JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Nicotine and vascular endothelial dysfunction in female ovariectomized rats: role of estrogen replacement therapy

Mohamed M. El-Seweidy, Hoda E. Mohamed, Mervat E. Asker and Hebatallah H. Atteia

Department of Biochemistry, Faculty of Pharmacy, University of Zagazig, Zagazig, Egypt

Keywords
atherogenic index; eNOS; ERT; nicotine; ovariectomy; VED

Correspondence
Hebatallah H. Atteia, Department of Biochemistry, Faculty of Pharmacy, University of Zagazig, Zagazig 44519, Egypt.
E-mail: hebahosany@yahoo.com

Received May 3, 2011
Accepted September 22, 2011

doi: 10.1111/j.2042-7158.2011.01377.x

Abstract

Objectives The protective effects of estrogen replacement therapy (ERT) against oxidative injury and endothelial dysfunction in the aortic tissues induced with nicotine in ovariectomized (OVX) rats were investigated.

Methods Female rats were divided into a sham-operated group ($n = 8$) and four groups in which OVX rats received either vehicle (0.1 ml sesame oil, i.m., $n = 8$), or nicotine (0.1 mg/kg, s.c., $n = 8$), or estradiol benzoate (0.1 mg/kg, i.m., $n = 8$), or both nicotine and estradiol benzoate ($n = 8$) starting at week 5 after the surgery and continuing for the following 6 weeks.

Key findings ERT was effective in preventing the rise in plasma lipid profile, atherogenic index and the level of induced endothelin-1 (ET-1) in nicotine-treated OVX rats. It also reduced aortic malondialdehyde, hydroxyproline levels, calcium content and caspase-3 expression induced in nicotine-treated OVX rats. ERT increased serum estradiol, high-density lipoprotein cholesterol and nitric oxide levels in nicotine-treated OVX rats. Furthermore, ERT was effective in restoring reduced glutathione and cyclic guanosine monophosphate contents and endothelial nitric oxide synthase expression in aortic tissues of nicotine-treated OVX rats.

Conclusions Short-term ERT could be a promising therapeutic strategy to minimize nicotine-induced oxidative stress and vascular endothelial dysfunction in menopausal women subjected to environmental smoke.

Introduction

Vascular endothelium is a thin monolayer of specialized epithelial cells comprised of simple squamous cells that covers the inner surface of the entire vasculature. Nitric oxide (NO) is a major mediator released from the endothelium, and it regulates a wide spectrum of cardiovascular functions such as vasodilation, inhibition of platelet adhesion and aggregation, prevention of smooth muscle cell proliferation and migration and vascular inflammation. NO is generated in the endothelium upon the conversion of L-arginine to L-citrulline by endothelial NO synthase (eNOS).[1] The partial loss of balance between endothelium-mediated vasodilation and vasoconstriction is described as vascular endothelial dysfunction (VED), which occurs mainly due to reduction in synthesis and release of NO, inactivation of eNOS, excessive generation of reactive oxygen species (ROS), eNOS uncoupling, reduction in endogenous antioxidant defence mechanism and upregulation of asymmetric dimethyl arginine (ADMA), an endogenous inhibitor of eNOS.[2] VED is an insidious condition as it is strongly associated with the pathogenesis of various cardiovascular disorders such as atherosclerosis, hypertension, heart failure and diabetic nephropathy. VED and atherogenesis also involves a general alteration of the unicellular layer of the vascular wall structure.[3]

Nicotine exposure via tobacco chewing or cigarette smoking is considered to be one of major risk factors involved in the induction and progression of cardiovascular disorders. The acute effects are observed immediately after smoking and are caused mainly by nicotine, while the chronic changes are correlated with a series of complex haemorheological modifications, as well as alterations of the vascular wall.[4] Accumulating evidence suggests that exposure to nicotine plays a key role in inducing VED among non-smokers. The mechanism of nicotine-induced endothelial dysfunction involves decrease in generation and bioavailability of NO. Furthermore, nicotine decreases

endothelium-dependent vasodilation and stimulates the adhesion of leukocytes to the endothelium.[5] Women who are exposed to secondhand smoke (SHS), may be at increased risk of earlier age at menopause.[6] SHS can achieve plasma nicotine levels equivalent to that produced by tobacco smoking and that are associated with nicotine-induced changes in behaviour. Tobacco smoke is a toxic air contaminant and a known human carcinogen responsible for up to 3000 annual lung cancer deaths among nonsmokers in the USA.[7] Cigarette smoke is a complex mixture of chemicals containing more than 4000 different constituents. Two major phases have been identified in cigarette smoke: a tar phase and a gas phase; both phases are rich in oxygen-centered, carbon-centered and nitrogen-centered free radicals as well as non-radical oxidants. From the analysis of each phase, it was estimated that a single cigarette puff contains approximately 1014 free radicals in the tar phase and 1015 radicals in the gas phase. These include various compounds that are capable of causing an increase in the generation of various ROS like superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH.) and peroxyl (ROO.) radicals. These ROS in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation, adding substantially to the oxidant effects of nicotine.[8] Since passive smoking, or SHS, is a causative factor in cardiovascular disease, appropriate assessment of passive smoking exposure in a variety of settings, as well as recommendations to avoid such exposure, is warranted. Policy-based public health initiatives to eliminate passive smoking in the workplace and other public areas are needed to decrease the risk of mortality and morbidity associated with earlier age at menopause.[9]

Aging is widely recognized as a risk factor for vascular disease and involves structural and functional alterations in vasculature. Vascular aging is associated with endothelial dysfunction, arterial stiffening and remodelling, impaired angiogenesis, defective vascular repair, and an increasing prevalence of atherosclerosis.[10] Premenopausal women have a lower risk and incidence of cardiovascular disease compared with age-matched men. In women, besides aging, a decline of sex hormone production accompanying the menopause has been associated with increased risk of cardiovascular disease. Estrogen has been largely described as a cardioprotective agent contributing to normal endothelial function in both experimental and observational studies.[11] However, the issue of hormone replacement therapy (HRT) remains highly controversial since some clinical trials failed to confirm that HRT affords cardioprotection. Two randomized prospective primary or secondary prevention trials showed that HRT (estrogen/progestin) may actually increase the risk and events of cardiovascular disease in postmenopausal women.[12] The controversy over the risks and benefits of HRT in primary prevention of cardiovascular disease continues, and much of the debate has focused on the age of postmenopausal women enrolled in these trials, when HRT is initiated and duration of the replacement.[13–15] Recent Women's Health Initiative (WHI) studies restricted to younger postmenopausal women illustrated that initiation of HRT closer to menopause reduced the risk of cardiovascular disease.[13] Furthermore, a later published study that was restricted to postmenopausal women aged 50–59 years, showed that HRT initiated in these younger women reduced coronary artery calcification and the prevalence of subclinical coronary heart disease.[16] More recently, a cohort study with long-term follow-up showed that women who underwent bilateral ovariectomy before age 45 had increased cardiovascular mortality and that this risk was significantly lowered by treatment with estrogen up until age 45 or longer.[17] Moreover, duration of estrogen treatment merits consideration when determining the outcome of HRT. Re-analysis of the WHI data-set shows that younger postmenopausal women given relatively short-term HRT (<10 years) tended to have a reduced risk and incidence of cardiovascular disease, but that this protection gradually disappeared in succeeding years.[13] It is reported that short-term HRT (2–3 years) reduced cardiovascular disease mortality by 30%, associated with significantly reduced severity of atherosclerotic lesions.[14] Klaiber *et al*. studied the effect of the length of menopause on serum estradiol level in women undergoing HRT; the levels were found to be higher after a long menopause and duration of HRT (average 78 months) than after a short menopause and duration of HRT (average 12.9 months).[15] It is known that some of the adverse effects of estrogen, such as increased breast and endometrial cancer and venous thrombosis, correlated positively with plasma estradiol levels. Taken together, these studies support the hypothesis that estrogen therapy might only be considered a short-term strategy having a cardiovascular benefit in younger postmenopausal women when initiated early after the onset of menopause. Cardiovascular protection by estrogen has been mostly associated with an increase of endothelium-derived vasodilator factors, including NO and prostacyclin.[18] Vasoconstrictor prostanoids, such as thromboxane A2 (TXA2), have also been implicated in the pathophysiology of vascular dysfunction during estrogen withdrawal and aging.[19] Previous studies suggest that the loss of endogenous estrogens leads to reduced bioavailability of endothelium-derived NO and impaired endothelial function.[20] The increased NO production due to estrogen replacement therapy (ERT) might explain why it has beneficial effects on endothelial function. Therefore, this study was undertaken to investigate the effect of ERT on ovariectomy-nicotine-induced VED in female rats exposed to nicotine at a lower dose level, similar to that received by passive smokers.

