Effects of 17β-Estradiol and Phytoestrogen α-Zearalanol on Tissue Factor in Plasma of Ovariectomized Rats and HUVECs

Wen Wang, Guang-jin Zhu, and Shu-Yu Zu

Department of Pathophysiology
Institute of Basic Medical Sciences
Chinese Academy of Medical Sciences (CAMS) & School of Basic Medicine
Peking Union Medical College (PUMC)
Beijing 100005, China

Abstract

In the present study, the effects of 17β-estradiol (E_2) and phytoestrogen α-zearalanol (ZAL) on tissue factor (TF) in plasma of ovariectomized (OVX) rats and human umbilical vein endothelial cells (HUVECs) were investigated. OVX rats were injected intramuscularly twice a week with E_2 (1mg/kg) or ZAL (1mg/kg) for five weeks. The concentrations of plasma E_2 were measured by radioimmunoassay. The TF levels of plasma were assayed by enzyme-linked immunosorbent assay (ELISA). HUVECs were exposed to E_2 or ZAL for 48 h. TF protein and mRNA were assayed by ELISA and RT-PCR, respectively. Nuclear proteins of HUVECs opposed to E_2 or ZAL for 8 h were extracted to assay transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) by Western blot. Both E_2 and ZAL were found to decrease plasma TF levels of OVX rats significantly. E_2 or ZAL were also found down-regulate the expression of TF protein and mRNA in HUVECs. Moreover, both E_2 and ZAL might decrease AP-1 and NF-κB levels. These results suggest that both E_2 and ZAL have inhibitory effects on TF in plasma of OVX rats and HUVECs, which may then contribute to their protective effects on cardiovascular diseases.

Key Words: 17β-estradiol, α-zearalanol, tissue factor, ovariectomized rats, HUVECs

Introduction

It is well known that premenopausal women have a lower risk than men at the same age for suffering cardiovascular disease (22). Therefore, supplements of different patterns of estrogen (estrogeare replacement therapy, ERT) are very popular in postmenopausal women in order to prevent or decrease the incidence of cardiovascular disease (7, 20). However, some collections of clinical reports indicated that the long-term ERT might increase the incidences of breast and endometrial cancers by statistical studies (14, 19). Problems are remained for further trials. Dai *et al*. (4) have reported that α-zearalanol (ZAL), a natural phytoestrogen (structural formula is shown in Fig. 1) has estrogenic properties in the form of showing positive effects on the prevention of experimental atherosclerosis to a certain extent, as well as lessening some adverse effects on mammary and uterine glands, suggesting that α-zearalanol might selectively target certain estrogen-related tissue, or possess some pharmacological properties different from 17β-estradiol (E_2). Such insight is encouraging for further study.

Tissue factor (TF) is a transmembrane glycoprotein that serves as primary initiator of the coagulation cascade by activating factor IX and X (17, 18). TF has been found higher in the blood from patients with cardiovascular disease, sepsis, hematologic disorders, and coagulation disorders such as disseminated intravascular coagulation. The presence of circulating TF has been associated with

Corresponding author: Dr. Guang-jin Zhu, Department of Pathophysiology, Institute of Basic Medical Sciences, Chinese Academy of Medical Science (CAMS), 5 Dong Dan San Tiao, Beijing 100005, China. Phone: +86-10-65296476 or +86-10-65284023; Fax: +86-10-65296476; E-mail: zhugj@pumc.edu.cn
Received: September 5, 2003; Revised: December 30, 2003; Accepted: January 9, 2004.

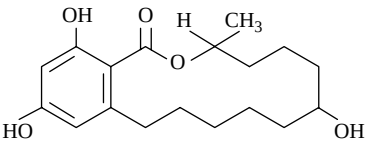

Fig. 1. Structural formula of α-zearalanol.

an increased blood thrombogenicity in these diseases (5). Within the vasculature, endothelial cells and monocytes can be activated to synthesize TF depending on the induction of transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (10,15). Endothelial cells in culture can rarely express TF in rest condition (18).

In the present study, we aim to further explore the effects of 17β-estradiol or α-zearalanol on TF in plasma of ovariectomized (OVX) rats and human umbilical vein endothelial cells (HUVECs). It might be a different avenue to peep the whole story of their effects on cardiovascular system.

