Anti-inflammatory effects of a Chinese herbal medicine in atherosclerosis via estrogen receptor β mediating nitric oxide production and NF-κB suppression in endothelial cells

L Wang1,3, X-M Qiu1,3, Q Hao1 and D-J Li*1,2

Bu-Shen-Ning-Xin Decoction (BSNXD) administration has alleviated the early pathologic damage of atherosclerosis by inhibiting the adhesion molecule expression and upregulating the estrogen receptor (ER) β expression in endothelial cells, and increasing the serum nitric oxide (NO) level without any effect on serum lipid status, endometrium and fat deposition in liver in ovariectomized rabbits. The BSNXD-derived serum increases ER β expression in the human umbilical vein endothelial cells (HUVECs), and decreases malondialdehyde (MDA) production, and upregulates eNOS expression then increases NO synthesis through ER/β-dependent pathway. NO not only suppresses the LPS-induced NF-κB transcription in HUVECs, but also decreases apoptosis of endothelial cells. The BSNXD-derived serum decreases monocyte chemoattractant protein-1 production, and suppresses cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) expression in HUVECs injured by oxidized low-density lipoproteins (ox-LDL), and these effects can be abolished by ER(β) antagonist (R,RTHC) and NO synthase inhibitor (L-NAME). The BSNXD-derived serum-treated HUVECs supernatant reduces CCR2, LFA-1 and VLA-4 expression in monocytes cell line U937 cells, which in turn inhibits adherence of U937 to injured endothelial cells. NO synthesis increases, and MDA production decreases through ERβ-mediated pathway that suppresses apoptosis and NF-κB activity in endothelial cells that downregulates adhesion molecules expression on endothelial cells via ERβ/NO/NF-κB pathway, and in turn leukocyte adhesion, which suggests BSNXD potential value in prophylaxis atherosclerosis.

Cell Death and Disease (2013) 4, e551; doi:10.1038/cddis.2013.66; published online 21 March 2013

Subject Category: Experimental Medicine

Atherosclerosis is one sort of inflammatory disease. An early step in the atherogenic process is transmigration of blood monocytes across an injured or dysfunctional endothelium into the subendothelial space where they differentiate into macrophages. The cell adhesion molecules have been implicated as one of the most important factors in atherosclerosis. The cytokine-mediated cell adhesion molecule expression is regulated by the activity of transcription factors, such as NF-κB (NF-κB). Nitric oxide (NO) is a widespread signaling molecule in the cardiovascular system, and functions in multiple ways to protect from the initiation and progression of atherosclerosis. NO prevents from the adhesion and aggregation of blood cells. It has also been shown that the induction and stabilization of IκBα by NO are important mechanisms by which NO inhibits NF-κB and attenuates atherogenesis. NO is synthesized by NO synthase (NOS) with L-arginine as a substrate. The promotion of NO production and prevention from cell adhesion may be an effective approach to control atherosclerosis.

Ovarian dysfunction has been recognized as a major risk factor for the accelerated atherosclerotic vascular disease. A lot of studies in vitro have identified mechanisms by which estrogen exerts beneficial effects on cardiovascular system. Epidemiological and experimental evidence indicates several atheroprotective effects of endogenous estrogen, which intervenes from atherosclerosis progression and inflammation. Estrogen effects are generally ascribed to transcriptional modulation of target genes through estrogen receptors (ERs), ERα and ERβ. The functional ERs are expressed in vascular endothelial cells. Normal coronary arteries of

1 Laboratory for Reproductive Immunology, Department of Obstetrics and Gynecology, Hospital and Institute of Obstetrics and Gynecology, IBS, Fudan University Shanghai Medical College, Shanghai, China and 2Department of Obstetrics and Gynecology, The Affiliated Hospital, Hainan Medical College, Haikou, China

*Corresponding author: D-J Li, Laboratory for Reproductive Immunology, Department of Obstetrics and Gynecology, Hospital and Institute of Obstetrics and Gynecology, IBS, Fudan University Shanghai Medical College, 413 Zhaozhou Road, Shanghai 200011, China. Tel: +86 21 63457331; Fax: +86 21 63457331; E-mail: djl@shmu.edu.cn

These authors contributed equally to this work.

Keywords: traditional Chinese medicinal formula; atherosclerosis; estrogen receptor; nitric oxide; inflammation

Abbreviations: BSNXD, Bu-Shen-Ning-Xin Decoction; CCR2, C–C motif chemokine receptor-2; CHD, coronary heart disease; CM, conditioned medium; ER, estrogen receptor; HRT, hormone replacement therapy; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; L-NAME, NG-nitro-L-arginine methyl ester; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; MPP, Methyl-piperidino-pyrazole; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; OXV, ovariectomy; ox-LDL, oxidized low-density lipoproteins; R,RTHC, R,R-tetrahydrochrysene; S.E.M., scanning electron microscopy; TCMs, traditional Chinese medicines; VCAM-1, vascular cell adhesion molecule-1; VLA-4, Integrin α4/β1 (Very Late Antigen-4)

Received 15.2.12; revised 13.1.13; accepted 29.1.13; Edited by M Federici
premenopausal women show normal ER expression, whereas in the atherosclerotic vessels of postmenopausal women, ERs are downregulated. ERs plays an important role in mediating estrogen’s vascular protection, but the precise mechanisms of ERβ in vascular homeostasis remains elusive. It is reported that the intimal ERβ expression correlates with atherosclerosis in postmenopausal women; polymorphisms in ERβ gene are associated with risk of cardiovascular disease in women; ERβ is the predominant mRNA transcript in normal human vascular smooth muscle cells and in the media of human arteries. The current understanding of ER does not allow one to clearly discern the importance of one isotype receptor over another. Indeed, important concepts are likely yet to be uncovered regarding receptor roles.

Traditional Chinese medicines (TCMs) have been used in Asian countries for over 5000 years to prevent and treat diseases. TCM uses a holistic and synergistic approach to restore the balance of Yin-Yang of body energy so that the body’s normal function and homeostasis is maintained.

