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Vasorelaxant effect of Salvia miltiorrhiza Radix extract on isolated rat aorta

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丹蔘 추출물의 흰쥐 흉부 대동맥 이완 효과

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丹蔘 (*Salvia miltiorrhiza*)은 꿀풀과(一科 Lamiaceae)에 속하는 다년생 초본으로, 중국이 원산지이며 약용하기 위해 우리나라에서도 널리 재배하고 있다. 神農本草經 上品에 收載되어 있으며, 祛瘀止痛, 凉血消腫, 淸心除煩, 活血調血 등의 효능이 있어 부인과 질환에 많이 사용되고 있는 약재에 속한다. 본 연구에서는 丹蔘 추출물이 흰쥐의 흉부 대동맥 절편에 어떠한 양상으로 작용하는지 확인하고자 하였으며, 그 결과 단삼 추출물 특히 헥산 분획에서 강력한 혈관 이완 작용이 나타났으며, 혈관 내피 세포의 존재 유무에 상관없이 농도 의존적으로 혈관을 이완시켰으나 혈관 내피 세포가 존재하는 상황에서 더욱 강력한 혈관 이완 작용을 보였다. 이러한 과정에 NO에 의한 cGMP 증가가 주요하게 작용하는 것으로 추정되었으며, 칼슘 통로 차단 효과에 의한 세포 내 Ca^{2+}의 감소도 관여하는 것으로 생각된다.

Ⅰ. Introduction

Salvia miltiorrhizae Radix (丹蔘, SR), commonly known as Dansam or Danshen, is from the root and rhizome of Salvia miltiorrhiza Bunge that belongs to the family of Labiatae, and it is a commonly and widely used traditional herbal medicine for the treatment of cardiovascular diseases such as stroke, angina pectoris and myocardial infarction.

The cardio-protective efficacy of SR has been studied in animal ischemia/reperfusion experiments[1-5] and regional cerebral blood flow6). And it's mechanism may involve the ability of SR to enhance antioxidant activities to decrease or abolish the production of free radicals[7].

SR has also been shown to attenuate the increase in intracellular calcium induced by anoxia/reoxygenation in isolated ventricular myocytes, which would decrease the transformation of xanthine oxidase from xanthine dehydrogenase to reduce the production of oxygen free radicals[8]. In addition, SR lowered the viscosity of whole blood, accelerated electrophoresis of red blood cells, and improved peripheral circulation[9]. The vasodilator and

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hypotensive actions of SR probably contributed to these effects[10-12].

In the present experiments, therefore, we examined the vasorelaxations induced by SR extract, and investigated the relaxation mechanisms in comparison with the effect of each constituent using rat aorta ring strip.

Ⅱ. Materials and Methods

1. Plant extracts

Dried root of Salvia miltiorrhiza (Salvia miltiorrhizae Radix, SR) was obtained from a local market and ground using a commercial food mixer. This powder was extracted consecutively under reflux with water for 1 h. The resulting water extract was evaporated under reduced pressure at 37-40℃ of temperature and lyophilized (SREx). This solid extract was stored at -20℃ until use. A solution was prepared with distilled water at a concentration of 100-300 ㎎/㎖ on the day of the experiment. Water extract of SR were extracted again three times with n-hexane in the sonicator. The suspension was filtered and evaporated under reduced pressure at low temperature and lyophilized. The residue of hexane extract was obtained. The remaining was extracted again with chloroform and methanol sequentially to yield chloroform and methanol extract fractions.