Materials and Methods

Materials

The ZAL was kindly provided by Prof. Ji-Lun LI (Chinese University of Agriculture). The E_2 and angiotensin II (AngII) were products of Sigma (St. Louis, MO, USA). The IMUBIND TF ELISA kit was obtained from American Diagostica Inc. (Greenwich, CT, USA). The bicinchoninic acid (BCA) total protein assay kit was purchased from Pierce (Rockford, IL, USA). The estradiol radioimmunoassay assay (RIA) kit was obtained from Jiuding Medical Biology Inc. (Tianjin, China). The medium199 (M199), fetal bovine serume (FBS) and Trizol reagent were purchased from Gibco BRL (Grand Island, NY, USA). The endothelial cells growth factor (ECGF) was obtained from Roche (Indianapolis, IN, USA). The rabbit polyclonal antibody for c-Jun/AP-1 and NF-κB p65 were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals and Treatments

Healthy adult female Wistar rats (12 weeks old, weighing 250±10g) were purchased from the Animal Center of Capital University of Medical Sciences (Beijing, China). All animals were housed in a temperature-controlled (22±1°C) room with free access to standard rat chow and tap water and received humane care in accordance with the animal care provisions. Rats were divided into four groups (nine rats in each group): not ovariectomized (sham), ovariectomized (OVX), ovariectomized and injected intramuscularly with E_2 two weeks after ovariectomy (1mg/kg, twice a week for five weeks) (OVX+E_2), ovariectomized and injected intramuscularly with ZAL two weeks after ovariectomy (1mg/kg, twice a week for five weeks) (OVX+ZAL). All rats were alive during the whole experiment.

Plasma Sample Collection and Assay

After the treatment, rats were anesthetized with sodium pentobabitone. Arterial blood was drawn from the common carotid artery. The blood was immediately distributed into plastic tubes containing 3.8% sodium citrate (9:1 ratio), then centrifugated at 3000g, 4°C for 10 min. The plasma samples were frozen at -70°C until analysis. The plasma E_2 levels were measured by radioimmunoassay with double antibodies. At first, plasma samples were incubated with ^{125}I labled E_2 derivate and rabbit monoclonal antibody specific for E_2 at 37°C for 90 min, then add donkey anti-rabbit antibody and centrifugated at 3600g for 20 min. After throwing away the supernatants, radioactivity intensity(cpm) in the sediments were measured by γ-counter. The plasma TF levels were assayed by the IMUBIND TF ELISA kit. In brief, plasmas samples were incubated in microtiter plates conjugated with a mab aganist human TF, detecting TF-apoprotein, TF and TF/FVII complex. A biotinylated antibody detected specifically the bound TF. Conjugation with a horseradish-POD converted tetra-methylbenzidine with a specific change of absorption at 450 nm. Results are expressed as pg of TF protein per ml plasma.

HUVECs Culture

HUVECs were isolated from fresh human umbilical cords by means of the collagenase perfusion technique as described previously (11). The cell pellet was resuspended in a culture medium M199 supplemented with 20% FBS, 2%ECGF and 1% penicillin/streptomycin. After reaching confluence, the cells were then incubated with medium containing only 5% dextran-charcoal-stripped FBS for 24 h prior to the experiments.

HUVECs Experimental Procedure

To explore the effects of E_2 or ZAL on TF protein and mRNA expression in HUVECs, cells were incubated with E_2 or ZAL at different concentration (10^{-9} mol/l~10^{-7} mol/l) for 48 h in the absence or presence of AngII (10^{-6} mol/l). In additional experiments, HUVECs were exposed to E_2 or ZAL for 8 h, then cells nuclear proteins were extracted for measurement of transcription factors

AP-1 and NF-κB (Western blot).

HUVECs TF Protein Assay

After the treatment, cells were washing with PBS, then lysated by 200 μl TSA(20 mmol/l TRIS, 130 mmol/l NaCl, 0.1% BSA). Lysates were centrifugated for 1 min at 4°C, 12000 xg and supernatants without nuclei were used for detection of TF protein. Total cellular protein concentrations were quantified by BCA method using bicinchoninic acid. Results were expressed as pg of TF protein per mg total protein.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

After the treatment, total RNA was isolated from HUVECs using Trizol reagent. Preparation of complementary DNA and subsequent PCR were performed as described before (9). Sequences of intron spanning TF-specific primers were sense 5'-CTACTGTTTCAGTGTTCAAGCAGTGA-3' and antisense 5'-CAGTGCAATATAGCATTTGCAGTAGC-3', and for the housekeeping gene β-actin sense 5'-GTGGGGCGCCCCAGGCACCA-3', and antisense 5'-GTCCTTAATGTCACGCACGATTTC-3'. The amplification length of TF and β-actin mRNA were 282 bp and 510 bp, respectively. PCR profiles consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 sec, primer annealing at 55°C for 45 sec and primer extension at 72°C for 45 sec. The final primer extension was performed at 72°C for 7 min. Negative controls were performed routinely by running PCR without cDNA to exclude false positive amplification products.

