Differential effects of oral conjugated equine estrogen and transdermal estrogen on atherosclerotic vascular disease risk markers and endothelial function in healthy postmenopausal women

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BACKGROUND: Recent studies have revealed that HRT may increase the risk for atherosclerotic vascular disease (ASVD). METHODS: We investigated the effects of HRT via different administration routes on the markers for ASVD and endothelial function in healthy postmenopausal women. The oral HRT group (n = 18) received conjugated equine estrogen 0.625 mg/day; the transdermal HRT group (n = 18) received 17β-estradiol (E2) gel 0.6 mg/day for 6 months. The control group (n = 30) had no treatment for 6 months. RESULTS: The C-reactive protein (CRP) rose from 0.129 ± 0.116 to 0.752 ± 0.794 mg/dl (P < 0.01) in the oral HRT group but remained unchanged in the transdermal HRT and control groups. The flow-mediated vasodilation (FMD) in the brachial artery was increased significantly by HRT from 6.0% before oral HRT to 14.7% after oral HRT (P < 0.001) and from 5.9% before transdermal HRT to 13.9% after transdermal HRT (P = 0.001). CONCLUSIONS: These data suggest that oral estrogen induces ASVD risk by increasing acute inflammation; however, transdermal estrogen avoids this untoward effect. Additionally, transdermal estrogen exerts a positive effect on endothelial function similar to that of oral estrogen. Therefore, the transdermal route might be favourable in terms of ASVD risks.

Key words: atherosclerotic vascular disease/C-reactive protein/estrogen/homocysteine/vasodilation

Introduction

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality in developed nations. It has long been known that the incidence of CVD increases in postmenopausal women (Castelli, 1984). On the basis of earlier epidemiological studies, HRT had been regarded as a strategy that could potentially reduce the incidence of CVD (Wenger et al., 1993). However, the Heart and Estrogen/Progestin Replacement Study (HERS) revealed that HRT in the form of continuous combined oral conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA) did not reduce the risk of coronary heart disease (CHD) in postmenopausal women with established coronary artery disease (CAD) (Hulley et al., 1998). Consistent with this, the same HRT regimen in the Women’s Health Initiative (WHI) study was associated with increased risk for stroke and CHD (Rossouw et al., 2002). A separate WHI study of oral unopposed estrogen, although it did not show the untoward effect on CHD, still demonstrated increased risk of stroke, which implicated increased atherosclerotic vascular disease (ASVD) risk (Anderson et al., 2004).

Ideally, increased safety was expected when surrogate markers served as predictors of ASVD in postmenopausal women undergoing HRT. However, the traditional markers such as high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total cholesterol and triglyceride failed to predict the lack of a beneficial vascular outcome in those women (Manson et al., 2003). More recently, inflammation has been implicated as a novel risk factor in the development and progression of atherosclerosis and CHD. Higher plasma C-reactive protein (CRP) has also been shown to be associated with increased CHD risk (Ridker et al., 1997). Furthermore, it has been reported that oral HRT increased plasma CRP (Pradhan et al., 2002), but little is known of the relationship between the transdermal estrogen therapy and the CRP level.

Plasma homocysteine level has been reported as an independent risk factor for CVD (Graham et al., 1997). Unlike inflammatory markers, oral HRT appeared to reduce homocysteine levels...
in several studies, in the forms of continuous combined estrogen and progestin or unopposed estrogen (Mijatovic et al., 1998; Walsh et al., 2000). However, this beneficial effect was also challenged by recent randomized controlled trials which investigated the homocysteine level changes after oral estrogen and progestin treatment (Bruschi et al., 2004; Barnes et al., 2005; Bukowska et al., 2005). Furthermore, because there have been few reports on the effect of different routes of estrogen administration on homocysteine, we sought to clarify the relationship. Elevated fibrinogen levels are related to higher risks for myocardial infarction (Kannel et al., 1987) and considered as an independent risk factor for CVD (de Maat et al., 1996; Maresca et al., 1999). However, the relationship between HRT and fibrinogen level remains unclear.

Impaired endothelial cell function per se is a direct cause of acute myocardial infarction and ischemic stroke. In a previous study, flow-mediated vasodilation (FMD) as an evaluation of endothelial function was shown to differentiate symptomatic carotid artery stenosis from asymptomatic stenosis (Hsu et al., 2002). Several studies have advocated that HRT enhances the endothelial function (Lieberman et al., 1994), but the effect by route of administration has not been previously investigated. Thus, we sought to demonstrate the effect of oral and transdermal estrogen on the endothelial function.

