately housed in the experiment.

1.3 Instruments

Grating spectrophotometer (mode 722) was made by Shanghai Analytical Instrument third Factory (Shanghai, China); platelet aggregometer (mode BS631)

by Beijing Biochemical Instrument Factory (Beijing, China); Double passageway whole automatic blood rheologometer (MDK-3200) by Chongqing Madike Science and Technical Develop Co. (Chongqing, China)

1.4 Determination of Coagulation Time in Mice (1)

Forty mice were divided into the control, aspirin (positive drug, 100ml/kg) and EM-X (20ml/kg and 10ml/kg) groups, and orally administered once a day for 7 days. The blood was drawn using a glass capillary (1.0mm internal diameter) to insert into retro-ocular venous plexus of mice, until the drawing blood column was raised to 5.0cm in the glass capillary, and then a small segment of glass capillary was cut in 30 sec. intervals, 40 min, after the last administration. After blood column movement was observed by eye, coagulated blood fiber was extracted. Also, the time for drawing blood from the glass.

1.5 Determination of Plasma Calcium-Recovering and Prothrombin Times of Rabbit *in vitro* (2)

the blood samples (the volume proportion of blood and 0.1 ml/L sodium oxalate = 9:1) were collected from the ear œntral artery of rabbit and then were centrifuged at 1500 rpm for 10min to obtain the plasma of sodium oxalate. In plasma calcium-recovering test, 0.1 ml of plasma and 0.1 ml of test sample s were added in test tube and cultivated in water bath at 37°C for 3 minutes. Then 0.2 ml of 0.025 mol/l CaCl2 (control tube added 0.1 ml of plasma and 0.1 ml of test samples 0.2 mol of calcium contained thrombase solution (free-dried powder of calcium contained thrombase per ampoule added 2.2 ml of distilled water) were added and the coagulation time was record.

1.6 determination of Fibrinolysin Activity of Rabbit Plasma *in vitro* (2)

The preparative method of plasma was the same as 1.5 mentioned above. In this test, 0.1 ml of plasma and 0.1 ml of calcium-contained thrombase (control tube added 0.1 ml of normal saline) were added to generate plasma coagulation lump. The coagulation lump with 0.2 ml of diluted reagent was cultivated at 37°C for 24 hours and the dissolved condition of plasma coagulation lump was examined.

1.7 Determination of Plasma Calcium-recovering and Prothrombin Times of Mice *in vivo* (2)

Forty mice were divided into the control, FDSP (0.66 g/kg), EM-X (20 ml/kg and 10 ml/kg) groups. The mice were orally given once a day for 7 days. 0.5 ml of blood samples (anticoagulating with 0.1% sodium oxalate) were collected form retroocular venous plexus of mice one hour after last administration. Blood samples were centrifuged at 1500 rpm for10 min to obtain the plasma. In plasma calcium-recovering test, 0.1 ml of plasma and 0.1 ml of test samples were added in test tube and cultivated in water bath at 37°C for 3 minutes. Then added 0.2 ml of 0.025 mol/1 CaCl2 (control tube added 0.2 ml of normal saline) was added and the coagulation time was recorded. In prothrombin time test, 0.1 ml of plasma and 0.1 ml of test samples were added in test tube and cultivated in water bath 37°C for 3 minutes. Then it was added 0.2 ml of calcium contained thrombase (freeze-

dried powder of calcium contained thrombase per ampoule added 2.2 ml of distilled water) was added and the coagulation time was recorded.

1.8 Test of Acute Pulmony Embolism Induced By Adenosse Diphosphate (ADP) in Mice (3)

The dividing group, doses and administered method were same with 1.7 above-mentioned. The mice were injected with ADP solution (200 mg/kg) in tail vein at one hour after last administration, occurred immediately short of breath and inability to move in mice. The times between the injection and recovering autonomic activity were recorded.

1.9 Lipid-Lowering Test

The hyperlipoidemia model method was described by Lin Suseng, et al [4] the preparation of yolk emulsion: the fresh egg of 1-2 days at birth was used and removed albumen, and yolk obtained was mixed with normal saline to prepare 75% yolk emulsion until use.

Fifty mice were divided into control, model, fenofibrate (30 mg/kg), and ME-X (20 ml/kg and 10 ml/kg) groups. The mice of the drug groups were orally given a dose of 0.2 ml/10g BW, the control and model groups were given with same volume of distilled water, once a day for 7 days. After oral administration on the seventh day, the mice were given only water, feeding food was stopped. 2 hours after last water administration, except the control group, all groups were intraperitoneally injected with the same volume of distilled water. Each mouse of these groups were intraperitoneally injected 0.5ml of 75% yolk emulsion in each mouse to make experimental hyperlipoidemia. The blood samples were collected from the retro-ocular venous plexus of mice after injecting for 20 hours, and total cholesterol (TC), trilyceride (TG) and high density lipoprotein cholesterol (HDL-C) levels in the serum were determined by enzyme methods.