Materials and Methods

Drugs and reagents

Estradiol benzoate was purchased from Misr Co. For Pharmceuticals Industries S.A.E. (Cairo, Egypt). Nicotine hydrogen bitartrate was obtained from Sigma (St Louis, MO, USA). The RIA kits for the 17β-estradiol and the cyclic guanosine monophosphate (cGMP) assays were purchased from Immunotech Company (Marseille, France) and IBL Com. (Hamburg, Germany), respectively. Endothelin-1 (ET-1) ELISA kit was obtained from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). The total cholesterol (TC), triacylglycerol (TAG) and high-density lipoprotein cholesterol (HDL-C) assay kits were purchased from Spinreact, Co. (Sant Esteve de Bas, Spain). Other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

Animals

The experiments were performed with female Wistar albino rats, 180–230 g (211 $\pm$ 20.67 g) body weight, at 4 months of age bred in the Egyptian Organization for Biological products and Vaccines (Cairo, Egypt). Rats were housed under climate-controlled conditions with a 12-h light–dark cycle and provided with free access to standard food and water. An acclimatization period of at least 1 week was allowed before initiating the experimental protocol. All procedures were in accordance with the recommendations of the ethical procedures approved by the Animal Care and Use Committee of the Biochemistry Department, Faculty of Pharmacy, Zagazig University.

Ovariectomy procedure

Ovariectomy was performed in the diestrus 2 stage of the estrous cycle, using sterile procedures. The rats were anaesthetized with (sodium thiopental, 50 mg/kg i.p.), and the ovaries were accessed aseptically through a small laparotomy and removed after the pedicle was ligated. The incision was sutured and the rats were kept warm until they recovered from anaesthesia. Rats were left for 4 weeks to simulate menopause.

Experimental protocol

Four weeks after the ovariectomy, the rats were divided into five age-matched groups ($n = 8$). Sham-operated group: rats were subjected to a surgical procedure without performing an ovariectomy and were treated with 0.1 ml vehicle (pure sesame oil, s.c.) (control). Ovariectomized (OVX) group: rats were subjected to ovariectomy and were treated with 0.1 ml vehicle (s.c.). OVX+Nic group: rats were subjected to ovariectomy in the diestrus 2 stage of the estrous cycle and treated with nicotine hydrogen bitartrate (0.1 mg/kg per day, s.c.) for 6 weeks (this low dose of chronic nicotine in a rat model is similar to those found in people exposed to environmental tobacco smoke 'passive smokers').[21] OVX+ERT group: rats were subjected to ovariectomy and received estradiol benzoate treatment at a physiological dose (0.1 mg/kg body weight every 2 days i.m.) throughout the treatment period.[22] OVX+Nic+ERT group: rats were subjected to ovariectomy and treated with both nicotine and estradiol benzoate using the aforementioned doses. All groups received treatment at the same time until termination of experiment. The mean plasma level of nicotine in the nicotine-treated rats was 20 ng/ml.

At the end of the 6 week treatment period, rats were fasted overnight. On the following morning, rats were anaesthetized with urethane (1.3 mg/kg) and blood samples were obtained from the orbital plexus into heparinized tubes. Blood samples were centrifuged (2683*g* for 5 min) and plasma was aspirated and stored at −50°C until analyzed. The chest was then opened by a midline incision and the thoracic aorta was immediately isolated, frozen in liquid N_2 and used for biochemical determinations.

Biochemical measurements

Plasma estradiol was determined using a solid-phase ^{125}I-radioimmunoassay technique according to the manufacturer's instructions.[23] The analytical sensitivity was <6 pg/ml. The plasma level of NO was measured indirectly as nitrite using Griess reagent.[24] Plasma ET-1 was assayed with ELISA kit. The assay procedure was carried out as previously described.[25] The levels of TC, TAG and HDL-C were estimated in plasma colorimetrically using commercial kits.[26–28] Low-density lipoprotein cholesterol (LDL-C) levels were calculated as previously described.[29] The atherogenic index was calculated using the formula: atherogenic index = LDL-C/HDL-C.[30]

Thoracic aorta reduced glutathione (GSH) was assessed spectrophotometrically using Ellman's reagent, according to the modified method of Ahmed *et al.*[31] Lipid peroxidation content in thoracic aorta was determined by thiobarbituric acid reaction with lipid peroxide end-product malondialdehyde (MDA). The concentration of MDA was estimated as thiobarbituric acid-reactive substances (TBARS) by spectrophotometric assay.[32] Aortic calcium content was evaluated by atomic absorption flame emission spectrophotometer (UNICAM 969; Unicam, Cambridge, UK) at 422.7 nm.[33] Collagen content in thoracic aorta can be estimated spectrophotometrically as hydroxyproline following acid hydrolysis of collagen using Ehrlich reagent. The colour formed can be detected at 550 nm using ELISA plate reader.[34] The cyclic guanosine monophosphate (cGMP level) in the aortic tissue extracts was determined using a radioimmunoassay kit according to the manufacturer's instructions.[35]

RNA expression analysis

Aortic caspase-3 and eNOS gene expression were detected by reverse transcription-polymerase chain reaction (RT-PCR). β-Actin expression was used as a loading control for each sample. About 30 mg of each aorta tissue was homogenized in RNA lysis buffer, which contains mercaptoethanol, and then centrifuged at 15 339*g* for 10 min. The supernatant was frozen at –80°C until examined for gene expression of caspase-3 and eNOS by RT-PCR. RNA was extracted from aorta homogenate using SV total RNA isolation system kit (Promega, Madison, WI, USA) according to manufacturer's recommendation and the extracted RNA concentration and purity were measured by determination absorbance using a UV spectrophotometer, at 260 nm and 280 nm. cDNA was prepared from RNA as follows: about 20 µg of mRNA was heated at 70°C for 5 min with 50 pmol of reverse primer of selected gene (caspase-3, eNOS) before adding 5× RT buffer (50 mM Tris-Cl, pH 8.3. 10 mM dNTPS and 200 units of murine leukaemia virus reverse transcriptase in a final volume up 36 µl). RT reaction was carried for 2 h at 37°C. For the PCR, 5 µl of cDNA was subjected to PCR under the conditions specified below (Table 1); PCR reaction was performed by adding 50 pmol of each of forward and reverse primer specific to each gene as detailed later. 10 mM dNTPS, 2–5 unit TACL, PCR 10× buffer (containing 100 mM Tris-Cl, HCl pH 8.3, KCl 10 mM to final volume 50 µl). The amplified PCR products of selected genes were electrophoresed on 1.5% agarose gels and were UV visualized after staining with ethidium bromide. UV-illuminated gels were photographed. A densitometry system using a standard DNA of known concentration was used for analysis (Syngene, Cambridge, UK).[36] The relative expression level of each gene (R) was calculated according to the following formula: R = Densitometric units of gene/densitometric units of β-actin.