2. Artery ring preparation

Male Sprague-Dawley rats (200-250 g each) were sacrificed by stunning and bleeding. The descending thoracic aorta was dissected free from surrounding connective tissues and cut into rings of 2-3 ㎜ in length. Rings were then transferred into 4 ㎖ horizontal type organ chambers, and were bathed in physiological salt solution (PSS) at 37℃ containing (mmol/l): NaCl, 136.9; KCl, 5.4; CaCl2, 1.5; MgCl2, 1.0; NaHCO3, 23.8; glucose, 5.5, and EDTA 0.01 (pH 7.4); and gassed with 95% O2 and 5% CO2. Rings were mounted on stainless steel hooks connected to a force-displacement transducer (FT03, Grass, Rhode Island, USA) connected to a polygraph system (RPS212, Grass, Rhode Island, USA) and a computer analyzer (Power Lab 400, MacLab System, Castle Hill, Australia) were used. A basal tension of 1 g was applied. Some segments were mechanically denuded of endothelium by gentle rubbing with a moistened cotton swab.

3. Experimental protocols

All rings were equilibrated for 60 min under a resting tension of 1 g and then exposed repeatedly to 72 mmol/l KCl PSS until responses became stable. Control contraction was produced using 300 nmol/l norepinephrine (NE). After sustained tension (60% or 80% of the maximal contraction to 72 mmol/l KCl PSS in endothelium-intact or -denuded rings) was obtained, SREx or vehicles were added sequentially to the bath solution. The high-potassium solution was prepared by replacing NaCl of PSS with equimolar KCl. In experiments where specific inhibitors were used, they were added 20 min before precontraction. The inhibitors tested were NG-nitro-L-arginine (LNNA, 10 mmol/l), NO-nitro-L-arginine methyl ester (NAME, 10 mmol/l) or NG-methyl-L-arginine (NMMA, 10 mmol/l) as an inhibitor of NO synthesis, methylene blue (1 mmol/l) or 1H-[1,2,3] oxadiazole

[4,3-a] quinoxalin-1-one (ODQ, 1 mmol/l) as a guanylate cyclase inhibitor, ICI 182,780 (10 mmol/l) as a specific estrogen receptor antagonist, or indomethacin (10 mmol/l) as a cyclooxygenase inhibitor, on SREx-induced endothelium-dependent relaxation. In some experiments, the endothelium-intact rings were first treated with 1 mmol/l L-arginine, then with L-NNA before addition of NE. To examine calcium antagonistic mechanism on the SREx induced vasorelaxation, endothelium-denuded arterial rings were washed three times (10-min interval) with calcium-free medium containing 1 mmol/l EGTA. Then arteries were stimulated with calcium-free, 72 mmol/l KCl medium (without or with SREx) and cumulative concentrations of CaCl2 (0.3-10 mmol/l) were added. For comparison, the L-type calcium channel blocker nicardipine (5 nmol/l) instead of SREx, were assayed in a separate series of experiments.

4. Reagents

Methylene blue, NE, L-NNA, NAME, NMMA, indomethacin, nicardipine, and ODQ were purchased from Sigma (St. Louis, MO, USA). ICI 182,780 was obtained from Tocris Cookson Ltd. (Bristol, UK). ICI 182,780 were dissolved in 100% ethanol at a concentration of 10 mmol/l.

5. Data analysis

Relaxation was expressed in terms of percentage decrease of the maximal contraction caused by NE (300 mmol/l). All results are expressed as mean±sem. The number of rings obtained from different rats was represented by n. The Student's t-test and one-way ANOVA with LSD post hoc test were used to evaluate between groups. The obtained p-values less than 0.05 were regarded as significant.

Ⅲ. Results

1. Relaxant effects of SREx

The aorta ring strip of the rat exhibited a strong contraction after an initial application of 5 uM NE. Water extract of SR (SREx, 0.03 to 3 mg/ml) applications potently relaxed the contraction induced by NE in a concentration-dependent manner in endothelium-intact aortas contracted with NE (Fig. 1).

Endothelium denudation significantly depressed this relaxation; in particular, relaxation was almost completely abolished at 0.1 and 0.3 mg/ml SREx, but not at the highest concentration of SREx (1.0 ㎎/㎖); endothelium-independent relaxation also occurred (Fig. 2).

The relaxation effects of hexane, chloroform and methanol subfractions of SREx were also examined. 0.3 ㎎/㎖ of hexane fraction showed most significant reduction of NE-induced vasoconstriction, but chloroform subfraction had no vasorelaxation effect.

