Combined oestrogen–progestogen replacement therapy does not inhibit low-density lipoprotein oxidation in postmenopausal women

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Aims The use of oestrogen containing hormone replacement therapy (HRT) is related to a significantly reduced atherosclerotic cardiovascular risk in postmenopausal women. Oestrogen is thought to be antioxidant and may inhibit low-density lipoprotein (LDL) oxidation *in vitro*. We investigated the effect of combined oestrogen and progestogen HRT on LDL oxidation in postmenopausal women.

Methods Eighteen healthy women were given oestrogen/progestogen, and the susceptibility of LDL to oxidation was measured as the level of autoantibody to oxidative modified LDL and the production of conjugated dienes during copper-dependent oxidation after 3 and 6 months HRT. The levels of vitamin E, the major antioxidant in LDL, were also measured.

Results After HRT, the anti-oxidatively modified LDL antibody level remained unchanged [1.58 ± 0.16 , 0.10 (-0.10 , 0.26), and 0.08 (-0.09 , 0.19), mean $\pm$ s.d. at baseline, and mean change with 95% confidence intervals for differences at 3 and 6 months, respectively, $P > 0.05$] as did the production of conjugated dienes when determined as lag phase [51.2 ± 7.5 , -0.3 (-3.9 , 3.3), and 1.5 (-3.4 , 6.4) min, $P > 0.05$]. The LDL vitamin E content, measured as α -tocopherol, was also not altered [2.34 ± 0.54 , -0.07 (-0.27 , 0.13), and -0.07 (-0.33 , 0.16) nmol mg⁻¹ LDL, $P > 0.05$] by treatment.

Conclusions Combined oestrogen and progestogen therapy for 6 months in postmenopausal women does not protect LDL against oxidation.

Keywords: postmenopause, hormone replacement therapy (HRT), antioxidant, oestrogen, vitamin E, LDL oxidation, atherosclerosis

Introduction

It is considered that the increased risk of coronary heart disease (CHD) in postmenopausal women may be due to a fall in female hormones and can be greatly reduced by oestrogen containing hormone replacement therapy (HRT) [1]. The major anti-atherosclerotic effect of oestrogen is associated with its beneficial influence on lipid metabolism, including increased high-density lipoprotein (HDL), especially HDL2, decreased low-density lipoprotein (LDL), lipoprotein(a) [Lp(a)] and total cholesterol concentrations [2]. Other proposed mechanisms include inhibition of endothelial hyperplasia, reduced

arterial impedance, enhanced production of prostacyclin and increased insulin sensitivity [1]. However, because of the increased risk of endometrial hyperplasia and carcinoma with oestrogen alone, most postmenopausal women who have not undergone hysterectomy are treated with progestogen in addition to oestrogen [3]. There have been concerns that progestogen may oppose some of the cardiovascular benefits of oestrogen. Although it has recently been shown that combined oestrogen and progestogen therapy maintains the beneficial effect of oestrogen in cardiovascular disease but lacks the proliferative effect on the endometrium [4], the long term effect of protective effect of combined HRT in CHD needs further investigation.

Another potential 'cardioprotective' effect of HRT is its antioxidant function [5]. Free radical mediated oxidised LDL may be readily taken up by scavenger receptors on macrophages leading to the formation of lipid-laden foam

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cells beneath the arterial endothelium, now recognised to be related to the initiation of atherosclerosis [6]. Supplementation of antioxidants inhibits *in vitro* LDL oxidation in man [7] and the progression of atherosclerosis in experimental animals [8]. Recently published studies have reported the significant benefits of supplementation of dietary antioxidants, mainly vitamin E, in the prevention of atherosclerotic disease [9–13]. Oestrogens have been shown to be free radical scavengers. The addition of oestrogens to isolated LDL solution effectively inhibited the *in vitro* oxidation of LDL induced by copper [14]. Sack *et al.* [15] reported that the acute use of oestrogen in the form of intra-arterial infusion and 3 weeks transdermal patch, resulted in a significantly reduced susceptibility of LDL to *in vitro* oxidation in postmenopausal women. While the addition of progestogen, alone or with oestradiol, does not affect LDL oxidation *in vitro* [16], the effect of a relatively long term of combined oestrogen and progestogen replacement therapy on LDL oxidation has not been documented.