Western Blot Analysis

Nuclear extracts were prepared as described previously (3). For Western blot analysis, 40 μg of nuclear extracts were separated on 12% SDS/PAGE and transferred to nitrocellulose membrane. Immunoblotting was done using rabbit polyclonal antibody specific for c-Jun/AP-1 and NF-κB p65 (1:500 dilution, 3 h incubation) and secondary peroxidase-conjugated goat anti-rabbit IgG (1:1500 dilution, 1h incubation). Immunoreactive proteins were quantified by BCA method.

Statistical Analysis

The statistical analyses were performed using SPSS 10.0 for Windows. Results were presented as mean±S.E.M. One-way ANOVA followed by Student-Newman-Keuls' multiple-range test was used to test the significance of difference among groups. $P < 0.05$ was considered as significant difference.

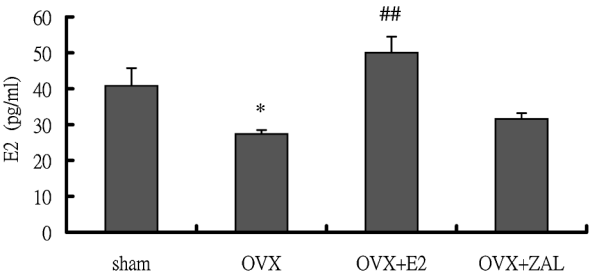

Fig. 2. Plasma E_2 levels of sham, OVX, OVX+E_2 (1mg/kg) and OVX+ZAL(1mg/kg) rats. Data are presented as mean±SEM from nine independent experiments. *$P < 0.05$ compared with sham; ##$P < 0.01$ compared with OVX.

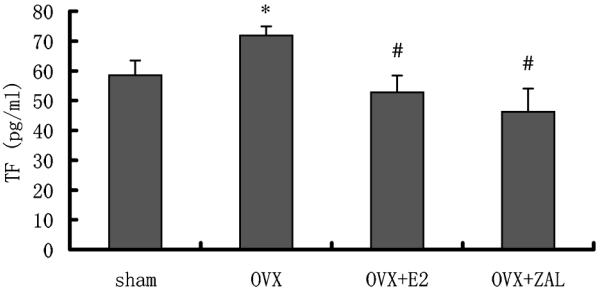

Fig. 3. Effects of E_2 (1mg/kg)or ZAL(1mg/kg) on Plasma TF levels in OVX rats. Data are presented as mean±SEM from nine independent experiments. *$P < 0.05$ compared with sham; #$P < 0.05$ compared with OVX.

Results

Changes of Plasma E_2 Levels

Seven weeks after ovariectomy, plasma E_2 levels in OVX rats decreased significantly ($P < 0.05$ compared with sham; Fig. 2). After treatment with E_2 for five weeks, plasma E_2 levels in OVX+E_2 rats increased significantly ($P < 0.01$ compared with OVX; Fig. 2). No significant difference in plasma E_2 levels between OVX+ZAL and OVX was observed ($P > 0.05$). These results implied that the ovariectomy model had been successfully established.

Effects of E_2 or ZAL on Plasma TF levels in OVX Rats

The plasma TF levels of OVX rats markedly increased after ovariectomy ($P < 0.05$ compared with sham; Fig. 3). TF levels of OVX+E_2 and OVX+ZAL were similar with sham, while they were significantly lower than that of OVX ($P < 0.05$ compared with OVX, Fig. 3).

Effects of E_2 or ZAL on TF Protein Expression in HUVECs

Table 1 illustrates the inhibitory effects of E_2 or ZAL on the expression of TF protein observed in

Table 1. Effects of E_2 or ZAL at different concentration on TF protein contents of HUVECs (pg/mg protein).

Treatment	TF (without AngII)	TF (with AngII)
control	89.66±3.99	419.47±25.27**
E_2 (10^{-9} mol/l)	88.82±5.47	375.99±22.78
E_2 (10^{-8} mol/l)	81.17±5.55	292.92±19.87#
E_2 (10^{-7} mol/l)	64.60±3.16**	187.16±17.22##
ZAL (10^{-9} mol/l)	82.74±5.18	346.13±23.94
ZAL (10^{-8} mol/l)	71.83±4.18*	268.92±18.23##
ZAL (10^{-7} mol/l)	57.08±5.04**	161.16±12.70##

Data are presented as mean±SEM from three independent experiments. *P < 0.05, **P < 0.01 compared with control (without AngII); #P < 0.05, ##P < 0.01 compared with control (with AngII).