1.10 Platelet Aggregative Test (5)

Fasted rats, weighing 250g – 300g were used. Blood samples were collected from the abdominal aorta under anesthesia with sodium pentobarbital (40mg/kg), anticoagulating with 3.8% sodium citrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by routine method. The range of light through rate was adjusted by PPP to 100%, then by PRP to 0% on platelet aggregometer. Each test tube was accurately added with 0.3 ml of PRP, then the control tube was added with 0.05 ml of 0.2 mol PBS, and the drug tube was added with 0.05 ml of EM-X both control and drug tubes were added with 0.02 ml of 2umol ADP (platelet aggregation-induced agent), and then traced platelet aggregative curve and millimeter number of curve lowering were determined. Inhibited percentage of aggregation was calculated by comparing drug group with control group.

1.11 Examination of Thrombus Formation in Rat body (6)

Forty rats, weighing 250 – 300g, were divided into the control (ig of same volume of distilled water, FDSP (0.66g/kg), and EM-X (20 ml/kg and 10 ml/kg) groups

and were orally administered once a day for 7 days. The rats were anesthetized with sodium pentobarbital (40mg/kg) at one hour after last administration. By method of thrombus formation in rat body (6), the neck skin in rat was incised and the extra-neck vein and neck total artery were separated, and casing pipe of polythene was filled with heparin- normal saline, joining from neck total artery-neck vena to form blood stream bypass *in vitro*. Blood stream was opened 5 min. after completion of surgical operation and suspended after 15 min. silk thread was taken from blood stream pipe immediately, blotted on a filter, and then weighed as soon as possible. The wet weight of thrombus was obtained by subtracting silk thread weight from total weight.

1.12 Test of Blood Rheology in Blood Stasis Syndrome Rats (7)

Forty rats, weighing 250g – 300g, were divided into the control, model, EM-X (20 ml/kg and 10 ml/kg) and DFSP (0.66 g/kg) groups. The rats were orally given once a day for 7 days, the control and model groups given with same volume of distilled water. On the seventh day, except the control group, all other groups were subcutaneously injected with 0.08 ml/100gDW of 0.1%epinephrine, once in four hours interval for twice. In this test, the rats were soaked in ice water for 5 min at 20 hours after first injection, and fasted but on fasting water. Blood samples (anticoagulating with heparin) were collected by cutting heads of rats in the second days and the indexes of hematocrit, whole blood viscosity, plasma viscosity, erythrocyte Electro-phoretic time, etc. were determined using a blood rheologomete.

1.13 Statistical Analysis

All data obtained are expressed as mean ± SD and the mean differences between groups were considered to be significant at $P<0.05$ with student's T-test.

Results

2.1 Effect of EM-X on coagulation Time (CT) in Mice

The results were shown in Table 1. After oral administration for 7 days, compared with the control group, EM-X at doses of 20 ml/kg and aspiring group could significantly prolonged coagulation time in mice. The results showed that EM-X had definite anticoagulation. (SeeTable1.)

2.2 Effect of EM-X on plasma Calcium-recovering and prothrombin Times of Rabbit *in vitro* (2) the results were shown in Table 2. Both EM-X at 0.25 ml/ml and DSI could significantly prolong plasma calcium-recovering time but on effect on plasma calcium recovering and prothrombin times of rabbit with EM-X of 0.125 ml/ml in vitro. (SeeTable2.)

2.3 Effect of EM-X on fibrinolysin Activity of Rabbits *in vitro*

The results showed that EM-X could not dissolve plasma coagulation lump, only DSI could partly dissolve plasma coagulation lump as compared with the control group. This suggests that EM-X did not influence on fibrinolysin activity *in vitro*.

2.3 Effect of EM-X on Fibrinolysin Activity of Rabbits *in vitro*.
The results showed that EM-X could not dissolve plasma coagulation lump, only DSI could partly dissolve plasma coagulation lump as compared with the control group. This suggests that ME-X did not influence on fibrinolysin activity *in vitro*.

2.4 Effect of EM-X on Plasma Calcium-recovering and Prothrombin Times of Mice *in vitro*.
The results were shown in Table 3. After oral administration for 7 days, compared with the control group, both EM-X at 20 ml/kg and DSI groups could significantly prolong plasma calcium-recovering and prothrombin times of mice *in vitro*. This suggests that EM-X had definite anticoagulation after oral given. (See Table 3).

2.5 Effect of ME-X on Acute Pulmonary Embolism Induced by Adenose Diphosphate (ADP) in Mice
The results were shown in Table 4. After oral given for 7 days, compared with control group, both EM-X at 20 ml/kg and 10 ml/kg and aspirin groups could significantly breathing difficulty the short time of breath in mice. The results showed that EM-X had definite action of anti-acute pulmony embolism induced by adenose diphosphate (ADP) in mice. (See Table 4.)