In this study, we acknowledged that WHI investigators postulated increased ASVD risks for postmenopausal women undergoing oral HRT, in the forms of opposed or unopposed estrogen. Nevertheless, the effects of transdermal HRT on the risk for ASVD remained undetermined. Therefore, our purpose for this randomized trial was to evaluate the effects of HRT through different administration routes on the markers for ASVD risk in healthy postmenopausal women.

Materials and methods

Subjects
With approval from the Institutional Review Board of Taichung Veterans General Hospital (IRB TC VGH No: 940327/490), we studied 66 Chinese female volunteers who satisfied the following criteria during this period: (i) age between 50 and 65 years; (ii) menopausal status confirmed by a serum FSH concentration >40 IU/l and a serum estradiol (E2) concentration <30 pg/ml; (iii) hysterectomized women; (iv) did not smoke or consume alcohol; (v) had no chronic diseases such as hypertension, hyperlipidaemia, diabetes mellitus, clinical manifestations of atherosclerosis (CHD, cerebrovascular disease or peripheral artery disease), venous thromboembolic disease, liver disorders, cancers, inflammatory diseases and autoimmune diseases; (vi) had not undergone estrogen replacement therapy previously and (vii) were not currently taking any medicine or nutrient supplement known to influence homocysteine metabolism. The participants received thorough written and verbal information on the purpose and procedures of the study, and an informed consent was obtained from all of them.

Thirty-six hysterectomized participants were randomly assigned in open, parallel-group fashion to two treatment groups. For 6 months, participants in the oral estrogen group received 0.625 mg of oral CEE daily (n = 18), whereas those in the transdermal estrogen group received 0.6 mg of transdermal 17β-E2 gel daily (n = 18). Thirty participants who did not receive HRT were assigned to the normal control group (n = 30).

Laboratory assays
The hormone, lipid and biochemical tests were performed using blood samples collected from the participants between 8 and 10 a.m. after a 12-h fast at the beginning and the end of each therapeutic period. Serum concentrations of E2 were determined by radioimmunoassay using commercial kits (Diagnostic Systems Laboratories, Webster, TX, USA). Serum FSH was determined as previously described (Ho et al., 2005) using a chemiluminescent immunosassay (IMMULITE 2000 for serum FSH, Diagnostic Products Corporation, Los Angeles, CA, USA). Serum concentrations of total cholesterol and triglyceride were measured by enzymatic methods in a Hitachi 7600 automated analyzer using commercial kits (Wako Pure Chemical Industries, Osaka, Japan). CRP and homocysteine serum concentrations were measured using a chemiluminescent immunosassay (IMMULITE 2000 for high-sensitivity CRP and homocysteine, Diagnostic Products Corporation). Plasma fibrinogen was measured with the Clauss method by automated Multi-Channel Discrete Analyzer using commercial kits (MDA Fibriquik and MDA Verify Reference Plasma, bioMérieux, Durham, NC, USA) within 1 h after blood sampling. The inter- and intra-assay coefficients of variation were, respectively, 5.3 and 8.1% for E2, 4.9 and 4.2% for CRP, 7.6 and 7.1% for homocysteine and <3% for fibrinogen. The minimum detection limits were as follows: E2 = 4.7 pg/ml, CRP = 0.01 mg/dl, homocysteine = 0.5 μmol/l.

Measurement of FMD
The vasodilator responses to reactive hyperaemia were performed as described in our previous study (Sheu et al., 1999). In brief, high-resolution Doppler ultrasonographic equipment (General Electric Voluson 730 Expert) with a 12-MHz linear array transducer was used to scan the brachial artery in the longitudinal section above the elbow after 20 min of rest at supine position. All the subjects were studied in the morning (9–11 a.m.) in a fasting state. After baseline images of the brachial artery were obtained, FMD was induced by increased arterial blood flow resulting from inflating a pneumatic cuff around the upper arm to 200 mmHg for 5 min and deflating suddenly. One minute after cuff deflation, the brachial artery was imaged. Diameter of the brachial artery was measured from the anterior to the posterior interface between the media and the adventitia at the end of diastole, coincident with the R-wave on electrocardiographic tracing. FMD was calculated as the percentage increase in arterial diameter during hyperaemia and was used as an index of endothelium-dependent vasodilation. In our study, all the ultrasonographic studies were performed by the same physician, and the intra-observer variability for repeated measurements of FMD was 2.3 ± 0.8%.