2. Influence of a variety of inhibitors on the endothelium-dependent relaxation of SREx

SREx-induced endothelium-dependent relaxation was reduced significantly by pre-incubation with L-NNA (10 mmol/l). L-Arginine (1 mmol/l) caused full reversal of the inhibitory effect of L-NNA on SREx induced relaxation. Pretreatment with ODQ (1 mmol/l) also strongly reduced the

SREx-induced endothelium-dependent relaxation at all concentrations. Indomethacin (10 mmol/l) significantly attenuated the relaxation at 0.3 ㎎/㎖ concentration of SREx. In addition, pretreatment with ICI 182,780 (1 mmol/l) also strongly inhibited the relaxation.

3. Effects of SREx on the calcium influx in endothelium-denuded rings

Since the data suggested that SREx conducts a direct effect on vascular smooth muscles, we investigated its effects on the cumulative concentration of CaCl2-induced (0.3-10 mmol/l) concentration-dependent contractions in a 72-mmol/l KCl depolarized calcium-free medium with endothelium-denuded aortic rings.

Different concentrations of CaCl2 (0.3-10 mmol/l) induced concentration-dependent contraction in rat aorta depolarized using 72-mmol/l KCl in calcium-free medium. The CaCl2-induced contraction curves shifted significantly to downward as compared to the control after pretreatment with SREx (0.3 or 1.0 ㎎/㎖) in a concentration-dependent manner (Fig. 5). The L-type calcium channel blocker nicardipine (5 nmol/l), as a positive control, strongly inhibited the CaCl2-induced contraction of the rat aorta.

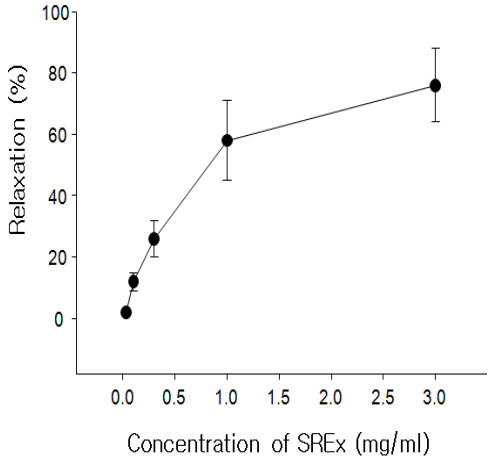

Fig. 1. Concentration-dependent relaxation of SREx. Values (%) represent mean±SEM.

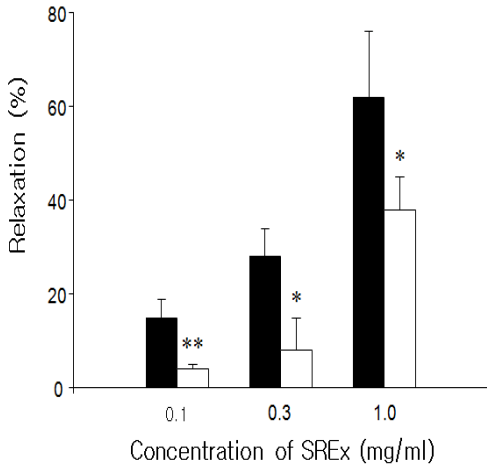

Fig. 2. The concentration-dependency of SREx (0.1-1.0 ㎎/㎖) in the endothelium-intact (■, n=6) and endotheliumdenuded (□, n=6) rat aorta. The relaxation response is expressed as the percentage relaxation of the NE-induced contraction (100% represent complete relaxation). Values are the mean±sem. *Significant differences compared with endothelium-intact rings; *p＜0.05; **p ＜0.01.