2.6 Effect of EM-X on Serum Lipid in Hyperlipoidemia Mice
The results were shown in Table 5. After oral given for 7 days, serum lipid levels in hyperlipoidemia mice were determined. Compared with the model group, EM-X at doses of 20 ml/kg and 10 ml/kg and fenobrate (positive drug) had no marked effect on TC and HDL-C levels, but could significantly lowered TG level. The results showed that EM-X has definite lipid-lowering action. (See Table 5.)

2.7 Effect of EM-X on platelet Aggregration in Rats
The results were shown in Table 6. Comparing with the control group, EM-X (0.28ml/ml and 0.14 ml/ml) and DSI (0.21 g/ml) could significantly inhibitedpaltelet aggregation induced by ADP to 21.8%, 15.7% and 37.7% and 37.7% respectively. The results showed that EM-X had definite anti-platelet aggregation. (See Table 6.)

2.7 Effect of EM-X on Thrombus Formation in Rats
The results were shown in Table 7. After oral given for 7 days, the most weight of thrombus in rats was weighted. Compared with the control group, EM-X at 20 ml/kg and FDSP groups could significantly decreased thrombus weight. This suggests that EM-X had definite anti-thrombus formation. (See Table 7).

2.9 Effect of EM-X on Blood Rheology in Blood Stasis Syndrome Rats
The results were shown in Table 8. Compared with model group, EM-X (20 ml/kg) and FDSP groups could lower the whole blood viscosity and plasma viscosity, and shorten the erythrocyte electrophoretic time, but these was no significant action in EM-X at 10 ml/kg group. The results showed that EM-X had

definite improvement action on blood viscosity of blood rheology in blood stasis syndrome rats. (See Table 8.)

Conclusion

The experimental study demonstrated that EM-X could significantly prolong the coagulation time of mice and the calcium-recovering time of plasma in rabbits and mice, but has no influence of prothrombin time and fibrinolysin activity *in vitro*. It shorts the time of acute pulmony embolism induced by ADP, and lowers TG contents, but no finluence on TC and HDL-C levels in serum of hypercholesterolemia mice. It has definite inhibition on platelete aggregation induced ADP on vitro and thrombus formation *in vivo*, and improves the blood viscosity and erythrocyte electrophoretic time of acute stasis syndrome rats. These suggest that EM-X has definite actions of anti-coagulant, lipid-lowering, anti-thrombus and improving blood rheology.

Table 1. Effect of EM-X on coagulation time (CT) in mice

Group	Doses	Coagulation times (min)
Control		4.51 ± 1.43
Aspirin	100mg/kg	7.50 ± 1.49**
EM-X	20ml/kg	6.56 ± 1.06*
EM-X	10ml/kg	6.01 ± 0.64*

Compared with Control group P>0.05, *P<0.01. (± SD, n = 10)

Table 2. Effect of EM-X on plasma calcium-recovering and porthrombin times of rabbit *in vitro*

Group	Doses	calcium-recovering times (s)	prothrombin times (s)
Control		92.0 ± 10.3	24.6 ± 0.8
DSI	1.5 g/ml	601.0 ± 1.9***	235.0 ± 4.1***
EM-X	0.25 ml/ml	153.7 ± 13.2***	24.4 ± 0.5
EM-X	0.125 ml/ml	94.0 ± 5.6	24.3 ± 0.5

Compared with Control group ***P>0.01. (± SD, n = 10)

Table 3. Effect of EM-X on plasma calcium-recovering and porthrombin times of rabbit *in vitro*

Group	Doses	calcium-recovering times (s)	prothrombin times (s)
Control		49.7 ± 0.7	16.6 ± 0.5
DSI	0.66 g/kg	63.8 ± 8.9***	17.7 ± 0.5*
EM-X	20 ml/kg	65.0 ± 5.1***	17.5 ± 0.5*
EM-X	10 ml/kg	60.2 ± 5.1	17.1 ± 0.7

Compared with Control group ***P>0.01, *P<0.05. (± SD, n = 10)

Table 4. Effect of EM-X on acute pulmony embolism by adenose diphosphate (ADP) in mice

Group	Doses	Short time of breath (min)
Control		3.27 ± 0.62
Aspirin	100 mg/kg	1.43 ± 0.31***
EM-X	20 ml/kg	256.42 ± 0.42*
EM-X	10 ml/kg	2.65 ± 0.34*

Compared with Control group *P>0.05, ***P<0.01. (± SD, n = 10)