Statistical analysis
Data are expressed as the mean ± SD. One-way analysis of variance test and Kruskal–Wallis test were used as appropriate to compare the baseline clinical characteristics, hormones, lipids, fibrinogen, CRP, homocysteine, baseline brachial artery diameter and FMD between the three groups. Treatment-induced changes in these parameters were analysed by Student’s paired t-test. At P < 0.05, the difference was considered to be statistically significant. Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS v11.0 for Windows, Chicago, IL, USA).

Results
Baseline clinical characteristics
There were no differences in baseline clinical characteristics between the oral estrogen group, the transdermal estrogen group and the untreated control group. No significant
differences were found between these groups for baseline concentration of CRP, fibrinogen, homocysteine and hormones. The baseline brachial artery diameter did not vary significantly between control and estrogen treatment groups (Table I).

**Effects of HRT on lipids, E2, CRP, homocysteine and fibrinogen**

After the 6-month experimental period, oral CEE significantly reduced serum concentrations of total cholesterol and increased the concentrations of triglyceride. Similarly, transdermal 17β-E2 also significantly decreased serum concentrations of total cholesterol, but serum concentrations of triglyceride did not change significantly (Table II).

Serum E2 concentrations were increased significantly and comparably in the two treatment groups (82.1 and 71.8 pg/ml, respectively) 6 months after estrogen therapy but remained unchanged in the control group (22.4 pg/ml). Oral CEE significantly increased the serum concentrations of CRP from 0.129 ± 0.116 mg/dl at pretreatment to 0.752 ± 0.794 mg/dl after treatment (P < 0.01). In contrast, transdermal 17β-E2 did not significantly change the CRP concentrations. In both the treatment groups, the concentrations of homocysteine and fibrinogen remained unchanged (Table II).

**Effects of HRT on endothelial function**

After the 6-month experimental period, the brachial artery diameter was 3.70 ± 0.53 mm in oral estrogen group, 3.77 ± 0.49 mm in transdermal estrogen group and 3.77 ± 0.44 mm in control group (each P > 0.05 versus pretreatment). There were no differences in the baseline FMD between the oral estrogen group, the transdermal estrogen group and the untreated control group (Figure 1A). After the 6-month experimental period, the FMD was greater in estrogen therapy groups than at pretreatment. Reactive hyperaemia caused a 14.7% [95% confidence interval (CI) = 11.6–17.9%] increase in brachial artery diameter compared with a 6.0% (95% CI = 3.1–8.9%) increase before oral estrogen therapy (P < 0.001) (Figure 1B). FMD was also greater after 6 months of transdermal estrogen therapy (13.9%, 95% CI = 10.1–17.7%) than that before treatment (5.9%, 95% CI = 3.9–8.0%, P = 0.001). In the control group, FMD was 6.7% (95% CI = 4.1–9.2%) before experimental period, and it did

| Table I. Baseline clinical characteristics and parameters of the control subjects and those who were to receive oral or transdermal HRT |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Control | Oral | Transdermal | P |
| Number | 30 | 18 | 18 | 0.99* |
| Age (year) | 55.83 ± 3.63 | 55.72 ± 7.58 | 55.89 ± 5.53 | 0.55* |
| Height (m) | 1.567 ± 0.049 | 1.567 ± 0.047 | 1.552 ± 0.044 | 0.97* |
| Weight (kg) | 57.78 ± 8.46 | 58.25 ± 7.24 | 57.71 ± 5.35 | 0.81* |
| Body mass index (kg/m²) | 23.47 ± 2.88 | 23.66 ± 2.08 | 23.97 ± 2.34 | 0.76 |
| Total cholesterol (mg/dl) | 197.2 ± 32.9 | 192.3 ± 30.0 | 190.6 ± 33.1 | 0.47 |
| Triglyceride (mg/dl) | 89.4 ± 44.2 | 107.2 ± 53.3 | 108.1 ± 49.1 | 0.34* |
| Serum FSH (IU/l) | 78.3 ± 47.1 | 79.2 ± 25.0 | 65.6 ± 10.1 | 0.41* |
| Serum estradiol (pg/ml) | 22.7 ± 6.0 | 22.8 ± 4.9 | 22.6 ± 6.3 | 0.99* |
| C-reactive protein (mg/dl) | 0.146 ± 0.166 | 0.129 ± 0.116 | 0.153 ± 0.144 | 0.88 |
| Homocysteine (μmol/l) | 6.69 ± 1.38 | 7.05 ± 2.61 | 7.15 ± 2.03 | 0.61* |
| Fibrinogen (mg/dl) | 292.4 ± 44.4 | 306.6 ± 71.6 | 296.5 ± 55.1 | 0.99* |
| Brachial artery diameter (mm) | 3.68 ± 0.43 | 3.76 ± 0.65 | 3.83 ± 0.59 | 0.64* |