The autoantibodies against oxidised-LDL are found in human plasma and atherosclerotic plaques [6] and the presence of antibodies to malondialdehyde-derived LDL has been demonstrated as an independent predictor of the progression of carotid atherosclerosis [17]. LDL oxidation is also commonly studied *in vitro*, for example, by following the generation of oxidized products during copper initiated oxidation. The resistance of LDL to *in vitro* oxidation has been found to be correlated with the extent of coronary atherosclerosis [18].

In this study, we assessed *in vivo* oxidative modification of LDL by measuring the titres of autoantibody to oxidised LDL and we also determined the susceptibility of LDL to copper induced oxidation in a group of healthy postmenopausal women receiving oestrogen and

progestogen combined hormone replacement therapy. As vitamin E is the major lipophilic antioxidant in LDL and since oestrogens, especially 2-hydroxyestrone, may regenerate oxidised vitamin E *in vitro* [19], LDL vitamin E content in these subjects was also measured.

Methods

The subjects studied comprised 18 healthy postmenopausal women aged 46–56 years attending the Menopause clinic at the Rotunda Hospital in Dublin. All the subjects were non-hysterectomised and amenorrhoeic for at least 1 year. The mean serum follicle-stimulating hormone (FSH) was $76.35 \pm 24.59 \text{ IU l}^{-1}$ and they all had oestradiol levels $< 40 \text{ pmol l}^{-1}$. Body mass index, blood pressure and plasma lipids were within the normal range and are presented in Table 1. All the subjects were free of any other medication and vitamin supplements. The subjects were instructed and agreed to maintain their usual dietary style and physical activity level during the period of the study. The study was approved by the ethics committee of the Hospital. Initially designed as a placebo controlled study subjects would not agree to a possibility of no treatment for symptoms of the menopause and the placebo arm was omitted.

The 18 postmenopausal women were given a combination of oestrogen/progestogen (Harmogen/Provera) therapy. Harmogen was taken 1.5 mg daily and on the 18th day Provera 5 mg daily was added for 12 days to this 30 day regimen. Blood samples were taken from all subjects after 12 h overnight fast at baseline and repeated at the end of the third and the sixth month of study. Blood (20 ml) was collected into two vacutainers with 10 ml capacity containing 0.12 ml of 15% ethylenediaminetetraacetic acid tri-potassium salt (EDTA K3) as

	Baseline	3 months HRT	6 months HRT
Body mass index (kg/m ²)	23.6 ± 4.3	23.7 ± 4.2	23.3 ± 4.5
Blood pressure (mmHg)	116 ± 11/77 ± 8	115 ± 10/73 ± 8	115 ± 13/75 ± 7
Plasma lipid profile (mmol l ⁻¹)			
Total cholesterol	5.67 ± 0.97	*5.35 ± 0.76	*5.10 ± 0.76
Triglyceride	1.2 ± 0.62	1.2 ± 0.71	1.3 ± 0.63
HDL	1.46 ± 0.19	1.55 ± 0.34	1.59 ± 0.26
HDL2	0.14 ± 0.06	0.15 ± 0.06	0.18 ± 0.12
HDL3	1.32 ± 0.16	1.25 ± 0.26	1.40 ± 0.27
LDL	3.51 ± 0.54	*3.07 ± 0.52	*2.92 ± 0.84
Lp(a) (mg l ⁻¹)	363.6 ± 349.9	337.6 ± 381.4	318.4 ± 283.1
Serum oestradiol (pmol l ⁻¹)	< 40	**138.4 ± 52.0	**123.1 ± 37.8

Table 1 Some clinical characteristics of postmenopausal women before and after HRT (mean ± s.d., *n* = 18).