The objective of doing this is to form specific formulae aimed to increase therapeutic efficiency and reduce adverse effects. According to the hypothesis of TCM, multiple active phytochemical components in the TCM formulae may simultaneously target multiple molecules/pathways, and thus potentially achieve superior effect to single compound. Traditional Chinese herbs have long been used for preventing atherosclerosis with less side-effect.

Bu-Shen-Ning-Xin Decoction (BSNXD) has long been used in the prophylaxis of the atherosclerosis associated with estrogen deficiency, which have already been proved to possess clear prophylactic action on human bodies in the clinical setting, but there is little information about its pharmacological properties and biochemical activities. In this paper, we summarized their anti-inflammatory effects in atherosclerosis animal models and some mechanisms at the cellular and molecular levels here. In order to clarify the effects of BSNXD on atherosclerosis in postmenopause, we use OVX in female rabbits to deplete ovarian function. The present study is to investigate the mechanisms of BSNXD ameliorating atherosclerosis in the OVX rabbits. In vitro, we have demonstrated that the BSNXD-derived serum increases ERβ expression in the human umbilical vein endothelial cells (HUVECs), and decreases malondialdehyde (MDA) production and upregulates eNOS expression then increases NO synthesis through ERβ-dependent pathway.

Results

BSNXD upregulates NO with no effect on E2 and lipid concentration in serum. A significant increase of body weight was observed in the sham group, OVX group and OVX + BSNXD group (ovariectomized rabbits treated with BSNXD) after high cholesterol chow diet. There was no significant difference in final body weight between sham group, OVX group and OVX + BSNXD group (P>0.05, data not shown). We examined whether there was a difference in plasma E2, NO and lipid levels among sham group, OVX and OVX + BSNXD group after 12 weeks of hypercholesterolic diet. As shown in Figure 1a, the serum E2 concentrations in the OVX group decreased significantly compared with the sham group (P<0.01). BSNXD treatment did not affect the serum E2 level. The ovariectomy resulted in a significantly decreased NO concentration in serum (P<0.01); OVX rabbits with BSNXD treatment exhibited significantly higher serum NO concentration than that of the OVX control (P<0.01, Figure 1b). Since the hypercholesterolemic diet led to hyperlipidemia, lipid concentrations were measured in plasma. As expected, we have not found any change in TC, TG, HDL-C and LDL-C levels in OVX + BSNXD rabbits compared with that of the OVX control or sham (Figure 1c).

BSNXD alleviates atherosclerotic lesions and reduces leukocytes adherent to the injured endothelial cells. High cholesterol diet led to atheromatous plaque formation in aorta luminal surface. A marked lipid deposition accompanied by continuous plaque formation was observed, and marked intimal thickening with focal endothelial injury in the aortas from OVX rabbits. All of the animals subjected to high cholesterol chow developed atheromatous plaque lesion, which consisted of lipid-rich deposits adhering to the aortic wall (Figure 1d). HE staining showed that the atheromatous plaque lesion was characterized by a hyperplastic transformation of the intima that contained elastic fibers, fibroblast-like cells, collagen deposits and foam cells (Figure 1e).

The extent and degree of atherosclerosis were estimated by calculating the percentage of the plaque area to total aorta....
Anti-inflammatory roles of TCM in atherosclerosis

L. Wang et al.

Cell Death and Disease
surface area; intimal hyperplasia in the aortic lesions that expressed as the intima cross-sectional thickness, intima/media thickness ratio and intimal/medial area ratio. It was shown that the OVX rabbit with high cholesterol chow showed severe atherosclerosis. Animals in the sham group showed less atherosclerosis compared with the OVX (P<0.01). The intima cross-sectional thickness, intima/media thickness ratio and intimal/medial area ratio at the site of aortic lesions in the OVX significantly increased when compared with the sham (P<0.01). The average percentage of aortic lesion area in inner surface, intima cross-sectional thickness, intima/media thickness ratio and intimal/medial area ratio in the aortic lesions was significantly decreased after having been treated with BSNXD (all P<0.01) (Figure 1f). The atherosclerosis degree in OVX with BSNXD was significantly decreased compared with the OVX.

S.E.M. analysis showed a wide spectrum of pathological alterations in the luminal surface of thoracic aorta from the OVX. In OVX rabbits, the aorta luminal vessel surface was covered by irregularly orientated endothelial cells; the endothelial cells showed cuboidal appearance protruding into the lumen of the vessel; some endothelial cells contained ‘craters’ disrupting the continuous surface of the endothelium and numerous microvilli; platelets and leukocytes adhering to endothelium were more frequently observed compared with the sham (P<0.01); in regions of the vessel with endothelial denudation, the underlying connective tissue was visible. After BSNXD treatment, vessel surface walls in endothelial disruption region were significantly ameliorated compared with the OVX, and the platelets and leukocytes sticking to endothelium were obviously decreased (P<0.01, Figure 1g).

BSNXD regulates ER\(\beta\), VCAM-1 expression in artery wall. Immunohistochemistry staining showed that the staining intensity of ER\(\beta\) was significantly lower in each artery wall layer (intima, media or adventitia) in the OVX than that of the sham (P<0.01); OVX with BSNXD administration significantly enhanced ER\(\beta\) expression (P<0.01) (Figure 1h), suggesting that ER mediates the effects of BSNXD on atherosclerosis. To provide further evidence regarding which ER isotype involved in the modulation of BSNXD on endothelial cells, we further checked the protein levels of ER\(\alpha\) and ER\(\beta\) expression. Our results showed that both ER\(\alpha\) and ER\(\beta\) expression in artery wall layer of OVX group were markedly lower than that in Sham animals. As shown in Figures 1i and j, BSNXD treatment did not alter the ER\(\alpha\) expression, while significantly increased ER\(\beta\) expression in artery wall layer of OVX animals. Furthermore, selective ER\(\alpha\) antagonist (Methyl-piperidino-pyrazole, MPP) and ER\(\beta\) antagonists (R,R-tetrahydrochrysene, R,RTHC) were used in an additional set of experiments of HUVECs in vitro.