HUVECs. HUVECs were either left unstimulated or stimulated with AngII (10^{-6} mol/l) in the absence or presence of varying concentrations of E_2 or ZAL. Treatment with either E_2 or ZAL for 48 h both led to a dose-dependent down-regulation of TF protein expression in HUVECs (P < 0.05, P < 0.01 compared with control; Table 1). AngII (10^{-6} mol/l) induced TF protein expression in HUVECs significantly (P < 0.01 compared with control; Table 1), whereas E_2 or ZAL might abrogate the TF response to AngII (P < 0.05, P < 0.01 compared with AngII; Table 1).

Effects of E_2 or ZAL on TF mRNA Expression in HUVECs

To explore whether E_2 or ZAL might be a regulatory mediator for the transcriptional activation of the TF gene, RT-PCR was performed with primers specific for the TF and β-actin gene. Consistent with the results obtained on TF protein expression, both E_2 and ZAL (10^{-7} mol/l) reduced TF mRNA expression in HUVECs (with or without AngII stimulation; Fig. 4)

Effects of E_2 or ZAL on AP-1 and NF-κB Levels of Nuclear Extracts in HUVECs

To determine whether E_2 or ZAL down-regulate TF gene transcription by reducing transcription factors AP-1 and NF-κB, c-Jun/AP-1 and NF-κB p65 levels of nuclear extracts in HUVECs were analyzed by Western blot. Treatment of HUVECs with E_2 or ZAL (10^{-7} mol/l) for 8 h resulted in reduction of both c-Jun/AP-1 and NF-κB p65 levels of nuclear extracts. Cells were also stimulated with AngII as a positive control (Fig. 5).

Discussion

Cardiovascular disease is the major cause of

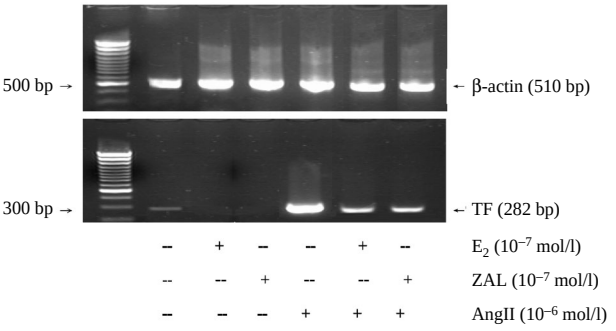

Fig. 4. Effects of inhibition of E_2 or ZAL(10^{-7} mol/l) on TF mRNA expression in HUVECs. A represntative photograph of PCR-amplified products of TF and β-actin mRNA is given. Similar results were obtained in three independent experiments. +: present ; --: absent

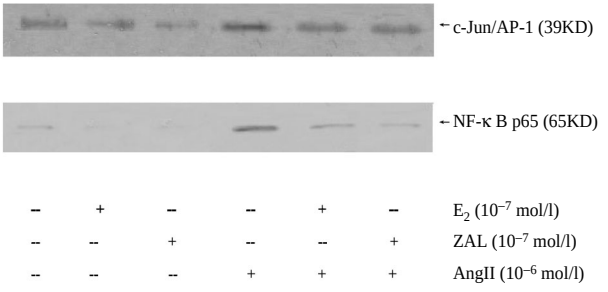

Fig. 5. Effects of E_2 or ZAL on AP-1 and NF-κB levels of nuclear protein extracts in HUVECs. A representative photograph of Western blot products of c-Jun/AP-1 and NF-κB p65 is given. Similar results were obtained in three independent experiments. +: present ; --: absent

mortality in postmenopausal women, half of which is due to coronary heart disease (CHD). A protective role of estrogen is suggested by the low risk of CHD in premenopausal women. The cardiovascular protective role of estrogen replacement therapy (ERT) is suggested by epidemiological studies. Estrogen has beneficial effects on lipid profile, vascular function, oxidative status, endothelial-dependent vasodilation and intimal hyperplasia. However, the effects of estrogen on coagulation are less clear and are controversial. ERT is associated with beneficial reductions in several coagulation factors such as fibrinogen and factor VII (2), whereas there are also reports that ERT users could have increased risk of thrombosis(16). Indeed, ERT may decrease or increase thrombosis risk depending on the coexistence of other risk factors, such as obesity, diabetes, advanced age, inherited traits and combined use of progestin (12, 23). The effects of estrogen on this phenomenon remain to be determined. Meanwhile, long-time ERT might raise the risk of other diseases such as breast and endometrial cancers. Of course great interest is growing about phytoestrogen such as ZAL, which

binds to the estrogen receptor and functions as antiestrogen in breast and uterine tissue but as an estrogen agonist in prevention of experimental atherosclerosis.