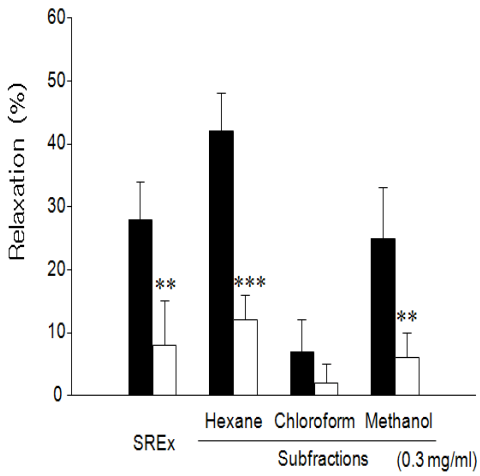

Fig. 3. The concentration-dependency of subfractions of SREx (0.1-1.0 ㎎/㎖) in the endothelium-intact (■, n=6) and endotheliumdenuded (□, n=6) rat aorta. The relaxation response is expressed as the percentage relaxation of the NE-induced contraction (100% represent complete relaxation). Values are the mean±sem. *Significant differences compared with endothelium-intact rings; **p＜0.01; ***p＜0.001.

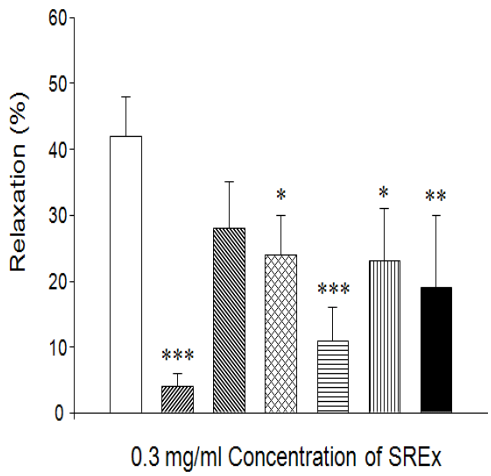

Fig. 4. Comparison of the responses to SREx on endothelium-intact and inhibitors pretreated rat aortas. The intact rings were contracted with NE and then SREx (□, 0.3 ㎎/㎖) was added to the muscle. The preparations were pretreated with L-NNA (▨, 10 umol/l), L-arginine (1.0 mmol/l) plus L-NNA (10 umol/l), (▧), methylene blue (▩, 1.0 umol/l), ODQ (▤, 1.0 umol/l), indomethacin (▥, 10 umol/l), ICI 182,780 (■, 10 umol/l) for 20 min. Then SREx (0.1-1.0 mg/ml) was added. *Significant differences compared with the corresponding controls; *p＜0.05, **p＜0.01, ***p＜0.001 (n=6, respectively).

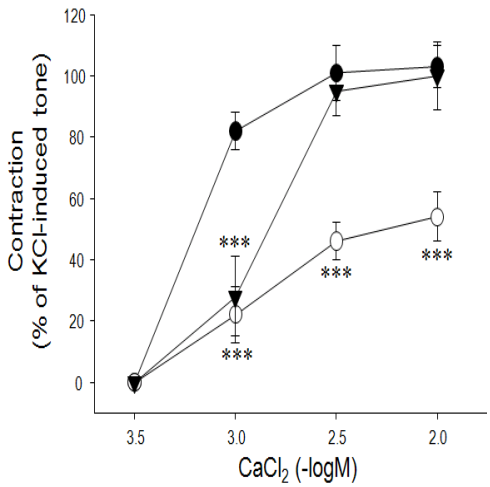

Fig. 5. The effects of SREx (▼, 0.3 ㎎/㎖) on the calcium concentration-dependent contraction curves of the rat thoracic aorta without endothelium compared with the effects of nicardipine (○, 5 nmol/l). Data are expressed as the percentage of the maximum contraction induced by Ca2+ in controls (●, mean±sem). *Significant differences compared with the corresponding controls; ***p＜0.001.