P* < 0.02; *P* < 0.001 in comparison with baseline.

anticoagulant. Plasma was obtained following centrifugation within 1 h of venepuncture. Aliquots were stored at -70°C and the measurements were carried out within 2 weeks.

Low-density lipoprotein isolation

LDL (density $1.019\text{--}1.063\text{ g ml}^{-1}$) was isolated within 2 h from plasma with single vertical spin gradient ultracentrifugation [20] using a Beckman Optima TLX ultracentrifuge. In brief, plasma was adjusted to density 1.30 kg l^{-1} by adding solid potassium bromide and 2 ml of adjusted plasma was placed in an ultracentrifuge tube (4.7 ml capacity) (Beckman, CA, USA). Plasma was then carefully overlaid with about 2.6 ml of salt solution (density $=1.019\text{ g ml}^{-1}$). The tubes were balanced and spun at $543\,000\text{ g}$ for 45 min at 4°C using a TLA 100.4 rotor. The LDL fractions were visible as yellow to orange coloured bands and were removed by puncturing the side wall of the tube with a syringe and needle. Agarose gel electrophoresis (Beckman Paragon, USA) was used to identify the isolated LDL. LDL fraction was immediately desalted using gel filtration (Sephadex G-25M, Pharmacia, Sweden). Desalted LDL was placed in ice under nitrogen in the dark. The concentrations of LDL protein were measured according to a modified version [21] of Lowry's assay and adjusted to 0.1 mg ml^{-1} with phosphate-buffered saline (PBS, pH 7.4) containing $2\text{ }\mu\text{mol l}^{-1}$ EDTA Na2.

Evaluation of anti-oxidised-LDL antibodies

An enzyme-linked immunosorbent assay (ELISA) method was employed to measure autoantibodies to oxidised-LDL in all subjects before and after HRT. The method was based on that described by Palinski *et al.* [22] and Maggi *et al.* [23]. Antigens used were native LDL containing and malondialdehyde (MDA)-derivatized LDL at the concentration of $50\text{ }\mu\text{g LDL protein ml}^{-1}$ in PBS [22]. Ninety-six well disposable polystyrene plates (Nunc, Denmark) were used for this assay. Each well was coated with $200\text{ }\mu\text{l}$ of antigen and incubated at 4°C for 16 h. The plate then was washed four times with PBS containing 0.05% Tween-20 (PBS-T) and blocked using 3% bovine serum albumin in PBS-T for 2 h at room temperature. After further washing with PBS-T, $220\text{ }\mu\text{l}$ of 1:11 diluted plasma the sample was added to each well and the plate was incubated overnight at 4°C . The plate was washed again. $100\text{ }\mu\text{l}$ of peroxidase-conjugated monoclonal antibody, specific for human Ig G (Sigma, St Louis, USA) 1:2000 diluted in PBS-T, was added to the wells and the plate was incubated for 1 h at 37°C . The peroxidase activity was developed by adding phenylenediamine dihydrochloride (Sigma P-9187) and read in

an automatic microplate reader. To calculate antibody titres, the ratio between the spectrophotometric readings of anti-oxidative antigen *vs* anti-native antigen wells was used. The readings of anti-native antigen wells represent the corresponding blank of anti-oxidative modified antigen wells.