Data analysis

The results are expressed as mean ± standard deviation (SD) for eight animals in each group. Differences between groups were assessed by one-way analysis of variance. Tukey–Kramer test was performed for inter-group comparisons using InStat 2.04 statistical package (GraphPad InStat). *P*-values <0.001, < 0.01 and < 0.05 were considered statistically significant.

Results

Effects of nicotine administration on body weight and plasma biochemical parameters in female OVX rats

OVX induced significant increases in body weight ($P < 0.01$) when compared with control, whereas nicotine administration (OVX+Nic) had a non-significant effect on body weight (Table 2). There was significant decrease in plasma estradiol level in the OVX group ($P < 0.001$) when compared with the control value. Nicotine administration (OVX+Nic) produced significant reduction in estradiol when compared with OVX group ($P < 0.001$). Plasma NO, a key component in endothelium-dependent regulation of vascular tone, was sig-

Table 1 The oligonucleotide primers sequence

Gene	Primer sequence	Annealing temperature	Product size
Caspase-3	Forward primer: 5′-GCTAGGCAAAGCCGTTTATG-3′ Reverse primer: 5′-AATTGCCTCCTTGTTTTGGT-3′	55°C	142 bp
eNOS	Forward primer: 5′-GATCAATAACCTGAAGCCCG-3′ Reverse primer: 5′-GCCCTTTTTTGCTCCATAAGG-3′	60°C	213 bp
β-actin	Forward primer: 5′-TGTTGTCCCTGTATGCCTCT-3′ Reverse primer: 5′-TAATGTCACGCACGATTTCC-3′	57°C	253 bp

Table 2 Effect of nicotine administration and estrogen replacement therapy on body weight of ovariectomized rats

Parameter	Control	OVX	OVX+Nic	OVX+ERT	OVX+Nic+ERT
Body weight (g)	211 ± 20.67	251 ± 22.84#	246 ± 22.16	219 ± 20.59*	217 ± 19.86[a]

ERT, estrogen replacement therapy; Nic, nicotine; OVX, bilateral ovariectomized. Nic injection (0.1 mg/kg, s.c.). ERT with estradiol benzoate (0.1 mg/kg, i.m.). The values are means ± SD ($n = 8$). Analysis of variance followed Tukey's post-hoc multiple comparison test, #$P < 0.01$ vs sham control, *$P < 0.05$ vs OVX, [a]$P < 0.05$ vs OVX+Nic.

Table 3 Effect of nicotine administration and estrogen replacement therapy on plasma estradiol, nitric oxide and endothelin-1 levels in ovariectomized rats

Parameter	Control	OVX	OVX+Nic	OVX+ERT	OVX+Nic+ERT
Estradiol (pg/ml)	135 ± 19.58	15.6 ± 1.87#	8.2 ± 0.86*	148.2 ± 22.23*	69.7 ± 9.9[a]
NO (μmol/L)	46.43 ± 7.43	23.3 ± 3.5#	10 ± 1.4*	42.89 ± 6.95*	18 ± 2.12[a]
ET-1 (pg/ml)	8.1 ± 1.05	17.3 ± 2.42#	38.8 ± 5.51*	10.7 ± 1.44*	27.4 ± 3.34[a]

ERT, estrogen replacement therapy; ET-1, endothelin-1; Nic, nicotine; NO, nitric oxide; OVX, bilateral ovariectomized. Nic injection (0.1 mg/kg, s.c.). ERT with estradiol benzoate (0.1 mg/kg, i.m.). The values are means ± SD ($n = 8$). Analysis of variance followed Tukey's post-hoc multiple comparison test, #$P < 0.001$ vs sham control, *$P < 0.001$ vs OVX, [a]$P < 0.001$ vs OVX+Nic.

Table 4 Effect of nicotine administration and estrogen replacement therapy on plasma lipid profile and atherogenic index in ovariectomized rats

Parameter	Control	OVX	OVX+Nic	OVX+ERT	OVX+Nic+ERT
TC (mg/dl)	78.1 ± 10.93	106.67 ± 16#	155.56 ± 22.08*	71.11 ± 11.73*	124.44 ± 16.17[a]
TAG (mg/dl)	56.7 ± 7.73	76.73 ± 9.79#	99.9 ± 11.98*	57.9 ± 6.94*	85.5 ± 10[a]
HDL-C (mg/dl)	28.12 ± 3.09	22.49 ± 2.36#	16.86 ± 1.71*	31.24 ± 3.56*	21.92 ± 2.27[a]
LDL-C (mg/dl)	38.64 ± 5.8	68.83 ± 8.94#	118.72 ± 17.21*	28.29 ± 3.82*	84.98 ± 10.62[a]
Atherogenic index	1.75 ± 0.19	3.06 ± 0.4#	7.04 ± 0.85*	0.91 ± 0.1*	3.88 ± 0.4[a]

ERT, estrogen replacement therapy; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Nic, nicotine; OVX, bilateral ovariectomized; TAG, triacyglycerol; TC, total cholesterol. Nic injection (0.1 mg/kg, s.c.). ERT with estradiol benzoate (0.1 mg/kg, i.m.). The values are means ± SD ($n = 8$). Analysis of variance followed Tukey's post-hoc multiple comparison test, #$P < 0.01$ vs sham control, *$P < 0.001$ vs OVX, [a]$P < 0.05$ vs OVX+Nic.

nificantly reduced in OVX group compared with control ($P < 0.001$). Injection of nicotine (OVX+Nic) induced an even greater reduction of NO than observed in the OVX group ($P < 0.001$). However, plasma ET-1, a vasoconstrictor and pro-inflammatory peptide in endothelium, was significantly increased after ovariectomy compared with control rats ($P < 0.001$). Nicotine administration (OVX+Nic) exerted an additive effect on ET-1 level compared with OVX group ($P < 0.001$) (Table 3). OVX rats demonstrated a significant increase in plasma lipid fractions (TC, TAG and LDL-C) and atherogenic index and decrease in HDL-C ($P < 0.01$). Nicotine treatment (OVX+Nic) resulted in enhancement of induced dyslipidaemia and atherogenesis risk (Table 4).