Adhesion molecule such as VCAM-1 plays an important role in the pathogenesis of atherosclerosis. To determine whether VCAM-1 was involved in the effect of BSNXD on atherosclerosis, expression of VCAM-1 in artery wall layer was detected. As shown in Figure 1k, endothelial cell, subendothelial and smooth muscle cell in the arterial wall of the OVX expressing higher level VCAM-1 than that of the sham (P<0.01); OVX with BSNXD treatment significantly decreased VCAM-1 expression than that of the OVX control (P<0.01).

**Figure 2** Effect of BSNXD on morphology of liver. (a) Representative liver images are shown. (b) fatty liver incidence of different group are represented. As evaluated by the pathological and morphological analysis of the liver from each group, OVX + BSNXD rabbits showed less incidence of fatty liver on the high-fat diet compared with OVX rabbits. Data are expressed as mean values ± S.E.M. (n = 6). **P<0.01 compared with the OVX group.

The effects of BSNXD on uterus and liver. Photomicrographs depicting OVX uterine columnar epithelium atrophyed, and uterine glands number reduced. Our previous data showed that the relative weights of uterus markedly decreased after ovariectomy. Administration of BSNXD for 12 weeks after ovariectomy resulted in no significant change (data not shown) in the relative uterus wet weight and endometrial glands compared with the OVX control.

We found that multifocal depositions of lipids in liver of the OVX rabbits after high cholesterol chow that showed more incidence of fatty liver compared with the sham (P<0.01). OVX with BSNXD treatment were less prone to high cholesterol chow-induced liver steatosis, as revealed by the pathological and histological analysis (data not shown). Moreover, BSNXD administration presented less incidence of fatty liver on the high-fat diet compared with the OVX control (Figure 2).

**BSNXD has prophylactic effect but not therapeutic effect on established atherosclerosis associated with estrogen deficiency.** The other in vivo experiment (treatment experiments) is to treat ovariectomized rabbit with established atherosclerotic plaques to see whether the BSNXD could revert the phenotype and not only prevent it. Determinations of lesion area by enface preparation of the aorta showed extensive lesion formation by the end of the inductive phase. There was a little reduction of lesions when animals were switched to a normal chow with or without BSNXD. Also, BSNXD group had similar aortic lesions when compared with the saline group (data not shown). So from our study, we can see BSNXD has prophylactic effect but not therapeutic effect on established atherosclerosis associated with estrogen deficiency.
BSNXD-derived serum increases ER\textbeta expression in HUVECs. Real-time PCR and western blot were used to analyze the transcription and translation levels of ER\textalpha and ER\textbeta in HUVECs. The results showed that ER\textalpha and ER\textbeta were steadily expressed in HUVECs. We tried to add the BSNXD drug directly to the culture medium of HUVEC in our \textit{in vitro} experiments, it has no effect on ER\textalpha or ER\textbeta expression (data not shown); while BSNXD-derived serum increased significantly the transcription and translation of ER\textbeta, but not ER\textalpha in HUVECs \((P<0.01, \text{Figures 3a and b}).

BSNXD-derived serum upregulates eNOS expression, increases NO and downregulates MDA production via ER\textbeta-dependent pathway in HUVECs. We further analyzed the modulation of the BSNXD drug directly on eNOS expression, NO and MDA production in HUVECs induced by ox-LDL. 

\begin{itemize}
\item eNOS expression, NO production was enhanced and MDA was decreased by the drug-derived serum \((P<0.05 \text{ or } P<0.01, \text{respectively.})\)
\item Meanwhile, we pretreated, respectively, the
\item BSNXD treatment increased ER\textbeta transcription and translation in HUVECs. Pretreatment of selective ER\textbeta antagonist (R, R-tetrahydrocrystene, R, RTHC) but not ER\textalpha antagonist (methyl-piperidino pyrazole, MPP) could block these effects on (eNOS, NO, MDA expression, apoptosis, NF-\kappaB transcription, etc.), which is induced by the drug-derived serum. Meanwhile the NO synthase inhibitor (NG-nitro-L-arginine methyl ester, L-NAME) significantly decreased the NO production, blocked the anti-apoptosis and NF-\kappaB activity inhibition effect of BSNXD in HUVECs. Data are expressed as mean values ± S.E.M. \((n=6). \,*P<0.05, \,**P<0.01\)
\end{itemize}
BSNXD-derived serum inhibits apoptosis and NF-κB activity in HUVECs through NO and ERβ pathway. To investigate the protective effects of BSNXD on HUVECs apoptosis induced by ox-LDL, we analyzed the percentage of the early apoptotic cells with the Annexin V-FITC/PI dual-labeling assay. The 10% drug-derived serum could relieve the ox-LDL-induced apoptosis (P<0.01). The anti-apoptosis effect of BSNXD could be blocked by NOS inhibitor (P<0.01) or ERβ antagonist (P<0.05), but not ERα antagonist (Figure 3f), which suggests that BSNXD inhibits the endothelial cells apoptosis in an ERβ and NO-dependent manner.

The activity of the transcription factor NF-κB in the LPS-stimulated HUVECs was also examined. It was found that NF-κB-luciferase activity decreased significantly after 10% drug-derived serum treatment (P<0.01); this effect of BSNXD-derived serum was significantly inhibited by ERβ antagonist or NOS inhibitor (P<0.01) (Figure 3g). It may be proposed that BSNXD exerts NF-κB activity suppression by increasing NO production via ERβ mediating pathway in endothelial cells.

BSNXD-derived serum suppresses adhesion molecules expression in HUVECs through ERβ/NOS/NF-κB pathway. We further investigated the effect of BSNXD on chemotactic factors such as monocyte chemoattractant protein-1 (MCP-1), adhesion molecules such as ICAM-1, VCAM-1 and E-selectin expression in HUVECs. The MCP-1 (Figure 4a), ICAM-1 (Figure 4b), VCAM-1 (Figure 4c) and E-selectin (Figure 4d) expression were greatly decreased after treatment with the 10% drug-derived serum (P<0.01); and ERβ antagonist (P<0.01) other than ERα antagonist (P>0.05) could block these effects of BSNXD-derived serum. Meanwhile, NOS inhibitor could also abolish these effects of BSNXD-derived serum upregulates eNOS expression, increases NO and decreases MDA production via ERβ other than α pathway in endothelial cells.