Copper induced LDL oxidation

The *in vitro* oxidation of LDL (0.1 mg ml^{-1}) was initiated by adding copper sulphate ($7\text{ }\mu\text{mol l}^{-1}$) and was carried out at 37°C for 4 h. Two indices of oxidation were used to assess the production of oxidative LDL. The amount of lipid peroxide formed in oxidised LDL was determined as thiobarbituric acid reactive substances (TBARS) at the end of every hour as described by Buege [24] and expressed as nmol malondialdehyde equivalents mg^{-1} of LDL protein compared with 1,1,3,3-tetramethoxypropane (TEP) standard. The production of conjugated dienes was assessed by measuring the 'lag phase' as described by Esterbauer [25] with continuous monitoring spectrophotometry (Unicam 8300) at 234 nm at 3 min intervals. The lag phase was determined as minutes before the polyunsaturated fatty acids contained in LDL begin to be oxidised at a maximum rate.

The measurement of LDL α -tocopherol

LDL vitamin E content was measured as α -tocopherol by using high-performance liquid chromatography (h.p.l.c., Shimadzu LC-6A) at 292 nm spectrophotometrically [26]. α -tocopherol in LDL was extracted with *n*-hexane and dried under nitrogen stream. The dried residue was then redissolved in methanol and injected into h.p.l.c. system. The peak areas were measured using a microcomputerized data processor (Shimadzu C-R3A) and calculated against a standard curve prepared by using d- α -tocopherol from Sigma.

The measurement of plasma lipid

Plasma total cholesterol was measured using automated enzymatic-colorimetric method on a Boehringer Mannheim analyzer (Mannheim, Germany). HDL cholesterol was determined after selective precipitation using manganese-heparin followed by enzymatic determination of cholesterol. Subclasses of HDL cholesterol were measured using the method by Gidez *et al.* [27]. HDL2 cholesterol was precipitated and calculated as the difference between total HDL cholesterol and HDL3 cholesterol. LDL was calculated using the formula by Friedewald *et al.* [28]. Plasma triglycerides were determined with an enzymatic kit from Biomerieux (Enzymatique PAP 500, Biomerieux, France).

Lipoprotein(a) concentrations were measured using an ELISA technique (TintElize Lp(a), Biopool, Sweden).

Data analysis

Results are expressed as mean $\pm$ s.d. and 95% confidence intervals for differences. The effect of HRT on measured parameters were compared with baseline levels using paired Student's *t*-test where the data were normally distributed and Wilcoxon rank sum test where it was not. The relationships between the variables were estimated with linear regression analysis. In all comparisons differences were considered significant at a *P* value of 0.05 or less.

Results

All of the subjects tolerated HRT well and experienced relief of symptoms related to oestrogen deficiency. All these women reported that they maintained a similar dietary style and level of physical activity throughout the study. Serum oestradiol level significantly increased after 3 and 6 months HRT (Table 1). Plasma total cholesterol, LDL, HDL, triglyceride and Lp(a) levels were within the normal range at their baseline. After 3 months HRT, significantly reduced levels of total cholesterol and LDL were observed and remained unchanged at 6 months. HDL levels were increased slightly and a small decrease in Lp(a) level (by 12%) was observed after 6 months but neither was statistically significant (Table 1).

After 3 and 6 months HRT, the oxidizability of LDL, when assessed either as copper induced oxidation (conjugated dienes production and TBARS formation) or the titre of plasma anti MDA-modified LDL antibodies, was not altered (Table 2).

LDL α -tocopherol did not change significantly during HRT. However, as the major antioxidant in LDL, α -tocopherol values correlated positively with the oxidative susceptibility of LDL at baseline ($r=0.68$, $P<0.01$, Figure 1) and after 3 ($r=0.54$, $P<0.05$) and 6 months ($r=0.56$, $P<0.05$) treatment with HRT. There was no correlation found between serum oestradiol level and oxidizability of LDL when determined either as lag phase of copper-dependent oxidation or autoantibodies to oxidised LDL at baseline, 3 and 6 months of HRT.