Values are presented as the mean ± SD.
*One-way analysis of variance for between-group differences.
*Kruskal–Wallis test for between-group differences.

| Table II. Changes in atherosclerotic vascular disease risk markers and brachial artery diameter before and 6 months after estrogen administration |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Control | Oral | Transdermal | P |
| Total cholesterol (mg/dl) | 197.2 ± 32.9 | 205.8 ± 29.7 | 192.3 ± 30.0 | 179.4 ± 32.4* | 54.6 ± 33.1 | 190.6 ± 32.4 | 176.4 ± 23.2* |
| Triglyceride (mg/dl) | 89.9 ± 44.2 | 96.8 ± 41.1 | 107.2 ± 53.3 | 133.4 ± 70.0* | 108.1 ± 49.1 | 114.7 ± 55.2 |
| Estradiol (pg/ml) | 22.7 ± 6.0 | 22.4 ± 4.7 | 22.8 ± 4.9 | 82.1 ± 50.3* | 22.6 ± 6.3 | 71.8 ± 39.7* |
| C-reactive protein (mg/dl) | 0.146 ± 0.166 | 0.147 ± 0.224 | 0.129 ± 0.116 | 0.752 ± 0.794* | 0.153 ± 0.144 | 0.209 ± 0.186 |
| Homocysteine (μmol/l) | 6.69 ± 1.38 | 6.93 ± 2.08 | 7.05 ± 2.61 | 7.55 ± 1.96 | 7.15 ± 2.03 | 7.24 ± 2.55 |
| Fibrinogen (mg/dl) | 292.4 ± 44.4 | 294.1 ± 36.1 | 306.6 ± 71.6 | 299.2 ± 66.3 | 296.5 ± 55.1 | 314.1 ± 60.8 |
| Brachial artery diameter (mm) | 3.68 ± 0.43 | 3.77 ± 0.44 | 3.76 ± 0.65 | 3.70 ± 0.53 | 3.83 ± 0.59 | 3.77 ± 0.49 |

Values are presented as the mean ± SD.
*P < 0.05 versus pretreatment. Student’s paired t-test.
**P < 0.01 versus pretreatment. Student’s paired t-test.
***P < 0.001 versus pretreatment. Student’s paired t-test.
Discussion

This is the first study in which the effects of oral and transdermal hormone therapy on endothelial function and ASVD risk markers, in terms of CRP, homocysteine and fibrinogen, in postmenopausal women have been concomitantly investigated. The major findings were a significant increase in FMD without untoward effects on ASVD risk markers during transdermal hormone therapy and, conversely, a significant increase in FMD and CRP during oral hormone therapy in postmenopausal women.

In the past decade, a number of new risk factors have been investigated and proven to be effective predictors of atherosclerosis and its related diseases. Of these markers, some have been highlighted because there is substantial evidence of their predictive abilities and available modifying treatments. Accordingly, CRP, homocysteine and fibrinogen are considered as representative risk markers for ASVD (Hackam and Anand, 2003).

Previous epidemiological studies and randomized controlled trials have showed that oral therapy with CEE or E₂ may alter the CRP, homocysteine or fibrinogen concentrations rapidly during early period of treatment (Davison and Davis, 2003). However, many of these studies were designed using estrogen combined with progestin as HRT, and progestin per se remains controversial because of its effects on the circulating concentrations of these markers. In the current study, by using unopposed estrogen in hysterectomized postmenopausal women, we were able to provide explicit evidence without confounding factors. Furthermore, by comparing the circulating concentrations of E₂ before and after the treatment period via either oral or transdermal route, we demonstrated that E₂ was elevated to the same level in both the groups and ensured that the difference did not originate from a dosage discrepancy.