BSNXD-derived serum-treated HUVECs supernatant inhibits CCR2, LFA-1, VLA-4 expression in U937 and U937 cell adhesion. We further examined the effects of HUVECs supernatant under different treatments on CCR2 (Figure 5a), LFA-1 (Figure 5b) and VLA-4 (Figure 5c) expression in U937. Our previous results showed that the supernatant from ox-LDL-treated HUVECs induced a significant increase of CCR2, LFA-1 and VLA-4 expression in U937; however, the ox-LDL-induced increasing could be inhibited by the 10% drug-derived serum (P<0.01). Thereafter, we investigated the BSNXD effect on U937 cell adhesion to HUVECs. The 10% BSNXD-derived serum could suppress U937 cell adhesion (P<0.01); ERβ antagonist (P<0.05) but not ERα antagonist (P>0.05) could block the effect of the 10% drug-derived serum. Simultaneously, NOS inhibitor (P<0.01) could also abolish this effect of BSNXD-derived serum (Figure 5d).

Discussion

The development of an atherosclerotic lesion requires a complex interplay between mononuclear cells and endothelia. Our histopathological analysis has demonstrated that BSNXD significantly relieves the extent and degree of atherosclerosis; S.E.M. examination also shows that BSNXD ameliorates vessel wall injury, and the platelets and leukocytes adhering to the endothelium are obviously decreased. BSNXD reduces lipids deposition in liver and consequently the incidence of fatty liver, and has no stimulating effects on the uterus. Disruptions of ovarian function are associated with increased risk of metabolic disease, it causes ectopic lipid deposition. In our experiments, there was no significant difference in final body weight and serum lipid levels between sham, OVX and OVX + BSNXD group. Meanwhile, multifocal depositions of lipids in the liver of the OVX rabbits showed more incidence of fatty liver compared with the sham. OVX with BSNXD treatment were less prone to liver steatosis and fatty liver. There is strong difference in liver steatosis, without relevant differences in circulating fatty acid; suggesting that E2 deficiency induced hepatic lipid accumulation. The present findings support a role for disruptions of ovarian function in the development of visceral adiposity, which in humans is known to precede the development of the metabolic syndrome. BSNXD could reduce E2 deficiency associated with ectopic lipid deposition. Some anti-atherosclerotic drugs can prevent from atherosclerosis by protecting LDL from oxidation and anti-hypercholesterolemic effect. Estrogen treatment prevents from the development of fatty streaks. Our data show that lipid parameters (TC, LDL-C, HDL-C) have not been changed by BSNXD. As other studies have showed, the changes in lipid parameters are too minor to explain the atheroprotective effect of the hormone. There are plasma lipid-dependent and -independent effects of anti-atherosclerotic drugs. Our results show that the effects of BSNXD on atheroma regression occur completely independent of systemic cholesterol levels. This effect seems to be a direct anti-inflammatory effect of BSNXD on the arterial wall. Previous research also showed the crucial role of intact endothelium, as the anti-atherogenic effect of E2 was abolished.

It is important to conclude whether the effects of BSNXD are specific or represent a combined effect with estrogen generated from BSNXD. The present study shows that BSNXD treatment does not change serum E2 concentration and ERβ expression in thoracic aortic tissues, but does increase ERβ expression. BSNXD may exert role directly through ERβ. BSNXD may enhance proliferation of estrogen-sensitive cells, and thereby increase endothelial reactivity to E2. Also, we can see that BSNXD has prophylactic effect but not therapeutic effect on established atherosclerosis associated with estrogen deficiency. In postmenopausal women with established atherosclerosis, E2 had no effect.
Figure 4  BSNXD suppresses adhesion molecules expression on HUVECs through ER/\(\kappa\)B/NF-\(\kappa\)B pathway. The primary HUVECs were exposed to control serum or 10% BSNXD-derived serum for 48 h; in the final 24 h culture the ox-LDL was added. The supernatants were collected, and MCP-1 concentrations were determined. The MCP-1 expression of HUVECs was determined by western blot analysis. The mRNA and protein expression levels of cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) were assessed by RT-PCR, western blot and FACS. The MCP-1(a), ICAM-1 (b), VCAM-1 (c) and E-selectin (d) expression was greatly decreased after treatment with the 10% drug-derived serum. NOS inhibitor (L-NAME), ER/\(\kappa\)B antagonist (R, RTHC) other than ER\(\alpha\) antagonist (MPP) could block these effects induced by the drug-derived serum. Data are expressed as mean values \(\pm\) S.E.M. (\(n=6\)). *\(P<0.05\), **\(P<0.01\)
Figure 5  BSNXD-treated HUVECs supernatant inhibits CCR2, LFA-1, VLA-4 expression in U937 and attenuates U937 cell adhesion. After HUVECs were treated as above, the supernatant was collected and used to treat U937 cells. CCR2 (a), LFA-1 (b) and VLA-4 (c) mRNA and protein expression levels in treated U937 cells were assessed by RT-PCR, flow cytometry and western blot. The ox-LDL-induced increase (Control group) could be inhibited by the 10% drug-derived serum. Furthermore, 10% BSNXD could suppress U937 cell adhesion; NOS inhibitor (L-NAME) and ERβ antagonist (R, RTHC) but not ERα antagonist (MPP) could block the anti-adhesion effect of the 10% drug-derived serum (d). Data are expressed as mean values ± S.E.M. (n = 6). *P<0.05, **P<0.01

Cell Death and Disease
on the progression of atherosclerosis. Cumulated data support a ‘window-of-opportunity’ for maximal reduction of CHD and minimization of risks with HRT initiation before age 60 and/or within 10 years of menopause.