Discussion

Our study showed that combined oestrogen/progestogen HRT for 6 months in postmenopausal women produced the expected alterations in lipids but did not alter the oxidative susceptibility of LDL to *in vitro* oxidation. We also found that HRT did not affect LDL α -tocopherol concentration and this finding seems to exclude the

possibility that oestrogen treatment may protect LDL against oxidation by regenerating the major lipophilic antioxidant, vitamin E. Allowing for differences in study design, these results from our study are in disagreement with those of Sack *et al.* [15].

Metal ion induced oxidation is a commonly used assay to assess indirectly the *in vivo* antioxidant protection of LDL against oxidative modification. An effective antioxidant in such an experimental procedure should be lipophilic and able to be present at a relatively high concentration in isolated LDL. O'Leary *et al.* recently demonstrated that the supplementation of antioxidants, vitamin E and probucol, massively increased their concentrations in LDL and consequently inhibited copper ion induced oxidation [29].

Addition of oestrogens, among which 17 β -oestradiol is the most effective one, to isolated LDL solutions significantly inhibits copper induced LDL oxidation. However, in such an assay the oestradiol level needed to inhibit LDL oxidation is $1 \mu\text{mol l}^{-1}$ or higher, which is one thousand fold higher than the mean physiologic serum oestradiol level which is usually 700 pmol l^{-1} in young women [30]. The level of oestrogen naturally present in LDL is unknown; this may be due to a lack of sensitivity in the methodology used to measure oestrogen in LDL.

Being highly lipophilic, oestrogen can be easily concentrated in LDL particle and may not be removed by isolating LDL from plasma with ultracentrifugation. However, more than 90% of the oestradiol in circulation is bound to sex hormone binding globulin (60%) and albumin (38%) and only about 2% is free (unbound and unconjugated) and biologically active [31]. The measurement of serum oestradiol levels reflects the total amount in blood, i.e. bound and unbound. The oestrogen used in this study was oestrone which may be converted to oestradiol *in vivo*, and such oral therapy for 3 and 6 months resulted in mean serum oestradiol level seen as 138 and 123 pmol l^{-1} , respectively (Table 1). It is therefore questionable that a limited increase of oestradiol in circulation, of which the majority is bound to proteins other than LDL, could result in an extremely high level of oestradiol in a diluted LDL solution. It is unlikely that increased serum oestradiol levels could influence the resistance of LDL to oxidation.

α -tocopherol is the most abundant and active antioxidant in LDL [32]. Greatly elevated α -tocopherol levels in LDL may increase its resistance to copper induced oxidation [26]. α -tocopherol may be oxidized to tocopheroxyl, a relatively stable radical and lose its antioxidant function. It has been shown that oestrogen may donate a hydrogen atom of the OH group at its aromatic ring to tocopheroxyl thereby reducing oxidized vitamin E and retarding the progression of LDL oxidation

Table 2 Oxidation of LDL and LDL α -tocopherol level before and after HRT ($n=18$). The antibody titres were expressed as the ratio between the spectrophotometric readings of anti-oxidative antigen *vs* anti-native antigen wells. Results were presented as mean $\pm$ s.d. at baseline, and as mean change with 95% confidence intervals at follow-up.

	Baseline	Change to 3 months HRT	6 months HRT
Autoantibodies to MDA-LDL:			
95% CI	1.58 ± 0.16	0.10 -0.10, 0.26	0.08 -0.09, 0.19
Lag phase (min)	51.2 ± 7.5	-0.3 -3.9, 3.3	1.5 -3.4, 6.4
TBARS formation (nmol MDA mg ⁻¹ LDL)			
1st hour	16.04 ± 5.83	1.46 -3.16, 6.08	1.47 -3.03, 5.6
95% CI			
2nd hour	58.08 ± 12.76	-2.19 -11.40, 7.18	-0.08 -10.33, 10.26
95% CI			
3rd hour	81.16 ± 13.0	-1.82 -8.64, 5.54	-4.13 -13.69, 6.06
95% CI			
4th hour	83.11 ± 11.54	-0.49 -7.99, 7.06	-0.73 -8.47, 7.20
95% CI			
LDL α -tocopherol (nmol mg ⁻¹ LDL)	2.34 ± 0.54	-0.07 -0.27, 0.13	-0.07 -0.33, 0.16
95% CI			

$P > 0.05$ in all comparisons with the baseline.