In the present study we analyzed the effects of E_2 or ZAL on TF both in plasma of OVX rats and cultured HUVECs. We found that ovariectomy could increase the plasma level of TF significantly, while either E_2 or ZAL might resume such change. Our recent study demonstrated that the prothrombin time (PT) of the OVX+E_2 and OVX+ZAL rats were significantly longer than that of the OVX rats, while plasma fibrinogen contents were lower than that of the latter (data not shown), implying that treatment with E_2 or ZAL might decrease coagulation activity of OVX rats, which was consistent with the change of plasma TF levels. We also investigated the effects of E_2 or ZAL on TF protein and mRNA expression in cultured HUVECs *in vitro*. Exposure to either E_2 or ZAL for 48 h both led to a dose-dependent down-regulation of TF protein in HUVECs. We further studied the HUVECs' potential to generate TF in reponse to AngII, an effective, natural inducing activator for TF in HUVECs. Previous study in our lab proved that AngII could induce TF expression in HUVECs significantly (6). In this study, either E_2 or ZAL might abrogate the TF response to AngII. The effects of E_2 or ZAL on TF mRNA were consistent with that of the TF protein. Our results are similar to those of the previous studies of Koh *et al*. (13) and Aune *et al*. (1), whereas in contrary to that of the studies of Holschermann *et al*. (8).

It is well known that TF gene expression in HUVECs is mediated by activation of transcription factors, including AP-1 (c-Fos/c-Jun) and NF-κB (c-Rel/p65). Molecular cloning of TF has provided evidence for AP-1- and NF-κB-binding domains in the promoter region of this gene (15). Lots of agonists for TF in HUVECs, such as LPS, AngII and TNFα, could activate these two factors (15). The inhibitory effects of E_2 on LPS-induced AP-1- and NF-κB-DNA binding activity have been reported by Simoncini *et al*. (21). However, the regulatory effects of ZAL on AP-1 and NF-κB have not been reported. In the present study, Western blot showed that E_2 or ZAL not only decreased c-Jun/AP-1 and NF-κB p65 levels of nuclear extracts in HUVECs but also inhibited AngII-induced activation of them. E_2 and ZAL had similar inhibitory effects. These results indicate that E_2 or ZAL might decrease TF gene expression in HUVECs through the inhibition of AP-1 and NF-κB, although the precise mechanism still awaits study.

In summary, our present study demonstrates that either E_2 or ZAL might decrease TF levels in plasma of OVX rats in vivo and HUVECs *in vitro*. The inhibitory effects of E_2 or ZAL on TF gene expression in HUVECs might result from the inhibition of transcription factors AP-1 and NF-κB. This might then contribute to their protective effects on cardiovascular diseases.

Acknowledgments

The authors are grateful to Prof. J.L. Li for providing purified α-zearalanol and to Prof. S.L. Dai for technical assistance. The study was supported in part by National Natural Science Foundation (No. 39730220) of China.