Effects of nicotine administration on thoracic aorta biochemical parameters in female OVX rats

The content of cGMP was significantly lower in the thoracic aorta of OVX rats compared with intact rats ($P < 0.001$). Likewise, the OVX+Nic group exhibited a marked reduction in aortic cGMP content with respect to the OVX group ($P < 0.001$). Besides, ovariectomy significantly elevated aortic calcium content when compared with control values ($P < 0.001$). Nicotine injection to OVX rats significantly increased aortic calcium content compared with their untreated (OVX) counterparts ($P < 0.001$). Aortic MDA, an oxidative stress and lipid peroxidation marker, and hydroxyproline, fibrosis marker, levels were significantly increased in the OVX group compared with control levels ($P < 0.001$). Nicotine administration (OVX+Nic) enhanced lipid peroxidation and fibrosis when compared with the untreated OVX group ($P < 0.001$). On the other hand, the aortic GSH content, which is the most important intracellular antioxidant, was markedly reduced in OVX rats compared with controls. Nicotine injection in the absence of ovarian hormones (OVX+Nic) resulted in further depletion of GSH content in aortic tissues with respect to the OVX group ($P < 0.001$) (Table 5). Furthermore, ovariectomy induced upregulation of aortic caspase-3 gene and downregulation of eNOS gene expression with respect to control value ($P < 0.001$). Moreover, nicotine administration (OVX+Nic) triggered a significant increase in aortic caspase-3 mRNA and decrease in eNOS mRNA expression when compared with the OVX group ($P < 0.001$) (Figure 1).

Effects of ERT on body weight and plasma biochemical parameters in nicotine-treated OVX rats

The OVX+ERT and OVX+Nic+ERT groups showed a marked decrease in body weight relative to the OVX and OVX+Nic rats ($P < 0.05$) (Table 2). Estradiol benzoate treatment (OVX+ERT) completely restored the plasma estradiol level in OVX rats. Although plasma estradiol levels were significantly increased in OVX+Nic+ERT, they did not reach normal levels ($P < 0.001$). Estrogen replacement (OVX+ERT and OVX+Nic+ERT) resulted in a significant increase in NO levels in plasma compared with the OVX and OVX+Nic rats, respectively ($P < 0.001$). Administration of estradiol

Table 5 Effect of nicotine administration and estrogen replacement therapy on aortic cyclic guanosine monophosphate, calcium, malondialdehyde, glutathione and hydroxyproline levels in ovariectomized rats

Parameter	Control	OVX	OVX+Nic	OVX+ERT	OVX+Nic+ERT
cGMP (pmol/g tissue)	27.71 ± 3.87	11.08 ± 1.44#	4.43 ± 0.42*	24.83 ± 3.35*	7.53 ± 0.71[a]
Aortic Ca content (ppm/g tissue)	115.81 ± 13.89	185.29 ± 20.38#	292.36 ± 29.01*	121.25 ± 12.37*	212.35 ± 18.66[a]
Aortic MDA level (nmol/g tissue)	31.58 ± 3.78	60.79 ± 7.11#	133.74 ± 13.77*	38.57 ± 4.71*	98.97 ± 9.69[a]
Aortic GSH level (μmol/g tissue)	117.66 ± 16.47	62.16 ± 8.14#	26.64 ± 2.93*	122.1 ± 15.26*	48.28 ± 4.82[a]
Aortic Hyp content (μg/g tissue)	128 ± 18.56	280 ± 38.64#	492.6 ± 62.56*	122 ± 14.76*	350.18 ± 41.62[a]

Ca, calcium; cGMP, cyclic guanosine monophosphate; ERT, estrogen replacement therapy; GSH, glutathione; Hyp, hydroxyproline; MDA, malondialdehyde; Nic, nicotine; OVX, bilateral ovariectomized. Nic injection (0.1 mg/kg, s.c.). ERT with estradiol benzoate (0.1 mg/kg, i.m.). The values are means ± SD ($n = 8$). Analysis of variance followed Tukey's post-hoc multiple comparison test, #$P < 0.001$ vs sham control, *$P < 0.001$ vs OVX, [a]$P < 0.001$ vs OVX+Nic.

benzoate significantly reduced plasma ET-1 level in both the OVX and the OVX+Nic groups ($P < 0.05$) (Table 3). TC, TAG and LDL-C, as well as atherogenic index, were markedly lowered in the plasma of estradiol-treated (OVX+ERT and OVX+Nic+ERT) rats compared with untreated (OVX) and OVX+Nic rats ($P < 0.05$). On the other hand, plasma levels of HDL-C were significantly elevated in rats treated with ERT (OVX+ERT and OVX+Nic+ERT) compared with untreated (OVX and OVX+Nic) rats ($P < 0.001$) (Table 4).

Effects of ERT on thoracic aorta biochemical parameters in nicotine-treated OVX rats

Estradiol-repleted OVX rats whether or not treated with nicotine (OVX+Nic+ERT and OVX+ERT) had significantly higher cGMP contents in their aortas than their OVX+Nic and OVX counterparts ($P < 0.001$). The OVX and OVX+Nic groups treated with ERT showed a significant decrease of calcium overload in their aortas in comparison with untreated groups (OVX and OVX+Nic) ($P < 0.001$). Treatment of OVX and OVX+Nic rats with estradiol significantly reduced both MDA and hydroxyproline levels in aortic tissues ($P < 0.001$). However, aortic GSH levels were significantly higher in the OVX+ERT and OVX+Nic+ERT groups than those in the corresponding OVX and OVX+Nic groups ($P < 0.001$) (Table 5). Caspase-3 mRNA expression was dramatically downregulated, while eNOS mRNA expression was upregulated, in the aortas of estradiol-treated (OVX+ERT and OVX+Nic+ERT) compared with untreated groups (OVX and OVX+Nic) ($P < 0.001$) (Figure 1).

Correlations between measured biochemical parameters

Analysis of the combined results from all tested rats indicated that the plasma estradiol level was positively correlated with NO ($r = 0.74$, $P < 0.0001$), HDL-C ($r = 0.77$, $P < 0.0001$), aortic eNOS mRNA expression level ($r = 0.8$, $P < 0.0001$) and cGMP content ($r = 0.77$, $P < 0.0001$). In contrast, it was negatively correlated with plasma ET-1 ($r = -0.65$, $P < 0.0001$), TC ($r = -0.68$, $P < 0.0001$), TAG ($r = -0.65$, $P < 0.0001$), LDL-C ($r = -0.76$, $P < 0.0001$), aortic caspase-3 mRNA expression level ($r = -0.7$, $P < 0.0001$) and calcium content ($r = -0.75$, $P < 0.0001$). Plasma NO showed negative correlation with plasma ET-1 ($r = -0.87$, $P < 0.0001$). Additionally, aortic eNOS mRNA expression level demonstrated negative correlation with caspase-3 mRNA expression level in the aorta ($r = -0.82$, $P < 0.0001$), calcium content in the aorta ($r = -0.86$, $P < 0.0001$) and plasma ET-1 ($r = -0.85$, $P < 0.0001$), and a positive correlation with aortic GSH ($r = 0.89$, $P < 0.0001$) and cGMP contents ($r = 0.91$, $P < 0.0001$).