Atherosclerosis is an inflammatory disease characterized by endothelial dysfunction, impairment of NO production, etc. NO is a crucial mediator in endothelial vasodilator function. It is synthesized from the terminal guanidine nitrogen of L-arginine by NOS enzymes. The bioavailability of NO is a representation of endothelial function. Our results show that eNOS expression and NO is increased by BSNXD, and the atheroprotective effect may be dependent on NO production. Adhesion molecule such as VCAM-1 plays an important role in the pathogenesis of atherosclerosis. Our results showed that VCAM-1 expression in artery wall is significantly decreased by BSNXD treatment, which suggests that BSNXD plays direct anti-inflammatory effects on arterial wall.

Our results showed that BSNXD-derived serum but not BSNXD raw herbs itself has effective action on HUVECs. After oral administration of BSNXD, compounds absorbed into the bloodstream then become active compounds. Compounds may be functional by oxidation, hydrolysis or reduction; primary metabolites may also undergo conjugation reactions to form secondary metabolites. Our results show that the BSNXD-derived serum increases ERβ expression in HUVECs, and results in an enhanced NO and reduced MDA production. ERβ other than ERα antagonist can block these effects induced by the drug-derived serum; meanwhile, NOS inhibitor can significantly decrease the NO production in HUVECs. BSNXD appears to increase NO and decrease MDA production via ERβ-dependent pathway. Furthermore, BSNXD treatment can inhibit the apoptosis of HUVECs in the ERβ-dependent manner.

Nuclear transcription factor NF-κB is known to be the key regulator for modulate functional genes expression including adhesion molecule. We have investigated whether NF-κB activity is involved in the inhibitory mechanism of BSNXD on adhesion molecule expression. As expected, BSNXD significantly inhibits NF-κB-luciferase transduction activity, which suggests that BSNXD attenuates the inflammatory reaction by inhibition of NF-κB in the vascular endothelium. It is reported that the induction and stabilization of IκBα by NO are important mechanisms by which NO inhibits NF-κB and attenuate atherogenesis. Our data also confirm that the effect of BSNXD-derived serum on NF-κB activity in endothelial cells is significantly inhibited by ERβ antagonist or NOS inhibitor. It is proposed that BSNXD exerts NF-κB activity downregulation by increasing NO production via ERβ-dependent pathway in endothelial cells, which subsequently attenuates atherogenesis.

Inflammatory cells play a crucial role in the pathogenesis of atherosclerosis. Penetration of atherogenic lipoproteins is the first step of the atheromatous process. Adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, mediate the binding of monocytes and lymphocytes to vascular endothelial cells through interactions with the counter-receptor LFA-1, VLA-4, etc. Chemokine MCP-1 may recruit monocytes to migrate into the intima of the arterial wall, and enhance the progression of the atherosclerotic lesions. Our results demonstrated that BSNXD decreases VCAM-1 expression in thoracic aortic tissues, inhibits the chemotactic factor such as MCP-1, adhesion molecules such as ICAM-1, VCAM-1 and E-selectin expression in HUVECs, and in turn attenuates the ox-LDL-induced inflammation in the endothelial cells. Inhibition of ERβ or NOS with selective antagonist or inhibitor in HUVECs can block these effects of BSNXD, which suggests that the anti-inflammatory and anti-atherogenic effect of BSNXD on endothelial cells is dependent on ERβ/NOS/NF-κB pathway. Furthermore, supernatant from the BSNXD-treated HUVECs can inhibit CCR2, LFA-1 and VLA-4 expression in U937 cells, and efficiently suppress the increased

Figure 6  Summary of anti-inflammatory roles of BSNXD in atherosclerosis. BSNXD upregulates estrogen receptor β (ERβ) pathway mediating nitric oxide synthesis and downregulates malondialdehyde (MDA) production. NO in turn suppresses apoptosis and NF-κB activity in endothelial cells. BSNXD suppresses monocyte chemoattractant protein-1 (MCP-1), and cell adhesion molecules expression in HUVECs via ERβ/NOS/NF-κB pathway. Moreover, BSNXD can induce HUVECs to downregulate CCR2, LFA-1 and VLA-4 expression in U937 cells, and in turn inhibit adherence of U937 to injured endothelial cells, which postpones the progression of atherosclerosis.
adhesion of U937 cells to HUVECs, suggesting that the effects of BSNXD on monocytes might participate in the anti-atherogenic effects. The NOS inhibitor or ER\(\beta\) antagonist could block the effect of BSNXD on U937 cell adhesion, suggesting that the anti-atherogenic effect of BSNXD on U937 cells adhesion is also through ER\(\beta\)/NO/NF\(-\kappa\)B pathway.

Taken together, the effect of BSNXD on endothelial cells seems to increase ER\(\beta\) expression, NO production, and decrease MDA production through ER\(\beta\)/mediated pathway. The newly formed NO suppresses apoptosis and NF\(-\kappa\)B activity of endothelial cells; thus, BSNXD suppresses adhesion molecules expression on HUVECs through ER\(\beta\)/NO/NF\(-\kappa\)B pathway, and attenuates leukocyte adhesion (Figure 6), which provides evidence that BSNXD can alleviate inflammation in endothelial cells, prevent from the migration and adherence of monocytes to endothelial cells, and postpone the progression of atherosclerosis. Consequently, BSNXD presents potential in clinical prophylaxis for atherosclerosis.

Materials and Methods

Chinese medicinal formula. Herbal formula BSNXD is composed of eight crude herbs that is prepared as seen in Table 1. The rule of compositions comes from Traditional Chinese Medicinal theory, and the compositions are due to our clinical experience.

Table 1 The composition and preparation of herbal formula BSNXD

| Crude herbs                                      | Content | Main components                  |
|--------------------------------------------------|---------|----------------------------------|
| Drying Rehmannia Root (Radix Rehmanniae)         | 15 g    | 0.11% Catalpol                   |
| Common Anemarrhena Rhizome (Rhizoma Anemarrhena) | 15 g    | Sarsasapogenin mangiferin        |
| Bank of Chinese Corktree (Phellodendron amurense Rupr.) | 9 g    | Berberine; palmatine             |
| Barbary Wolfberry Fruit (Fructus Lycii barbari)  | 15 g    | Betaine Lycium barbaram polysaccharide (LBP) |
| Chinese Dodder Seed (Semen Cuscutae Chinesis)     | 12 g    | Rutin                            |
| Shorthomed Epimedium Herb (Herba Epimedii)       | 12 g    | Icarin total flavonoids of Epimedium (TFE) |
| Spina Date Seed (Semen Ziziphi spinosae)          | 9 g     | Betulinic acid                   |
| Oriental Waterplantain Rhizome (Rhizoma Alismatis)| 12 g    | Alisol B monoacetate             |

*Note: based on the traditional method. The crude herbs above (\(\times\) 100) were mixed, immersed in deionized water (10 times of the herbs total weight) overnight, and then boiled at 90°C for 60 min for the first decoction. The soluble extract was recovered, and repeated the same process. The three extracts were combined and concentrated by rotary evaporator. The yield of the BSNXD extract was 2675.7 ml with the total raw herbs 3.7 g/ml.