Table 5. Effect of EM-X on serum lipid in hyperlipoidemia mice

Group	Doses	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)
Control		2.16 ± 0.69	2.01 ± 0.28	1.56 ± 0.58
Model		6.16 ± 1.51##	7.10 ± 2.77###	1.70 ± 0.29
Fenobrate	30 mg/kg	5.94 ± 0.51	5.53 ± 1.56	1.63 ± 0.42
EM-X	20 ml/kg	5.37 ± 1.35	5.82 ± 1.64*	1.73 ± 0.36
EM-X	10 ml/kg	6.19 ± 1.59	6.00 ± 1.67	1.53 ± 0.51

Compared with Control group ##*P>0.01; ###P<0.01. Compared with Model control group *P<0.05, ***P<0.001. (± SD, n = 10)

Table 6. Effect of EM-X on platelet aggregation in rats

Group	Doses	Concentration Platelet aggregation curve (mm)	Inhibition (%)
Control		140.9 ± 22.8	
DSI	0.21 g/ml	87.7 ± 26.8***	37.7
EM-X	0.28 ml/ml	110.2 ± 19.1**	21.8
EM-X	0.14 ml/ml	118.8 ± 22.7*	15.7

Compared with Control group **P<0.05, ***P<0.001. (± SD, n = 10)

Table 7. Effect of EM-X on thrombus formation in rats

Group	Doses	Moist wt of thrombus (mg)	Inhibition (%)
Control		11.8 ± 5.8	
FDSP	0.66 g/kg	7.4 ± 2.1*	37.3
EM-X	20 ml/kg	8.1 ± 2.8*	31.4
EM-X	10 ml/kg	9.7 ± 3.4	15.8

Compared with Control group *P<0.05. (± SD, n = 10)

Table 8. Effect of EM-X on blood rheology in blood stasis syndrome rats

Group	Doses	Whole blood viscosity	Plasma	Erythrocyte electro-hematocirt	
	High shear (200s-1)	Low shear (3s-1)	viscosity	PHoretic time (s)	(%)
Control	5.79 ± 0.40	13.94 ± 1.43	1.33 ± 0.15	21.6 ± 1.5	46.3 ± 3.2
Model	6.98 ± 0.51###	17.22 ± 1.41###	1.89 ± 0.11###	26.3 ± 2.2###	49.8 ± 1.7##
FDSP 0.66 g/kg	6.33 ± 0.66*	14.94 ± 0.79***	1.66 ± 0.18**	23.9 ± 1.6**	46.7 ± 2.5**
EM-X 20 ml/kg	6.14 ± 0.59**	15.84 ± 1.90	1.75 ± 0.12*	24.4 ± 1.1*	48.4 ± 3.1
EM-X 10 ml/kg	6.84 ± 0.44	16.38 ± 1.69	1.81 ± 0.16	25.1 ± 1.4	49.5 ± 3.1

Compared with Control group ##P<0.01; ###P<0.001; Compared with Model control group P<0.05; *P<0.05. ***P<0.001. (± SD, n = 10)

Reference

1 Jin Chunhua, Jiang Xiulian, Wang Yingun, et al. Experimental Study of PolysaCharide of Gaqoderma Lucidum on Promoting Blood Circulation to Remove Blood Stasis. Chinese Traditional and Herbal Drugs, 1998, 29 (7): 470

2 Zhu Yan, Chen Ninghong, Liu Guoquing. Effects of the Mixture of Shengmai Injection and Mailuoning Injection on Anticoagulant and Fibrinolytic Function in vitro and in vivo. Journal of China Pharmaceutical University 1999, 30 (2): 130

3 M Shipin, Liu Baolin, Zhou Sudi, et al. Pharmacological Studies of Glycosides of Saffron Crocus. Chinese Traditional Herbal Drugs, 1999, 30 (3): 196

4 Lin Suseng, Zhang Ju, Sun Weilin. Study of Hypercholesterolemia Model Method. Quarterly Pharmaceutic Bulletin, 1989, 70 (2): 15

5 Li Yikue. Pharmacological Experiment Methodology of Chinese Materia Medica Shanghai: Shanghai Science and Technology Press. 1991. 83-84

6 Chen Qi. Pharmcological Research Methodology of Chinese Materia Medica. Beijing: Beijing People's Press. 1993.614

7 Chen Qi. Pharmacological Research Methodology of Chinese Materia Medica. Beijing: Beijing People's Press. 1993.564-565

Research designer: Zhong Zheng Xian[1] (associate professor), and Kebin[2] (research fellow)
Experimenter: Zhong Zhengzian[1], Zhou Guifen[1] (high-ranking laboratory technician), and Chen Experimental data: June 1, 2000 – December 20, 2000
Research affiliation:
[1]: Guangzi Institute of Traditional and Pharmaceutical Sciences, Nanning, China
[2]: EM Research Organization, Okinawa, Japan.