References

1. Aune, B., Oian, P., Omsjo, I. and Osterud, B. Hormone replacement therapy reduces the reactivity of monocytes and platelets in whole blood-A beneficial effect on atherogenesis and thrombus formation? *Am. J. Obstet. Gynecol.* 173: 1816-1820, 1995.
2. Chae, C.U., Ridker, P.M. and Manson, J.E. Postmenopausal hormone replacement therapy and cardiovascular disease. *Thromb. Haemost.*78: 770-780, 1997.
3. Chiu, J.J., Lee, P.L., Lee, C.I.,Chen, L.J., Chen, C.N., Ko, Y.C. and Lien, S.C. Shear stress attenuates tumor necrosis factor-α-induced monocyte chemotactic protein-1 expressions in endothelial cells. *Chinese J. Physiol*. 45: 169-176, 2002.
4. Dai, S.L., Duan, J., Lu, Y., Cheng, J., Ren, J., Zhang, Y. Zhao, X., Zhang, L.,Wu, Y. and Ge, Q.A new phytoestrogen α-zearalanol markedly inhibits progression of atherogensis in overiectomized cholesterol-fed rabbits(Abstract A01). The 24th Annual scientific sessions, North American section of the international society for heart research, Madison, USA, 2002, p. 3.
5. Falciani, M., Gori, A.M., Fedi, S., Chiarugi, L., Simonetti, I., Dabizzi, R.P., Prisco, D., Pepe, G., Abbate, R., Gensini, G.F. and Neri Serneri, G.G. Elevated tissue factor and tissue factor pathway inhibitor circulating levels in ischaemic heart disease patients. *Thromb. Haemost.* 79: 495-499, 1998.
6. Gao, Y.M. and Zhu, G.J. Effects of tumor necrosis factor α and angiotensin II on expression of tissue factor in human vascular endothelial cells. *Basic Med. Clin.* 20: 126-129, 2000.
7. Grimes, D.A. and Lobo, R.A. Perspectives on the women's health initiative trial of hormone replacement therapy. *Obstet. Gynecol.* 100: 1344-1353, 2002.
8. Holschermann, H.,Terhalle, H.M., Zakel, U., Maus, U., Parviz, B., Tillmanns, H. and Haberbosch, W. Monocyte tissue factor expression is enhanced in women who smoke and use oral contraceptives. *Thromb. Haemost*. 82: 1614-1620, 1999.
9. Iochmann, S., Reverdian-Moalic, P., Beaujean, S., Rideau, E., Lebranchu, Y., Bardos, P. and Gruel, Y. Fast dection of tissue factor and tissue factor pathway inhibitor messenger RNA in endothelial cells and monocytes by sensitive reverse transcription-polymerase chain reaction. *Thromb. Res.* 94: 165-173, 1999.
10. Ishii, H., Takada, K., Higuchi, T. and Sugiyama, J. Verotoxin-1 induces tissue factor expression in human umbilical vein endothelial cells through activation of NF-kappaB/Rel and AP-1. *Thromb. Haemost*. 84: 712-721, 2000.
11. Jaffe, E.A., Nachman, R.L., Becker, C.G.and Minick, C.R.Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52: 2745-2756, 1973.
12. Koh, K.K. Effects of hormone replacement therapy on coagulation and fibrinolysis in postmenopausal women. *Int. J. Hematol*.76 Suppl 2: 44-46, 2002.
13. Koh, K.K., Kang, M.H., Jin, D.K., Lee, S.K., Ahn, J.Y., Hwang,

H.Y., Yang, S.H., Kim, D.S., Ahn, T.H. and Shin, E.K. Vascular effects of estrogen in type II diabetic postmenopausal women. *J. Am. Coll. Cardiol*. 38: 1409-1415, 2001.
14. Lobo, R.A. Benefits and risks of estrogen replacement therapy. *Am. J. Obstet. Gynecol*. 173: 982-990, 1995.
15. Mackman, N. Regulation of the tissue factor gene. *Thromb. Haemost.* 78: 747-754, 1997.
16. Mammen, E.F. Oral contraceptive pills and hormonal replacement therapy and thromboembolic disease. *Hematol. Oncol. Clin. North Am*. 14: 1045-1059, 2000.
17. Martin, D.M.,Wiiger, M.T. and Prydz, H. Tissue factor and biotechnology. *Thromb. Res*. 90: 1-25, 1998.
18. Rauch, U. and Nemerson, Y. Circulating tissue factor and thrombosis. *Curr. Opin. Hematol*. 7: 273-277, 2000.
19. Reid, R.L. Progestins in hormone replacement therapy:Impact on endometrial and breast cancer. *J. SOGO*. 22: 677-681, 2000.
20. Rosano, G.M., Sarrel, P.M., Poole-Wilson, P.A. and Collins, P. Beneficial effect of oestrogen on exercise-induced myocardial ischaemia in women with coronary artery disease. *Lancet* 342: 133-136, 1993.
21. Simoncini, T., Maffei, S., Basta, G., Barsacchi, G., Genazzani, A.R., Liao, J.K., and Caterina, R. Estrogens and glucocorticoids inhibit endothelial vascular cell adhesion molecule-1 expression by different transcriptional mechanisms. *Circ. Res.* 87: 19-25, 2000.
22. Stampfer, M.J. and Coldity, G.A. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Prevent. Med*. 20: 47-63, 1991.
23. Young R.L., Goepfert, A.R. and Goldzieher, H.W. Estrogen replacement therapy is not conducive of venous thromboembolism. *Maturitas* 13: 189-192, 1991.