Experiment and drug administration. The experimentation of animals was carried out according to the Principles of Laboratory Animal Care (NIH publication number 85-23, revised 1985). Twelve female New Zealand rabbits underwent bilateral oophorectomy, which were purchased from the Laboratory Animal Facility of Chinese Academy of Sciences (Shanghai, China). In 2 weeks after OVX, the rabbits were then randomly divided into two groups (OVX and OVX + BSNXD) of six rabbits each. The OVX group serving as controls received saline treatment and high cholesterol chow made of the standard rabbit chow and additives of 0.5% cholesterol, 15% egg yolk and 5% pork liver.6 Those from group OVX + BSNXD received high cholesterol chow plus 5 ml mixed row herbs (BSNXD) per kilogram body weight daily, a dosage equivalent to the human adult dose based on an established formula for human–rabbit drug conversion.35 The other animals were killed after the last treatment, and the blood samples and tissues were harvested for further investigation. The other in vivo experiment (treatment experiments) is to treat ovarioectomized rabbit with established atherosclerotic plaques to see whether the BSNXD could revert the phenotype and not only prevent it. Twelve female New Zealand rabbits underwent bilateral oophorectomy. In 2 weeks after OVX, the rabbits were fed an atherogenic high-fat, high cholesterol diet for 3 months. At the end of the 3 months (influenz phase), four rabbits were killed, and surface area of the lesions in the aorta were measured. The remaining rabbits (\(n = 8\)) were divided into two groups, and placed on a normal chow diet and saline (\(n = 4\)), or normal chow and BSNXD (\(n = 4\)) for >3 months (regressive phase). The lesion areas were measured in these animals.

The drug-derived serum preparation. At 1 h after the last intragastric administration of BSNXD, the serum was acquired from auricular artery of rabbits, inactivated at 56°C for 30 min, and filtrated by 0.2 μm of filter and put into use with different concentrations, or followed by storage at –70°C until application.

Serum lipids, NO and E2 determination. Blood samples were acquired from rabbits’ ear marginal vein in the morning after an overnight fast. Serum lipids (including TC, TG, HDL-C and LDL-C) were measured once before beginning the hypercholesterolemic diet again after treatment for 12 weeks, through standard enzymatic techniques on an automated analyzer (Hitachi 911, Tokyo, Japan) by using Boehringer Mannheim reagent kits according to the manufacturer’s protocol. NO was determined as NO2/NO3 (including aortic arch) were excised, cut longitudinally, and fixed in 4% paraformaldehyde for 48 h. The aortas were then washed briefly in 70% alcohol and immersed in a solution of Sudan III (4% w/v in 70% alcohol) for 45 min. The images of the aorta inner surface segment were processed with an image analysis application.
were isolated, grown and identified as described previously.45 The passage 3 of Cell apoptosis analysis. U937 cells were treated with the different CM from HUVECs for 24 h chemotactic factor MCP-1 or adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and incubated with biotinylated secondary antibody for 30 min at room temperature antibody or rabbit polyclonal to ERα. The samples were then rinsed in PBS and incubated with biotinylated secondary antibody for 30 min at room temperature followed by peroxidase-conjugated streptavidin for 30 min, and developed with 3,3'-diaminobenzidine. Sections were counterstained with Mayer’s hematoxylin for 1 min and mounted. Negative controls were obtained by substituting the primary antibodies with isotype. The aortic expressions of VCAM-1, ERα, ERβ and ERβ/α were determined by quantitative immunohistochemistry by using ImagePro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA).44

Uterus and liver histomorphometry. After euthanasia, the uterus and liver were removed from the rabbits. The wet weights of the organs were measured. Macroscopic examination followed, and representative sections were taken. Samples were fixed in 4% formalin, embedded in paraffin. Routine histotechnique was performed on sections 4 μm in thickness, including staining with H&E.

HUVECs culture and the drug-derived serum treatment in vitro. Ox-LDL was prepared as described previously.22 The primary HUVECs were isolated, grown and identified as described previously.45 The passage 3 of the cells in EBM was for experiments. When cells were at 75% confluency, changed culture medium to phenol red-free EBM-2 medium supplemented with 10% charcoal/dextran-treated FBS, and maintained for 24 h. The starved cells in serum-free EBM for further 24 h, then exposed to control serum from the 10% charcoal/dextran-treated FBS, and maintained for 24 h. Finally, the cells were treated with ox-LDL for 24 h. The supernatant was collected as conditioned medium (CM) or for measurement of NO, MDA and MCP-1. NO and MDA production were determined by using commercially available kits. NO was detected as described above; MDA was measured at a wavelength of 532 nm by reacting with thiobarbituric acid (TBA) to form a stable chromophoric product. MCP-1 was quantified with a sandwich ELISA, and a curve calibrated from MCP-1 standards according to the manufacturer’s instruction.

The HUVECs were collected for the analysis of apoptosis, ERα/ERβ, chemotactic factor MCP-1 or adhesion molecules (ICAM-1, VCAM-1 and E-selectin) expression. U937 cells were treated with the different CM from HUVECs for 24 h prior to analyzing CCR2, LFA-1 and VLA-4 mRNA and protein expression.

Cell apoptosis analysis. For cell apoptosis assays, according to the manufacturer’s instructions, HUVECs were washed with PBS, stained with Annexin V-FITC and propidium iodide, and then analyzed by FACS Calibur (Becton Dickinson, Palo Alto, CA, USA). In every sample, 1 × 10^5 cells were counted.