Ⅳ. Discussion

SR is the dried root of Salvia miltiorrhiza that belongs to the family of Labiatae. It is a traditional Chinese medicine commonly used for the treatment of cardiovascular diseases such as angina pectoris, myocardial infarction and stroke[1]. The cardio-protective efficacy of danshen has been studied in animal ischemia/reperfusion experiments[1-5]. The mechanism may involve the ability of SR to enhance antioxidant defense enzymes activities to decrease or abolish the production of free radicals[7].

The actions of SR were known as vigorates bood and dispels stasis, clears heat and soothes irritability, cools blood and reduces abscesses, nourishes the blood and balms the spirit. But it still has many contra-indications and cautions, such as do not use during pregnancy, do not use in cases with hyper-menorrhea or hematuria, do not use for early menses or obstructed menses due to lack of blood, do not use for restless fetus due to lack of blood unable to nourish etc.

It also has herb-drug interactions such as decreases warfarin clearance and increases its bioavailability. When it comes with toxicity and overdose, allergic reactions affecting the skin and respiratory system have been reported. Possible side effects include: dry mouth, dizziness, general weakness, numb and swollen feeling in the hands, shortness of breath, anxiety, tachycardia, nausea, vomiting, and gastrointestinal symptoms. These symptoms gradually disappear, usually without terminating treatment.

Salvianolic acid, isolated from SR, showed improvement of rCBF in the ischemic hemisphere and inhibit platelet aggregation in rats[6]. And in above study6), it was reported that the rCBF decreased immediately after bilateral carotid artery occlusion and that it decreased to 48 % of pre-occlusion levels 20 min after arteries' occlusion.

SR has also been shown to attenuate the increase in intracellular calcium induced by anoxia-reoxygenation in isolated ventricular myocytes, which would decrease the transformation of xanthine oxidase from xanthine dehydrogenase to reduce the production of oxygen free radicals[8]. In addition, SR lowered the viscosity of whole blood, accelerated electrophoresis of red blood cells, and improved peripheral circulation[9]. The vasodilator and hypotensive actions of danshen probably contributed to these effects[10-12].

The content of SR can be separated into lipid-soluble and water-soluble fractions. Its lipid-soluble fraction contains more than 30 diterpenoid tanshinones; the major active constituents include tanshinone I, IIA, B, cryptotanshinone, dihydrotanshinone I, methylenetanshinone, and isotanshinone IIA [13]. The putative active components of its aqueous extract are salvianolic acid B, danshensu, lithospermic acids, protocatechuic acid, and rosemarinic acid [10,14-16].

However, since the water decoction is the commonly used method in preparing asian traditional medicine for human consumption, current interest in studying traditional medicine is on the aqueous fraction and ingredients. Hence, SR was reported to

possess hepatoprotective and antifibrogenic effects[17], inhibit platelet aggregation and improves cerebral blood flow[6], enhance angiogenic processes[18-19], and protect against injury in the skin, heart and brain caused by ischaemia-reperfusion; possibly by reducing lipid peroxides, scavenging free radicals and improving the energy metabolism[19-21].

The present study has demonstrated that the water extract of SR and one of its water-soluble hexane subfraction have dilatory action on rat thoracic aorta. Their vasorelaxant action was produced primarily by inhibition of Ca2+ influx in the vascular smooth muscle cells and a small component was mediated by the opening of K+ channels (Fig. 1-3). The present findings do not support the involvement of endothelium dependent mechanisms in their action.

These results demonstrate for the first time that the relaxation of rat aorta caused by SREx is mediated partially by an endothelium-dependent mechanism (Fig. 2).

In particular, the relaxation induced by lower concentrations of SREx (0.1 and 0.3 ㎎/㎖) was largely endothelium-dependent, and was essentially abolished by pretreatment with the nitric oxide synthase inhibitor L-NNA or by a soluble guanylate cyclase inhibitor, methylene blue or ODQ. Indomethacin also significantly attenuated the SREx-induced relaxation, implying a role for prostacyclin in this response (Fig. 4).