Only sparse randomized controlled studies have addressed the CRP concentrations in healthy postmenopausal women receiving transdermal estrogen without progestin (Vehkavaara et al., 2001; Vongpatanasin et al., 2003; Zegura et al., 2003). Conducting a randomized, double-blind, cross-over trial on the CRP between oral and transdermal unopposed estrogen therapy in 21 subjects, Vongpatanasin et al. (2003) reported that oral estrogen induced an increase in CRP, which was not changed by a transdermal route of administration. In the present study, we demonstrated that oral CEE increased acute inflammatory CRP, whereas transdermal estrogen did not change the level of this biomarker. Our data concurred with the results of Vongpatanasin et al. and supported the hypothesis postulated by the WHI observational study that HRT-associated increases in CRP are caused by a direct hepatic pass effect of oral estrogen and not by any effect of systemic inflammation (Pradhan et al., 2002).

Homocysteine is considered as an important independent risk factor for ASVD and increases after menopause in healthy women. Unlike the effect on CRP, HRT in forms of oral estrogen has been shown to decrease homocysteine levels, which implies a decreased ASVD risk in HRT users. However, most studies have used progestin in combination with estrogen, and different kinds of progestin have been reported to enhance or attenuate the effects on plasma homocysteine. To accurately assess the effect of estrogen on homocysteine, we used unopposed estrogen through different administration routes in the current study. We demonstrated that neither oral CEE nor transdermal 17β-E₂ caused a significant change in fasting serum homocysteine level during the 6 months of treatment.

Figure 1. (A) The baseline flow-mediated vasodilation (FMD) of the brachial artery from control (n = 30) and oral (n = 18) or transdermal (n = 18) HRT groups. (B) Comparison of changes in the FMD of the brachial artery before (open column) and after (closed column) 6 months of administration of oral conjugated equine estrogen (0.625 mg/day) or transdermal 17β-estradiol gel (0.6 mg/day). NS indicates not significant.
We also showed that the changes in fasting serum homocysteine levels during the study period did not differ between HRT groups and control group. To our knowledge, Smolders et al. (2003) reported the only randomized controlled trial on the effect of unopposed transdermally administered 17β-E2 on homocysteine. Our results are partly in agreement with those of Smolders et al. who reported that unopposed transdermal 17β-E2 did not significantly reduce homocysteine levels.

Unopposed oral estrogen was reported to decrease homocysteine levels in postmenopausal women in some studies (Mijatovic et al., 1998; Smolders et al., 2003). However, our results did not show such a benefit on this ASVD risk marker. In a Netherlands study (van Baal et al., 1999), the benefit of lowering the homocysteine levels from oral HRT was indicated to be attributable to the high baseline homocysteine levels, which implies that a different subject characteristic may influence study results. The baseline homocysteine levels in the European study are higher than those in the current study, which may have been affected by our subject selection criterion that limited the study to healthy postmenopausal women. Moreover, people of Chinese ethnicity have been reported to have lower homocysteine levels because of higher serum folate levels, which maintain the activity of methylenetetrahydrofolate reductase (Kelemen et al., 2004). As a result, estrogen therapy provided no benefit in reducing homocysteine levels for postmenopausal women in our study.

Fibrinogen, like CRP, is an acute-phase reactant and is regarded as an important independent risk factor for CVD and ischemic stroke. Recent meta-analysis studies demonstrated that it represents a strong statistically significant risk for cardiovascular mortality and morbidity. Fibrinogen appeared to be increased after menopause in healthy women in one observational study; however, the association between fibrinogen levels and HRT remains debatable because some studies reported a decrease in fibrinogen levels by HRT, whereas others reported no change. In our study, neither route of HRT administration significantly changed the fibrinogen levels.

Endothelial dysfunction, especially reduction of the bioavailability of endothelium-derived nitric oxide (NO), is present in increased acute inflammatory CRP, transdermal estrogen was not associated with this effect. Additionally, transdermal and oral estrogen increased FMD of the brachial artery to the same extent. The WHI study indicated that oral estrogen therapy, whether combined with MPA or not, may increase ASVD risk. Our study using surrogate markers reveals that the transdermal administration route might be favourable in terms of ASVD risks; however, further studies are required to investigate whether transdermal estrogen therapy changes CHD or stroke incidence in healthy postmenopausal women in the long term.

Acknowledgement
This work was supported by research grant No 92–21 from Yen Tjing Ling Medical Foundation, Taipei, Taiwan.

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Submitted on February 15, 2006; resubmitted on April 6, 2006; accepted on April 14, 2006