Cell adhesion assay. U937 cells were labeled with the fluorescent dye 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Acros, Geel, Belgium) at a 10 μM final concentration in RPMI1640 complete medium containing 10% FBS at 37°C for 1 h. After HUVECs were stimulated and washed, the labeled U937 cells were added to each well, and allowed to interact for 1 h at 37°C. U937 cells bound to HUVECs were lysed with 50 mM Tris-HCl, pH 8.0, containing 0.1% SDS. The quantitative results were obtained by using a Fluoroscan ELISA plate reader at 485 nm excitation and 535 nm emission wavelengths. The detailed methodology was previously reported.22

RT-PCR. RT-PCR was carried out to evaluate HUVECs adhesion molecules (ICAM-1, VCAM-1, E-selectin) and U937 cells adhesion molecules (CCR2, LFA-1, VLA-4) mRNA expression. Total RNA was isolated from cells by using TRIzol reagent. The forward and reverse primers for these genes are as our previous reports.47 All the reactions were normalized using β-actin as control. PCR products were detected by electrophoresis on a 1.5% agarose gel with ethidium bromide. The annealing temperature was for ICAM-1 was 62°C, for VCAM-1 and E-selectin was 55°C, for CCR2, LFA-1, VLA-4 and β-actin was 57°C. The number of PCR cycles was 20–35 for each reaction.

Quantitative real-time PCR. Real-time PCR was carried out to evaluate ERα and ERβ mRNA in HUVECs by using the method described in the previous study.49–51 RNA samples were extracted by using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). ERα and ERβ expression were analyzed by using TaqMan probes (Hs00174860 and Hs0023095, respectively; Applied Biosystems, Warrington, UK) according to the manufacturer’s description. GAPDH was used as endogenous reference gene (Hs00266705). Standard curves for all analyzed genes were run on each plate, by using serial diluted cDNA to normalize the runs. The data obtained from GAPDH were used to normalize the sample variation in the amount of input cDNA.

Flow cytometry. HUVECs adhesion molecules (ICAM-1, VCAM-1, E-selectin) and U937 cells adhesion molecules (CCR2, LFA-1, VLA-4) protein expression were analyzed by flow cytometry. HUVECs were incubated with PE-conjugated monoclonal antibodies against human ICAM-1 and VCAM-1, and U937 cells were incubated with FITC-labeled monoclonal antibodies against human CCR2, CD11a and CD49d for 30 min at 4°C. The labeled U937 cells were added to each well, and allowed to interact for 1 h at 37°C. U937 cells bound to HUVECs were lysed with 50 mM Tris-HCl, pH 8.0, containing 0.1% SDS. The quantitative results were obtained by using a Fluoroscan ELISA plate reader at 485 nm excitation and 535 nm emission wavelengths. The detailed methodology was previously reported.22

Western blot. Western blot analysis was carried out to evaluate ERα, ERβ, eNOS, MCP-1, ICAM-1, VCAM-1 and E-selectin protein levels in HUVECs, also adhesion molecules (CCR2, LFA-1, VLA-4) protein expression in U937 cells. A volume of 155 μl of lysis buffer (0.1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS, 0.1% PMSF, in 100 ml of PBS containing protease inhibitors: 1 mg/ml leupeptin, 0.5 mg/ml pepstatin and 1 mg/ml bestatin) was added and maintained at 4°C for 30 min. Then, the cells were collected, boiled for 5 min and sonicated for 10 s. Protein content was measured by using Bradford protein assay. Equal amounts of proteins were then separated by 10% SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to a PVDF membrane by electrotransfer for 1.5 h. The membranes were blocked with 5% non-fat milk in TBS-T, and then incubated with anti-ERα (sc-8002; Santa Cruz Biotechnology), anti-ERβ (sc-8002; Santa Cruz Biotechnology), anti-eNOS (Cell Signaling Technology Inc., Beverly, MA, USA), anti-MCP-1 (Santa Cruz Biotechnology), anti-ICAM-1 (Cell Signaling Technology), anti-VCAM-1 (Santa Cruz Biotechnology), anti-E-selectin (Santa Cruz Biotechnology), mouse monoclonal antibodies for human CCR2 (Abcam), anti-CD11a antibody (LFA-1 (Abcam), Anti-Integrin α 4 antibody (VLA-4 (Abcam)) or anti-β-actin (Cell Signaling Technology) overnight at 4°C followed by secondary horseradish peroxidase-conjugated antibody (Rockland Co., Gilbertsville, PA, USA). The proteins of interest were identified by detection of HRP-labeled antibody complexes with
chemiluminescence with ECL Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK). The intensity of the band was scanned, and analyzed with (Alpha Innotech Corporation, San Leandro, CA, USA). Data were presented as a ratio of ERα, ERβ or ENOS versus β-actin, respectively.

Transfection and luciferase assay. Transient transfection with NF-κB-luciferase was constructed by using Lipofectamin (Gibco BRL, Gaithersburg, MD, USA); HUVECs cell cultures, luciferase transfection and control experiments, LPS stimulation, luciferase activity assay were done by using dual luciferase kit (Promega, Madison, WI, USA) and TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). HUVECs cell cultures, luciferase transfection and control experiments, LPS stimulation, luciferase activity assay were done by using dual luciferase kit (Promega, Madison, WI, USA) and TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) that have been extensively described. Briefly, cells were transfected with 2 μg of NF-κB-luciferase, allowed to recover for 24 h, and then 0.1 mM L-NMMA (NOS inhibitor), 10 μM MPP (ERα antagonist) or 10 nM RTHC (ERβ antagonist) was administered 1 h before stimulated with LPS (10 μg/ml) in the presence of the drug-derived serum or the control. The cells were harvested 8 h after treatment, and luciferase activity was determined.

Statistical analysis. All values are expressed as the mean ± S.E.M. Data were analyzed with aid of SPSS database. The difference between experimental groups of equal variance was analyzed by using Student’s t-test with P < 0.05 being considered significant.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements.