Figure 1 The correlation between lag phase of LDL oxidation and LDL vitamin E content, $r=0.68$. $P<0.01$.

[19]. We investigated whether such an effect may happen by oral HRT and found that the LDL content of α -tocopherol remained unchanged after 6 months HRT as shown in Table 2. On the other hand, decreased vitamin E level has been observed in plasma from young women taking oral contraceptives containing oestrogen [33]. It has been suggested that high levels of oestrogen, particularly diethylstilbestrol, may be associated with the generation of oxygen active species [34]. Our results show that oestrogen containing HRT in contrast to oral contraceptives did not modify the oxidizability of LDL by affecting its main antioxidant, vitamin E, level.

There is no doubt that oestrogen containing HRT favours a healthier lipid profile. However, HRT may be associated with an elevated proportion of small and dense LDL in LDL subfractions [35, 36]. The pathogenic role of small and dense LDL in coronary heart disease has been well established [37]. LDL particles containing more small and dense LDL are more oxidizable and are less protected by antioxidants than larger, more buoyant LDL [38, 39]. Because HRT is associated with a reduction in plasma total cholesterol and LDL levels, the overall effect on atherosclerotic event is seen as a reduced risk. When LDL is isolated and diluted according to a standard concentration, however, the increased proportion of small and dense LDL in a given LDL sample may possibly promote the process of oxidative modification. The present study did not investigate the effect of HRT on the size and dense of LDL but we could not exclude the possibility of such a minor effect which may be counteractive to any protection obtained from HRT.

Copper induced oxidation of isolated LDL is an *in vitro* assessment of LDL oxidation and does not take into consideration the *in vivo* interaction between LDL and other protective factors, e.g. water soluble antioxidants. The measurement of autoantibodies to oxidised LDL however is an *in vivo* indicator to evaluate the antioxidant role of oestrogen and reflects more accurately the natural milieu. Our results suggest that *in vivo* HRT is unlikely to affect LDL oxidation.

Nenseter and colleagues recently reported that 12 weeks HRT with combined 17 β -oestradiol and norethisterone acetate did not show a protective effect

on LDL oxidation in hypercholesterolaemic postmenopausal women [40]. Our additional findings indicate that combined oestrogen/progestogen HRT does not protect LDL against oxidation when investigated as either copper induced oxidation or antibodies to oxidised LDL in healthy women, nor does such treatment affect *in vivo* vitamin E status. However, we do not rule out the possibility that oestrogens may inhibit free radical damage to tissue in other ways. Rifici *et al.* [41] suggested that free or protein bound oestradiol may scavenge free radicals in the subendothelial space of artery wall therefore inhibiting the formation and progression of fatty streaks.

The effect of combined oestrogen/progestogen on blood lipids has been controversial. There is a suggestion from our results that the addition of progestogen appears to oppose the beneficial effects of oestrogen therapy alone. HDL level was not significantly affected, although total cholesterol and LDL levels fell. But the relatively small number of the subjects with the low dose preparation employed in the study makes it difficult to draw definite conclusions.

In summary oestrogen/progestogen HRT does not affect oxidizability of LDL. This may be due to the levels of oestradiol contained in LDL which does not reach concentrations required to influence oxidizability of LDL. HRT does not seem to protect or preserve the major natural antioxidant, vitamin E, in LDL. There may be increased small and dense LDL particles after HRT and LDL samples with such pattern may possibly counteract the protective effect gained from the relatively minor increase in oestradiol level. We conclude that oral administration of combined oestrogen/progestogen HRT for 6 months in healthy postmenopausal women does not affect LDL oxidation.

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