Incubation with SREx shifted the calcium concentration-dependent contraction curve to downwards in high-potassium depolarization medium and reduced the maximal contraction, suggesting that action via a calcium antagonistic mechanism relaxed the aorta (Fig. 5). The influx of calcium is an important mediator of excitation-contraction coupling in smooth muscle cells.

Therefore, inhibition of the calcium influx may be an important mechanism of SREx action. Phytoestrogens interact with the estrogen receptor[22-24], although the cellular and vasoactive mechanisms are dependent on the individual phytoestrogens [25-29].

In our study, ICI 182,780 significantly reduced the relaxation induced by lower concentrations of SREx (0.3 ㎎/㎖) (Fig. 4), implying the involvement of vascular estrogen receptors.

Ⅴ. Conclusion

This study investigated the vasorelaxant effect of SREx on isolated rat aortic rings and its possible mechanisms. The mechanism of this effect involves enhancement of the NO-cGMP system, and inhibition of the extracellular calcium influx into vascular smooth muscle, resulting in vasodilation. This study may provide a clue to the role of SR, which has long been used to promote the vascular health of postmenopausal women. The isolation and identification of the active ingredients of SREx and the investigation of possible in vivo effects of SREx are underway.

References

1. Ji XY, Tan BK, Zhu YZ. Salvia miltiorrhiza and ischemic diseases. Acta Pharmacol. Sin. 2000;21:1089-1094.

2. Fung KP, Zeng LH, Wu J, Wong HN, Lee CM, Hon PM, Chang HM, Wu TW. Demonstration of the myocardial salvage effect of lithospermic acid B isolated from the aqueous extract of Salvia miltiorrhiza. Life Sci. 1993; 52:239-244.
3. Kuang P, Tao Y, Tian Y. Radix Salviae miltiorrhizae treatment results in decreased lipid peroxidation in reperfusion injury. J. Tradit. Chin. Med. 1996;16:138-142.
4. Wu TW, Zeng LH, Fung KP, Wu J, Pang H, Grey AA, Weisel RD, Wang JY. Effect of sodium tanshinone IIA sulfonate in the rabbit myocardium and on human cardiomyocytes and vascular endothelial cells. Biochem. Pharmacol. 1993;46:2327-2332.
5. Zhou W, Ruigrok TJ. Protective effect of danshen during myocardial ischemia and reperfusion: an isolated rat heart study. Am. J. Chin. Med. 1990;18:19-24.
6. Tang MK, Ren DC, Zhang JT, Du GH. Effect of salvianolic acids from Radix Salviae miltiorrhizae on regional cerebral blood flow and platelet aggregation in rats. Phytomedicine. 2002;9:405-409.
7. Ji X, Tan BK, Zhu YC, Linz W, Zhu YZ. Comparison of cardioprotective effects using ramipril and danshen for the treatment of acute myocardial infarction in rats. Life Sci. 2003; 73:1413-1426.
8. Cao CM, Xia Q, Zhang X, Xu WH, Jiang HD, Chen JZ. Salvia miltiorrhiza attenuates the changes in contraction and intracellular calcium induced by anoxia and reoxygenation in rat cardiomyocytes. Life Sci. 2003;72:2451-2463.
9. Chen KJ. Certain progress in the treatment of coronary heart disease with traditional medicinal plants in China. Am. J. Chin. Med. 1981;9:193-196.
10. Kamata K, Iizuka T, Nagai M, Kasuya Y. Endothelium-dependent vasodilator effects of the extract from Salviae miltiorrhizae radix. A study on the identification of lithospermic acid B in the extracts. Gen. Pharmacol. 1993;24:977-981.
11. Lei XL, Chiou GC. Studies on cardiovascular actions of Salvia miltiorrhiza. Am. J. Chin. Med. 1986 ;14:26-32.
12. Li CP, Yung KH, Chiu KW. Hypotensive action of Salvia miltiorrhiza cell culture extract. Am. J. Chin. Med. 1990;18:157-166.
13. Cheng YY, Fong SM, Chang HM. Protective action of Salvia miltiorrhiza aqueous extract on chemically induced acute myocardial ischemia in rats. Zhong Xi Yi Jie He Za Zhi. 1990;10:582, 609-611.
14. Chan K, Chui SH, Wong DY, Ha WY, Chan CL, Wong RN. Protective effects of danshensu from the aqueous extract of Salvia miltiorrhiza (danshen) against homocysteine-induced endothelial dysfunction. Life Sci. 2004;75:3157-3171.
15. Kohda H, Takeda O, Tanaka S, Yamasaki K, Yamashita A, Kurokawa T, Ishibashi S. Isolation of inhibitors of adenylate cyclase from danshen,