Discussion

The major finding of this work was that ERT at a physiological dose produced a decrease in nicotene-induced oxidative stress and atherogenic index followed by a significant decrease in aortic calcium content and endothelial injury in OVX rats. This is probably due to a direct improvement of estradiol level, antioxidant concentration and to an upregulation of eNOS gene expression leading to an increase in basal aortic endothelial NO and hence cGMP production. Therefore, estradiol appears to be a promising pharmacological agent capable of improving endothelial function in menopausal women in whom second-hand smoke produces the aforementioned deleterious effects.

As expected, our experimental results demonstrated that ovariectomy caused a significant decrease in serum estradiol level. Furthermore, treating OVX rats with nicotine promoted further reduction in estradiol level. However, ERT either alone or in combined form with nicotine, restored serum estradiol level. Consistent with this, previous studies indicated that nicotine could interfere directly or indirectly with the effects of estradiol. For example, nicotine reduces circulating estrogen levels and leads to early onset of menopause in women. Also, women who smoke present lower levels of estradiol in the middle of the cycle and in the luteal phase, in comparison with nonsmokers. Experimental models using animals have suggested that nicotine increases

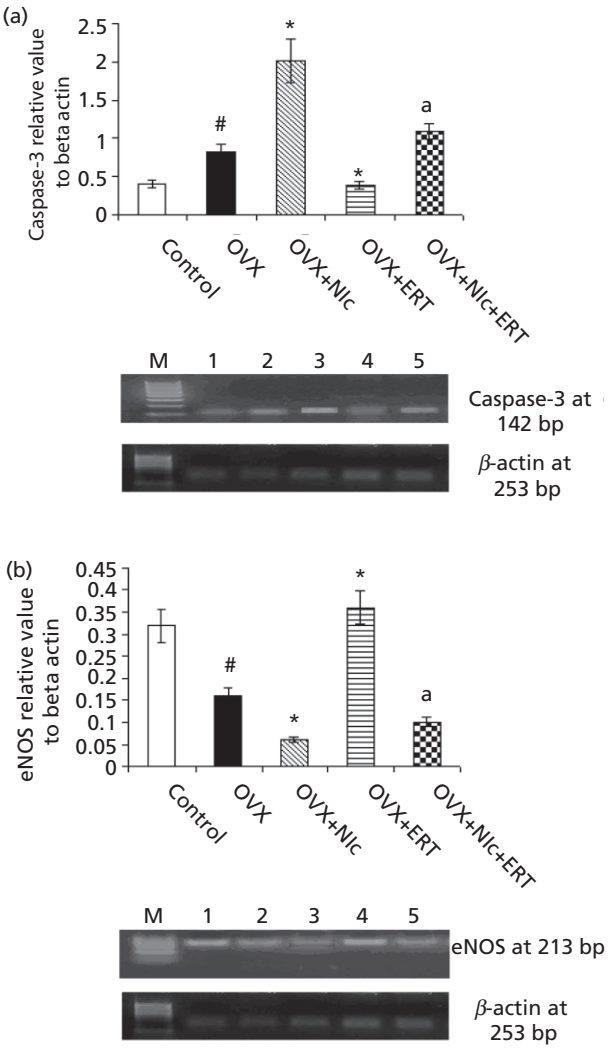

Figure 1 Representative agarose gel electrophoresis profiles of mRNA amplification stained with ethidium bromide of caspase-3 (a) and endothelial nitric oxide synthase (eNOS) (b), normalized to β-actin, obtained by reverse transcription-polymerase chain reaction in thoracic aorta from control (1), OVX (2), OVX+Nic (3), OVX+ERT (4) and OVX+Nic+ERT (5), groups of rats. OVX, bilateral ovariectomized; Nic, nicotine injection (0.1 mg/kg, s.c.); ERT, estrogen replacement therapy (estradiol benzoate 0.1 mg/kg, i.m.). The images were merged; M represents 100 bp DNA marker. Quantitative results of these bands are represented in bar graph showing the effects of ERT on mRNA expression of caspase-3 and eNOS in nicotine-treated ovariectomized rat aortas. Values are means ± SD. #$P < 0.001$ compared with control, *$P < 0.001$ compared with OVX, [a]$P < 0.001$ compared with OVX+Nic.

the loss of follicles in the ovary and blocks the enzyme aromatase, which is responsible for converting androgens into estrogens. Furthermore, these models have demonstrated that the peak level of luteinizing hormone in the middle of the cycle is delayed or nonexistent.[37] Diminished ovarian reserves are more common among women who smoke than among those who do not, which would at least partially explain why infertility is more common among smokers. Smoking has also been associated with increased use of the 2-hydroxylation pathway for estradiol metabolism in the liver. This produces increased levels of 2-hydroxyestrogen, which almost totally lacks peripheral activity.[38] Smoking also seems to increase the quantity of androgens produced by the suprarenal glands, which contributes towards an anti-estrogen effect.[39] Nicotine exposure directly antagonizes estrogen signalling by reducing the number of estrogen receptor(s) and increasing post-ischaemic neurodegeneration from nicotine exposure, suggesting that chronic nicotine use alters the beneficial effects of estrogen and thus might be responsible for the increased risk of stroke pathophysiology and cardiovascular disorders in female smokers.[40]

Chronic exposure to nicotine often leads to cardiovascular disorders such as atherosclerosis, hypertension and ischaemic heart disease. In addition, menopause has been considered a risk factor for cardiovascular pathogenesis. In this study, administration of nicotine in OVX rats impaired vascular endothelial function as manifested by decreased plasma concentration of NO, aortic expression of eNOS and consequently reduced aortic cGMP content. This suggests that both nicotine and estrogen hormone depletion produced VED, which is consistent with recent studies and reports by others.[41,42] Nicotine has been documented to reduce the activity of eNOS and decrease the generation and availability of NO. Furthermore, nicotine has been noted to upregulate ADMA, which is an endogenous eNOS inhibitor.[5] It has been suggested that the loss of endogenous estrogens leads to reduced expression of eNOS and thus reduced production of endothelium-derived NO, leading to impairment of endothelial function. This may be due to severe injury of the endothelium resulting in decreased NO synthesis by eNOS.[43] Furthermore, a previous study indicated that menopausal women had significantly lower plasma estradiol and NO levels than premenopausal women. Similarly, c-GMP levels were significantly lower in the postmenopausal women than in the premenopausal ones. Since NO modulates its vasodilatory effects via c-GMP-dependent mechanisms,[44] the observed difference in c-GMP levels in the groups could be attributed to significant difference in NO levels. Accordingly, NO levels demonstrated a significant positive correlation with estradiol levels. Thus, the observed nicotine and ovariectomy-induced VED may be due to downregulation of eNOS and subsequent reduction in generation of NO and cGMP in the vessel wall. Estrogens are thought to contribute to the maintenance of normal endothelial function. The vasculoprotective effects of estrogen are mediated through genomic and non-genomic mechanisms, and may be attributed to their ability to increase the availability of NO through activation of

eNOS.[20] This contention is supported by the results obtained in this study that ERT increases the plasma concentration of NO and aortic eNOS expression and hence cGMP production.