This work was supported by National Natural Science Foundation of China No. 30801052 (to L Wang), Shanghai Pujiang Program No. 11PJ1401000 (to L Wang), by National Key Research Program of China 2006CB844007, National and Shanghai Leading Academic Discipline Project 211XK22, and Program for Outstanding Young Academic Leader (to D-J Li).

1. Toussoulis D, Kampoli AM, Papageorghiou N, Androutakis E, Antoniades C, Toutouzas K et al. Pathophysiology of atherosclerosis: the role of inflammation. Curr Pharm Des 2011; 17: 4089–4110.

2. Libby P. Inflammation in atherosclerosis. Nature 2002; 420: 688–687.

3. Van der Heiden K, Cuhmann S, Luong A, E. Zaiakar M, Evans PC. Role of nuclear factor-kappaB in cardiovascular health and disease. Clin Sci (Lond) 2010; 118: 593–605.

4. Ignarro LJ, Napoli C. Novel features of nitric oxide, endothelial nitric oxide synthase, and atherosclerosis. Curr Atheroscler Rep 2004; 6: 281–287.

5. Ignarro LJ, Buga GM, Wei LH, Bauer PM, Wu G, del Soldato P. Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. Proc Natl Acad Sci USA 2001; 98: 4202–4208.

6. Dement L, Trinti Y. The role of lipid oxidation products and receptor activator of nuclear factor-kappaB signaling in atherosclerotic calcification. Circ Res 2011; 108: 1482–1493.

7. Mendelsohn ME. Protective effects of estrogen on the cardiovascular system. Am J Cardiol 2002; 89: 12E–17E; discussion 17E-18E.

8. Meyer MR, Haas E, Barton M. Gender differences of cardiovascular disease: new insights into endothelial function. Biochem Biophys Res Commun 2006; 345: 1075–1082.

9. Makis H, Matsui-Hirai H, Miyazaki-Akita A, Fukatsu A, Funami J, Ding QF et al. Anti-inflammatory roles of TCM in atherosclerosis. Curr Drug Discov Technol 2012; 9: 257–266.

10. Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. Proc Natl Acad Sci USA 2008; 105: 40–45.

11. Lee S, Lim H, Park H, Lee KS, Park JH, Jiang Y. Berberine inhibits rat vascular smooth muscle cell proliferation and migration in vitro and improves neointima formation after balloon injury in vivo. J Int Cardiol 2002; 15: 29–37.

12. Wang L, Hao Q, Wang YD, Wang WJ, Li QJ. Protective effects of dehydroepiandrosterone on atherosclerosis in ovxated rabbits via alleviating inflammatory injury in endothelial cells. Atherosclerosis 2011; 214: 47–57.

13. Hayaishi T, Matsui-Hirai H, Miyazaki-Akita A, Fukatsu A, Funami J, Ding QF et al. Anti-inflammatory roles of TCM in atherosclerosis. Curr Drug Discov Technol 2012; 9: 257–266.

14. Chow MS, Huang Y. Utilizing Chinese medicines to improve cancer therapy—fiction or reality? Curr Drug Discov Technol 2012; 7: 1.

15. Liu PY, Christian RC, Ruan M, Miller VM, Fitzpatrick LA. Correlating androgen and estrogen steroid activity expressed by using dual luciferase and PCR. Prostate in men without known coronary artery disease. J Clin Endocrinol Metab 2005; 90: 1041–1046.

16. Efferth T, Li PC, Konkimalla VS, Kaina B. From traditional Chinese medicine to rational cancer therapy. Trends Mol Med 2007; 13: 353–361.
43. Ducasse E, Chevalier J, Cosset JM, Creusy C, Eschwege F, Speziale F et al. Ionizing radiation to prevent arterial intimal hyperplasia at the edges of the stent: induces necrosis and fibrosis. J Surg Res 2006; 135: 331–336.

44. Liu XD, Chen HB, Tong Q, Li XY, Zhu MJ, Wu ZF et al. Molecular characterization of caveolin-1 in pigs infected with Haemophilus parasuis. J Immunol 2011; 186: 3031–3046.

45. Huang FY, Mei WL, Li YN, Tan GH, Dai HF, Guo JL et al. Tocoroside A inhibits tumor growth and angiogenesis: involvement of TGF-beta/Endoglin signaling. PLoS One 2012; 7: e50351.

46. Zhong W, Zou G, Gu J, Zhang J. L-arginine attenuates high glucose-accelerated senescence in human umbilical vein endothelial cells. Diabetes Res Clin Pract 2010; 89: 36–45.

47. Chen YJ, Lee MT, Yao HC, Hsiao PW, Ke FC, Hwang JJ. Crucial role of estrogen receptor-alpha interaction with transcription coregulators in follicle-stimulating hormone and transforming growth factor beta1 up-regulation of sterodogenesis in rat ovarian granulosa cells. Endocrinology 2008; 149: 4658–4668.

48. Somponpun S, Sladek CD. Role of estrogen receptor-beta in regulation of vasopressin and oxytocin release in vitro. Endocrinology 2002; 143: 2899–2904.

49. Yu X, Ling W, Mi M. Relationship of impairment induced by intracellular S-adenosylhomocysteine accumulation with DNA methylation in human umbilical vein endothelial cells treated with 3-deazadenosine. Int J Exp Pathol 2009; 90: 638–648.

50. Wang L, Wang YD, Wang WJ, Li DJ. Differential regulation of dehydroepiandrosterone and estrogen on bone and uterus in ovariectomized mice. Osteoporos Int 2009; 20: 79–92.

51. Toth B, Saadat G, Geller A, Schulz C, Schulze S, Friese K et al. Human umbilical vascular endothelial cells express estrogen receptor beta (ERbeta) and progesterone receptor A (PR-A), but not ERalpha and PR-B. Histochem Cell Biol 2008; 130: 399–405.

52. Sahara M, Sato M, Monia T, Hirata Y, Nagai R. Nicorandil attenuates monocrotaline-induced vascular endothelial damage and pulmonary arterial hypertension. PLoS One 2012; 7: e33367.