the root of Salvia miltiorrhiza. Chem. Pharm. Bull. (Tokyo). 1989;37:1287-1290.
16. Liu GT, Zhang TM, Wang BE, Wang YW. Protective action of seven natural phenolic compounds against peroxidative damage to biomembranes. Biochem. Pharmacol. 1992;43:147-152.
17. Lin YL, Wu CH, Luo MH, Huang YJ, Wang CN, Shiao MS, Huang YT. In vitro protective effects of salvianolic acid B on primary hepatocytes and hepatic stellate cells. J. Ethnopharmacol. 2006;105:215-222.
18. Lay IS, Chiu JH, Shiao MS, Lui WY, Wu CW. Crude extract of Salvia miltiorrhiza and salvianolic acid B enhance in vitro angiogenesis in murine SVR endothelial cell line. Planta Med. 2003;69:26-32.
19. Lay IS, Hsieh CC, Chiu JH, Shiao MS, Lui WY, Wu CW. Salvianolic acid B enhances in vitro angiogenesis and improves skin flap survival in Sprague-Dawley rats. J. Surg. Res. 2003;115:279-285.
20. Chen YH, Du GH, Zhang JT. Salvianolic acid B protects brain against injuries caused by ischemia-Nreperfusion in rats. Acta Pharmacol. Sin. 2000;21:463-466.
21. Zhao BL, Jiang W, Zhao Y, Hou JW, Xin WJ. Scavenging effects of Salvia miltiorrhiza on free radicals and its protection for myocardial mitochondrial membranes from ischemia-Rreperfusion injury. Biochem. Mol. Biol. Int. 1996;38:1171-1182.
22. Martin PM, Horwitz KB, Ryan DS, McGuire WL. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology 1978;103:1860-1867.
23. Miksicek RJ. Commonly occurring plant flavonoids have estrogenic activity. Mol. Pharmacol. 1993;44:37-43.
24. Wang TT, Sathyamoorthy N, Phang JM. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis. 1996;17:271-275.
25. Mishra SK, Abbot SE, Choudhury Z, Cheng M, Khatab N, Maycock NJR, Zavery A, Aaronson PI. Endothelium-dependent relaxation of rat aorta and main pulmonary artery by the phytoestrogens genistein and daidzein. Cardiovasc. Res. 2000;46:539-546.
26. Latonnelle K, Menn FL, Kaushik SJ, Bennetau-Pelissero C. Effects of dietary phytoestrogens in vivo and in vitro in rainbow trout and siberian sturgeon: interest and limits of the in vitro studies of interspecies differences. Gen. Comp. Endocrinol. 2002;126:39-41.
27. Nevala R, Korpela R, Vapaatalo H. Plant-derived estrogens relax rat mescenteric artery in vitro. Life Sci. 1998;63:95-100.
28. Tomioka H, Hattori Y, Fukao M, Sato A, Liu M, Sakuma I, Kitabatake A, Kanno M. Relaxation in different-sized rat blood vessels mediated by endothelium-derived hyperpolarizing factor: importance of processes mediating precontractions. J. Vasc. Res. 1999;36:311-320.
29. Nagao T, Illiano S, Vanhoutte PM. Heterogeneous distribution of endothelium-dependent relaxations

resistant to NG-nitro-L-arginine in rats. Am. J. Physiol. 1992;263:1090-1094.