Our study showed that depletion of ovarian hormones by OVX and nicotine administration resulted in oxidative injury, antioxidant depletion and aortic damage in rats. The pharmacological treatment with estradiol at physiological dose markedly prevented oxidative stress-induced aortic injury by improving the antioxidant status which is consistent with recent studies and reports by others.[41,42] Oxidative stress plays a key role in the development of VED by inhibiting eNOS activation and reducing the generation and availability of NO. Nicotine exposure was shown to generate ROS excessively by activating NADPH oxidase and reducing the antioxidant defence mechanisms(s) via downregulation of catalase and superoxide dismutase.[45] The reduction of estrogen by OVX produced oxidative stress by generating ROS and inducing peroxynitrite formation. A previous study showed that OVX induced a reduction of antioxidant status (GSH, superoxide dismutase and glutathione peroxidase) and elevated lipid peroxides and carbonyl protein content in plasma and erythrocytes. In concordance, a rise of lipid peroxides in serum of postmenopausal healthy women has been observed. Thus, it can be suggested that nicotine-mediated and ovariectomy-mediated development of oxidative stress and antioxidant depletion may be responsible for inducing VED. It was found that the administration of 17β-estradiol counteracts the lipid peroxidation and antioxidant depletion in plasma and erythrocytes observed in estrogen-deficient (OVX) rats.[46] It has been suggested that the antioxidant properties of estrogens are related to the presence of a phenolic A ring in the molecule. The hydroxyphenolic structure facilitates the transfer of hydrogen atoms from its hydroxyl phenolic group to lipid radicals, blocking the lipid peroxidation chain reaction. 17β-Estradiol is able to prevent the induction of conjugated diene formation in LDL induced by copper. In addition, estrogens have been shown to exert numerous receptor-mediated antioxidant effects. In this sense, 17β-estradiol enhanced the expression of NOS-3 and NO production through receptor-mediated signal in endothelial cells.[47] The antioxidant activity of 17β-estradiol may be also related to its capacity to enhance the expression of antioxidant enzymes, as well as to reduce the secretion of inflammatory cytokines and adhesion molecules.[48]

A significant increase was observed in plasma TC, TAG, LDL-C and atherogenic index, coupled with decrease in HDL-C, in the OVX+Nic and OVX groups compared with the control. This effect was reversed by ERT. In postmenopausal women, estrogen deficit has a negative influence on lipid profile, and ERT has favorable effects on lipoproteins that could reduce the progression of atherosclerosis.[49] Accumulating evidence suggests that an elevated level of circulating lipids impairs the integrity of the vascular endothelium and induces VED. It has been shown that nicotine administration alters the lipid profile by increasing the levels of TC and TAG and consequently decreasing the levels of HDL-C.[50] Thus, it appears possible that in this study the alteration in lipid profile was additionally involved in the induction of VED in OVX rats administered nicotine. However, ERT prevented nicotine-induced and OVX-induced alteration in lipid profiles by reducing the circulating levels of TC, TAG, LDL-C and elevating HDL-C levels, which may be primarily due to its favourable effect on hepatic lipid metabolism.[51] In our study, a positive correlation was seen between estradiol levels, HDL-C and NO. A significant negative correlation was observed between estradiol levels and serum TC, TAG and LDL-C. The fact that premenopausal women tend to have a better cardiovascular risk factor profile than men of the same age group also seems to prove the role of estrogen in maintaining a favorable lipid profile. Augmented release of NO by estrogen might account not only for enhancement of endothelium-dependent vasodilation but also for much of the anti-atherogenic effects of estrogen. The beneficial changes in lipids and lipoproteins observed during HRT in several previous studies have been assumed to explain 25–50% of the reduced cardiovascular risk.[51] Additionally, ERT in OVX and OVX+Nic rats resulted in a marked reduction of body weight, adding another beneficial metabolic effect on weight loss.[52]

Nicotine exposure due to chronic cigarette smoking exposure (passive smoking) is a known cause of cardiovascular disorders, such as atherosclerosis, hypertension and ischaemic heart disease, in most industrialized nations. Indeed, VED is a hallmark of the various cardiovascular disorders.[53] Our results confirm and extend the previous data showing that the administration of nicotine for 6 weeks to OVX rats induces oxidative injury and alterations in the major vessel (i.e. thoracic aorta of rats). These alterations include changes in the aorta with increased plasma levels of the vasoconstrictor ET-1, atherogenic index, aortic calcium content, hydroxyproline level and caspase-3 expression. However, ERT restored the aforementioned changes in the aortic tissues. Many risk factors associated with endothelial dysfunction share etiologies related to endothelial apoptosis. Caspase-3 has a central role in the apoptotic cascade. This study revealed enhanced expression of aortic caspase-3 in nicotine-treated OVX rats only, suggesting apoptosis, but is not evidence for apoptosis, and estradiol exerted an opposite effect. Clinically, nicotine in cigarettes might contribute to endothelial dysfunction, whereas ambient estradiol may provide cellular protection against nicotine-induced injury through its functional membrane receptor via mitogen activated protein kinase (MAPK) pathway downregulation.

This might be attributed to reduction of calcium influx, consequently attenuating nicotine-induced apoptosis. In concordance, recent research demonstrated that in-vitro exposure of endothelial cells to nicotine increased endothelial apoptosis and that nicotine's pro-apoptotic effects were reversed by a calcium-sequestering reagent suggesting a causal role for calcium stress.[54] The concept that nicotine causes endothelial dysfunction via an increase in calcium overload is supported by the significantly higher aortic calcium content in nicotine-treated OVX rats. Whether the observed changes in expression of caspase-3, which is synthesized as an inactive procaspase, in the current experiment translates into more cell death in response to apoptotic signals remains to be determined. However, consistent with this finding, it has been reported previously that ovariectomy increased Bid3, a BH3-interacting domain death agonist. Bid regulates apoptotic cascades in that when it is cleaved by death receptor-activated caspases generating truncated Bid (tBid), it then functions as an operational BH3 domain-only protein causing cell death.[55] Collectively, the OVX-associated increases in caspase-3 and bid suggested an overall increase in the pro-apoptotic gene expression, which could be halted by ERT.[56] In concordance, a recent study indicated that gene silencing of caspase-3 genes with siRNAs provided profound protection against vascular endothelial cell apoptosis.[57] Therefore, it seems that controlling caspase-3 gene expression in the aortic tissues (downregulation of this effector pro-apoptotic gene target in the apoptotic pathway) by ERT will reduce translation to protein products and aid in the protection from apoptosis and hence endothelial dysfunction. A previous study demonstrated estradiol's regulatory effect on nicotine toxicity in endothelial cells. Estrogen has been shown to be a survival factor in toxin-induced endothelial cell death, and such protection may represent an unreported alternative mechanism for inhibition of formation of atherogenic lesions.[58]

We found a rise in ET-1 level in the plasma of nicotine-treated OVX rats with a reduction of aortic eNOS expression. Thus, considering the inverse relationship between ET-1 and eNOS, we suggest that ET-1 is a key factor and an initial step in the development of nicotine-mediated injury. ET-1 is released continuously, mostly from endothelial cells, by a constitutive and regulated pathway and contributes to the maintenance of vascular tone. ET-1 and NO are functionally closely interdependent, with a strong inhibitory effect of ET-1 on NO-mediated dilation in human coronary and cerebral arteries.[59] There is also evidence that ET-1 contributes to increased oxidative stress by stimulating the production of ROS, particularly via membrane-bound NAD(P)H oxidase. The rise in ROS may lead to excess radicals thereby diminishing the levels of active antioxidant species. It was indicated that estrogen downregulates plasma ET-1 level by inhibiting the preproET-1 mRNA expression and functional endothelin converting enzyme (ECE) activity.[60] We suggest that the main role of ERT is to reduce the ET-1 level, which in turn, leads to a decrease in NO by downregulating eNOS expression. These data are consistent with previous findings showing that ET-1 decreases eNOS expression.[61]

