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Lipoproteins and low-dose estradiol replacement therapy in post-menopausal Type 2 diabetic patients: the effect of addition of norethisterone acetate

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Received 9 March 1999; revised 20 October 1999; accepted 6 February 2000

Abstract

Aims Low-dose continuous oestrogen/progestogen may increase patient compliance long-term but the cardioprotective effects in diabetes are unknown. The aim of this study was to compare the effect of low-dose oral oestrogen (1 mg, 17-β-estradiol) treatment with oestrogen (1 mg 17-β-estradiol) in combination with low-dose (0.5 mg) continuous norethisterone acetate (NETA) on lipoproteins in Type 2 diabetic patients.

Methods Thirty-four post-menopausal Type 2 diabetic patients in moderate control (mean haemoglobin A_{1c} 7.7%) who had a serum oestradiol level of < 50 pg/ml were examined over a 6-month period. Serum lipids, and lipoprotein composition of very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) were measured. Serum lipoprotein(a) was determined by an ELISA method, LDL fatty acids by gas–liquid chromatography and LDL oxidizability by thiobarbituric acid reactive substances (TBARS assay). Cholesteryl ester transfer protein (CETP), and cell cholesterol were measured.

Results There was a reduction in serum cholesterol on both treatments but no significant difference between treatment groups. LDL cholesterol decreased by 17% in each group. There was a no significant difference between the groups in serum VLDL or HDL cholesterol or serum triglycerides during the study. The change in lipoprotein(a) during the study was not significantly different between the groups. There was no significant difference in 4 h LDL oxidizability between groups. Although CETP increased with time in both groups there was no significant difference in the change between the groups.

Conclusion In this small study, the addition of continuous low-dose NETA did not reduce the potentially beneficial effects of low-dose 17-β-estradiol on the progression of atherosclerosis in diabetes.

Diabet. Med. 17, 308–315 (2000)

Keywords 17-β-estradiol, cell cholesterol, cholesteryl ester transfer protein, hormone replacement therapy, LDL fatty acids, lipoprotein composition, norethisterone acetate, plasma cholesterol, Type 2 diabetes mellitus

Abbreviations CETP, cholesteryl ester transfer protein; FSH, follicle stimulating hormone; HDL, high density lipoprotein; HRT, hormone replacement therapy; LDL, low density lipoprotein; Lp(a), lipoprotein(a); NETA, norethisterone acetate; TBARS, thiobarbituric acid reactive substances; VLDL, very low density lipoprotein

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Introduction

Type 2 diabetic patients have up to a fivefold increase in cardiovascular disease. The major abnormalities in lipoproteins in Type 2 include raised serum triglyceride, low high density lipoprotein (HDL) cholesterol and abnormal composition of low density lipoprotein (LDL). This abnormal composition leads to increased oxidizability which is mostly a result of increased linoleic acid in the LDL fraction [1,2]. These abnormalities are considered to be potentially atherogenic. There is some suggestion that patients with diabetes lose the protective effect of oestrogen on cardiovascular disease seen in non-diabetic women of childbearing age [3]. This has prompted research into the effect of oestrogen and progestogen in diabetes after the menopause when diabetic subjects are at most risk of cardiovascular events.

The beneficial effects of oestrogen on lipid profiles include lowering total cholesterol and LDL cholesterol and raising HDL cholesterol. Some of these effects may be neutralized by the addition of progestogen, which is necessary to prevent endometrial hyperplasia and the risk of endometrial cancer [4–6]. Recently, hormone replacement therapy in post menopausal women has been administered as combined oestrogen/progestogen in a continuous instead of cyclical manner in an effort to inhibit menstruation. The dose of oestrogen replacement which has most benefit and least side-effects has yet to be defined. A low-dose preparation combining estradiol/norethisterone acetate has been investigated in non-diabetic women and found to have high patient acceptability with beneficial effects on lipids [6] bone loss [7] and vasomotor symptoms as well as providing good endometrial protection [8]. There is little data on the effect of combined oestrogen/progestogen hormone replacement in diabetes. In a recent case-controlled study of post-menopausal oestrogen use and incident myocardial infarction (MI) in diabetic women, current use of oestrogens was associated with a reduced risk of MI relative to never use although the 95% confidence interval for the relative risk was wide. The sample was small and did not include enough hormone users to examine separately various oestrogen doses or combined oestrogen/progestogin therapy [9]. Short-term estradiol replacement in post-menopausal women with Type 2 diabetes has been shown to improve insulin sensitivity in the liver and glycaemic control. It also reduced LDL cholesterol and increased HDL cholesterol [10]. Estradiol *in vitro* has an inhibitory effect on the oxidation of low density lipoprotein [11–13] but this may not occur *in vivo* [14].

The effect of progestogen is variable: most progestogens appear to attenuate the oestrogen-induced increase in HDL [15] or even reduce HDL levels [5,6]. Some progestogens lower LDL cholesterol and raise triglyceride levels [4] whereas others have been reported to decrease triglycerides [16] or leave them unchanged [5,6]. Hypertriglyceridaemia is a major lipid disturbance in diabetes and may be associated with small dense LDL [17]. Small dense LDL is potentially more atherogenic because it is more easily oxidized. Thus the specific type, dose and method of administration of HRT may have very different effects particularly in diabetes

The purpose of this study was to investigate the effect of six months treatment with low dose estradiol as compared to continuous low dose estradiol together with low dose norethisterone acetate on lipoprotein composition and LDL oxidizability in post-menopausal subjects with Type 2 diabetes mellitus.