These results also demonstrated a positive association between ET-1 and aortic calcium content. Thus, we cautiously speculate that long-term exposure of aortic tissue to higher ET-1 levels might partly contribute to calcium overload in nicotine-treated OVX rats, which is in agreement with an earlier study.[62] Furthermore, ET-1 antagonists prevent increased calcium uptake into arterial tissue of nicotine-treated male rats and suppress increased calcium content in calcified vascular smooth muscle cells.[63]

It has been reported that increased ET-1 may be associated with promotion of cardiovascular hypertrophy and fibrosis, resulting from the deposition of extracellular matrix and fibroblast recruitment, and this endothelial-to-mesenchymal transition process is stimulated by ET-1.[64] Additionally, ETA receptor antagonist prevented cardiac and aortic collagen deposition and aortic hypertrophy. This suggests a role for ET-1 in fibrosis of the heart and large vessels.[65] In accordance, our results revealed significant elevation of plasma ET-1 and aortic fibrosis marker content in OVX rats administered nicotine, whereas ERT offsets aortic fibrosis. We hypothesize that the anti-fibrotic effect of ERT may be mediated via reducing ET-1 level.

Conclusions

This study, using a combined approach (menopause and passive smoking models), demonstrated that short-term ERT lowered markers of aortic injury, and improved markers of endothelial function in nicotine-treated OVX rats. Whatever could be the underlying additional mechanisms, the results shown here suggest that the cytoprotective action of estradiol could be a consequence of both a decrease in oxidative stress and atherogenic index. Decrease in oxidative stress was probably due to both direct antioxidant effect of estradiol and indirect reduction of calcium overload that might be a consequence of the inhibition of calcium entry to smooth muscle cells by voltage-dependent calcium channels. Moreover, the substantial decrease in body weight achieved by ERT in OVX rats, implies effectiveness of ERT in targeting postmenopausal metabolic symptoms.

The results of our study indicate that ERT causes improvement of vascular endothelial function, probably as a consequence of the upregulation of aortic eNOS expression, hence NO generation and cGMP release, as well as reduction of plasma ET-1, which seems to be the main mechanisms of its vasodilatory action in preventing nicotine-induced and ovariectomy-induced experimental VED.

Declarations

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

Funding

We wish to thank the Biochemistry Department, Faculty of Pharmacy, University of Zagazig, for their financial support in the form of grants.

Acknowledgements

We thank Mr Samir El-Sayed Moussa, Pharmacology Department, Faculty of Medicine, Zagazig University, for technical assistance.

References

1. Balakumar P *et al*. Ameliorative effect of combination of benfotiamine and fenofibrate in diabetes-induced vascular endothelial dysfunction and nephropathy in the rat. *Mol Cell Biochem* 2009; 320: 149–162; doi:10.1007/s11010-008-9917-z.
2. Balakumar P *et al*. Potential target sites to modulate vascular endothelial dysfunction: current perspectives and future directions. *Toxicology* 2008; 245: 49–64.
3. Ciurea E *et al*. Nutritional antioxidant effects on endothelial dysfunctions in experimental atherosclerosis by ovariectomy. *Rev Med Chir Soc Med Nat Iasi* 2009; 113: 826–831.
4. Nicita-Mauro V *et al*. Smoking, aging and the centenarians. *Exp Gerontol* 2008; 43: 95–101.
5. Jiang DJ *et al*. Involvement of DDAH/ADMA/NOS pathway in nicotine-induced endothelial dysfunction. *Biochem Biophys Res Commun* 2006; 349: 683–693.
6. Fleming LE *et al*. Earlier age at menopause, work, and tobacco smoke exposure. *Menopause* 2008; 15: 1103–1108.
7. Okoli CT *et al*. Secondhand smoke and nicotine exposure: a brief review. *Addict Behav* 2007; 32: 1977–1988.
8. Pasupathi P *et al*. Cigarette smoking – effect of metabolic health risk: a review. *Diabetes Metab Syndr Clin Res Rev* 2009; 3: 120–127.
9. Ahijevych K, Wewers ME. Passive smoking and vascular disease. *J Cardiovasc Nurs* 2003; 18: 69–74.
10. Erusalimsky JD. Vascular endothelial senescence: from mechanisms to pathophysiology. *J Appl Physiol* 2009; 106: 326–332.
11. Reckelhoff JF, Maric C. Sex and gender differences in cardiovascular-renal physiology and pathophysiology. *Steroids* 2010; 75: 745–746.
12. Yang XP, Reckelhoff JF. Estrogen, hormonal replacement therapy and cardiovascular disease. *Curr Opin Nephrol Hypertens* 2011; 20: 133–138.
13. Rossouw JE *et al*. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA* 2007; 297: 1465–1477.
14. Alexandersen P *et al*. The long-term impact of 2–3 years of hormone replacement therapy on cardiovascular mortality and atherosclerosis in healthy women. *Climacteric* 2006; 9: 108–118.
15. Klaiber EL *et al*. Relationships of serum estradiol levels, menopausal duration, and mood during hormonal replacement therapy. *Psychoneuroendocrinology* 1997; 22: 549–558.
16. Manson JE *et al*. Estrogen therapy and coronary-artery calcification. *N Engl J Med* 2007; 356: 2591–2602.
17. Rivera CM *et al*. Increased cardiovascular mortality after early bilateral oophorectomy. *Menopause* 2009; 16: 15–23.
18. Mendelsohn ME. Estrogen actions in the cardiovascular system. *Climacteric* 2009; 12(Suppl. 1): 18–21.
19. Dantas AP *et al*. Relative contribution of estrogen withdrawal and gonadotropins increase secondary to ovariectomy on prostaglandin generation in mesenteric microvessels. *J Cardiovasc Pharmacol* 2004; 43: 48–55.
20. Gimenez J *et al*. Effects of oestrogen treatment and angiotensin-converting enzyme inhibition on the microvasculature of ovariectomized spontaneously hypertensive rats. *Exp Physiol* 2006; 91: 261–268.
21. Micu AL *et al*. Rat hepatic CYP2E1 is induced by very low nicotine doses: an investigation of induction, time course, dose response, and mechanism. *J Pharmacol Exp Ther* 2003; 306: 941–947.
22. Chen Y *et al*. Estrogen-replacement therapy promotes angiogenesis after acute myocardial infarction by enhancing SDF-1 and estrogen receptor expression. *Microvasc Res* 2009; 77: 71–77.
23. Korde A *et al*. A radioimmunoassay for serum estradiol. *J Radioanal Nucl Chem* 1996; 206: 219–226.
24. Moshage H *et al*. Nitrite and nitrate determination in plasma. A critical evaluation. *Clin Chem* 1995; 41: 892–896.
25. Porstmann T, Kiessig ST. Enzyme immunoassay techniques, an overview. *J Immunol Methods* 1992; 150: 5–21.
26. Meiattini F *et al*. The 4-hydroxybenzoate/4-aminophenazone chromogenic system used in the enzymatic determination of serum cholesterol. *Clin Chem* 1978; 24: 2161–2165.
27. Grove TH. Effect of reagent pH on determination of HDL cholesterol by precipitation with sodium phosphotungestate-magnesium. *Clin Chem* 1979; 25: 560–564.

28. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem* 1982; 28: 2077–2080.
29. Friedewald WT *et al*. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; 18: 499–502.
30. Sagud M *et al*. Lipid levels in female patients with affective disorders. *Psychiatry Res* 2009; 168: 218–221.
31. Ahmed AE *et al*. Studies on mechanism of haloacetonitrile induced gastrointestinal toxicity, interaction of dibromoacetonitrile with glutathione as glutathione-s-transferase in rats. *J Biol Toxicol* 1991; 612: 115–121.
32. Buege J, Aust S. Microsomal lipid peroxidation. *Methods Enzymol* 1978; 52: 302–306.
33. Campillo N *et al*. Selenium determination in biological fluids using Zeeman background correction electrothermal atomic absorption spectrometry. *Anal Biochem* 2000; 280: 195–200.
34. Fujita M *et al*. Over expression of tumor necrosis factor β diminishes pulmonary fibrosis induced by bleomycin or transforming growth factor β. *Am J Respir Cell Mol Biol* 2003; 29: 1–7.
35. Kojda G *et al*. Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. *Circ Res* 1996; 78: 91–101.
36. Wang S *et al*. Fluorescein provides a resonance gate for FRET from conjugated polymers to DNA intercalated dyes. *J Am Chem Soc* 2004; 126: 5446–5451.
37. Biegon A *et al*. Nicotine blocks brain estrogen synthase (aromatase): in vivo positron emission tomography studies in female baboons. *Biol Psychiatry* 2010; 67: 774–777.
38. Parente RC *et al*. The relationship between smoking and age at the menopause: a systematic review. *Maturitas* 2008; 61: 287–298.
39. Tanko LB, Christiansen C. An update on the antiestrogenic effect of smoking: a literature review with implications for researchers and practitioners. *Menopause* 2004; 11: 104–109.
40. Raval AP *et al*. Chronic nicotine exposure inhibits 17beta-estradiol-mediated protection of the hippocampal CA1 region against cerebral ischemia in female rats. *Neurosci Lett* 2009; 458: 65–69.
41. Balakumar P *et al*. Benfotiamine attenuates nicotine and uric acid induced vascular endothelial dysfunction in the rat. *Pharmacol Res* 2008; 58: 356–363.
42. Novella S *et al*. Gathering of aging and estrogen withdrawal in vascular dysfunction of senescent accelerated mice. *Exp Gerontol* 2010; 45: 868–874.
43. Fenoy FJ *et al*. Acute effects of 2-methoxyestradiol on endothelial aortic No release in male and ovariectomized female rats. *Nitric Oxide* 2010; 23: 12–19.
44. Salhotra S *et al*. Influence of menopause on biochemical markers of endothelial dysfunction – a case–control pilot study in North Indian population. *Maturitas* 2009; 62: 166–170.
45. Fang Q *et al*. Inhibition of NADPH oxidase improves impaired reactivity of pial arterioles during chronic exposure to nicotine. *J Appl Physiol* 2006; 100: 631–636.
46. Muñoz-Castañeda JR *et al*. Estradiol and catecholestrogens protect against adriamycin-induced oxidative stress in erythrocytes of ovariectomized rats. *Toxicol Lett* 2006; 160: 196–203.
47. Hayashi T *et al*. Estrogen increases endothelial nitric oxide by a receptor-mediated system. *Biochem Biophys Res Commun* 1995; 214: 847–855.
48. Strehlow K *et al*. Modulation of antioxidant enzyme expression and function by estrogen. *Circulation Res* 2005; 93: 170–177.
49. Emre A *et al*. Effect of hormone replacement therapy on plasma lipoproteins and apolipoproteins, endothelial function and myocardial perfusion in postmenopausal women with estrogen receptor-α IVS1-397 C/C genotype and established coronary artery disease. *Cardiology* 2006; 106: 44–50. doi:10.1159/000092598.
50. Chattopadhyay K, Chattopadhyay BD. Effect of nicotine on lipid profile, peroxidation & antioxidant enzymes in female rats with restricted dietary protein. *Indian J Med Res* 2008; 127: 571–576.
51. Vassalle C *et al*. Effects of the estrogen replacement therapy on different biochemical markers of endothelial reactivity in recent postmenopausal healthy women. *Immuno-Anal Biol Spec* 2005; 20: 181–185.
52. Babaei P *et al*. Effects of ovariectomy and estrogen replacement therapy on visceral adipose tissue and serum adiponectin levels in rats. *Menopause Int* 2010; 16: 100–104.
53. Balakumar P, Kaur J. Is nicotine a key player or spectator in the induction and progression of cardiovascular disorders? *Pharmacol Res* 2009; 60: 361–368.
54. Wang LL *et al*. Estradiol pretreatment attenuated nicotine-induced endothelial cell apoptosis via estradiol functional membrane receptor. *Int Immunopharmacol* 2011; 215: 309–315 [Epub ahead of print].
55. Hamilton KL *et al*. Effect of ovariectomy on cardiac gene expression: inflammation and changes in SOCS gene expression. *Physiol Genomics* 2008; 32: 254–263.
56. Pechenino AS *et al*. Impact of aging vs. estrogen loss on cardiac gene expression: estrogen replacement and inflammation. *Physiol Genomics* 2011; 43: 1065–1073 [Epub ahead of print].
57. Matsuda N *et al*. Silencing of caspase-8 and caspase-3 by RNA interference prevents vascular endothelial cell injury in mice with endotoxic shock. *Cardiovasc Res* 2007; 76: 132–140.
58. Subramanian M, Shaha C. Oestrogen modulates human macrophage apoptosis via differential signalling through oestrogen receptor-alpha and beta. *J Cell Mol Med* 2009; 13: 2317–2329.
59. Rodella LF *et al*. Endothelin-1 as a potential marker of melatonin's therapeutic effects in smoking-induced vasculopathy. *Life Sci* 2010; 87: 558–564.
60. Tan Z *et al*. Mechanisms of 17β-estradiol on the production of ET-1 in ovariectomized rats. *Life Sci* 2003; 73: 2665–2674.

61. Zemse SM *et al*. Restoration of endothelin-1-induced impairment in endothelium-dependent relaxation by interleukin-10 in murine aortic rings. *Can J Physiol Pharmacol* 2008; 86: 557–565.
62. Park JH *et al*. Relationship between arterial calcification and bone loss in a new combined model rat by ovariectomy and vitamin D(3) plus nicotine. *Calcif Tissue Int* 2008; 83: 192–201.
63. Wu SY *et al*. Endothelin-1 is a potent regulator in vivo in vascular calcification and in vitro in calcification of vascular smooth muscle cells. *Peptides* 2003; 24: 1149–1156.
64. Widyantoro B *et al*. Endothelial cell-derived endothelin-1 promotes cardiac fibrosis in diabetic hearts through stimulation of endothelial-to-mesenchymal transition. *Circulation* 2010; 121: 2407–2418. Epub 2010 May 24.
65. Park JB, Schiffrin EL. Cardiac and vascular fibrosis and hypertrophy in aldosterone-infused rats: role of endothelin-1. *Am J Hypertens* 2002; 15 (2 Pt 1): 164–169.