Patients and methods

Patients

Forty-two postmenopausal, Type 2 diabetic patients were recruited for a randomized, parallel, double-blind study. The subjects were chosen from patients attending the diabetic clinic. Post-menopausal Type 2 diabetic women, under the age of 70 years, who were treated with diet alone or diet and oral agents and who had had a spontaneous menopause more than 1 year previously or hysterectomized women, were asked to take part in the study. People suffering from liver or thyroid disease, hypertension, proliferative retinopathy and those on lipid-lowering therapy or β-blockers were excluded from the study. Women with carcinoma of the breast or a family history of carcinoma of the breast were excluded. Women with any known or suspected estradiol-dependent neoplasia and those with deep venous thrombosis, thromboembolic disorders and cerebrovascular accidents associated with estradiol use were also excluded. Only subjects with plasma estradiol < 50 pg/ml, follicle stimulating hormone (FSH) > 40 mIU/ml, plasma cholesterol 8.5 mmol/l, blood glucose < 10 mmol/l and body mass index (BMI) = 30 kg/m^2 were included in the study. Other exclusion criteria were myocardial infarction in the past year and history of hypoglycaemia unawareness. The study was approved by the Hospital Ethics Committee and all subjects gave informed consent.

A screening visit was performed to evaluate the inclusion criteria and subjects underwent an endometrial biopsy or vaginal ultrasound to measure endometrial thickness. Thirty-eight subjects with normal screening tests were randomized (1:1 in blocks of 4) to 6 months daily treatment with either 1 mg 17-β-estradiol (n = 19) or 1 mg 17-β-estradiol + 0.5 mg norethisterone acetate (NETA) (Activelle) (Novo Nordisk, Bagsvaerd, Denmark) (n = 19). Women entering the study were given the lowest free randomization number. Each woman's treatment assignment was available in a sealed envelope and the code was not broken for any women during the study. Tablets were identical and were dispensed in identical 28-day packs. Patients diet was monitored throughout the study as was exercise to ensure that no major changes took place.

Four patients, all of whom had been randomized to estradiol + NETA, withdrew from the study before completion, one as a result of breast tenderness, two through non-

compliance and one through intermittent pain in the pelvis. These four patients were excluded from analysis. Nineteen of those included were therefore taking 17-β-estradiol alone and 15 were taking 17-β-estradiol + NETA. All subjects continued with their usual anti-diabetic treatment during the study. Of the estradiol group, six were treated with a sulphonylurea, four with sulphonylurea + metformin, two with metformin alone, one with guar and the remaining six with diet alone. Of the estradiol + NETA group, one woman was treated with sulphonylurea, one with sulphonylurea and guar, three with sulphonylurea and metformin, four with metformin alone and the remaining six with diet alone.

Methods

Blood was taken at the beginning of the study for baseline measurements and again at 3 and 6 months. Waist-to-hip ratio was determined at baseline and at 6 months. BMI (kg/m^2) and indices of glycaemic control were measured at baseline, 3 and 6 months. Blood haemoglobin A_{1c} (HbA_{1c}, normal value < 7.0%) was determined using an affinity binding assay (Abbott IMx, IL) and fructosamine was measured using a kit from Boehringer Mannheim (Mannheim, Germany). Serum insulin was determined by a microparticle enzyme immunoassay (Abbot Diagnostic Laboratories) which does not cross-react with proinsulin.

Lipoprotein isolation

Blood samples for lipoprotein isolation and mononuclear leucocyte preparation were taken into heparinized tubes and centrifuged within 1 h to separate plasma and cells. Ethylenediaminetetraacetic acid (1 mg/ml) and butylated hydroxytoluene were added to the plasma to prevent LDL oxidation. VLDL (density < 1.019 g/ml) LDL (density 1.019–1.063 g/ml) and HDL (density 1.063–1.21 g/ml) were isolated from plasma by sequential ultracentrifugation [18]. Plasma and lipoprotein total and free cholesterol and triglycerides were determined using enzymatic colorimetric assays (Boehringer Mannheim). LDL protein was measured by a modification of the Lowry technique [19]. Plasma LDL cholesterol was determined using the Friedewald equation and plasma HDL cholesterol was determined following precipitation of the apo B-containing lipoproteins with HDL reagent A (Immuno GmbH, Heidleberg, Germany). Lipoprotein(a) (Lp(a)) was measured using an enzyme-immunoassay IMMUNOZYM Lp(a) (Immuno, Sevenoaks, Kent, UK). Samples from the same patient were stored frozen at –70°C and all assayed on the same plate

LDL oxidizability

The susceptibility of LDL to *in vitro* oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) as previously described [20]. LDL, which had been stored frozen at –70°C was diluted with phosphate-buffered saline (PBS) immediately prior to oxidation to give a concentration of 300 μg protein/ml and dialysed at 4°C for 24 h against 2 × 5 l of PBS to remove antioxidants. LDL was oxidized by incubating with 10 μmol/l $CuSO_4$ at 37°C for up to 4 h. The oxidation was terminated by addition of EDTA (0.1 mmol/l) at 4°C. The degree of oxidation was assessed by measuring malondialdehyde (MDA) [21]. The intra-assay variation for the TBARS method was 3.4%. Baseline, 3 and 6 month samples from each patient were determined in the same assay.

LDL fatty acids

Heptadecanoic acid (100 μg) was added as an internal standard to LDL (1 mg/ml LDL protein) and the lipids were extracted by a modification of the method of Folch *et al* [22]. The organic fraction was dried with anhydrous sodium sulphate. The samples were dried under nitrogen and transmethylated [23]. Following transmethylation, the fatty acids methyl esters were extracted into hexane, dried under nitrogen and reconstituted in iso-octane immediately prior to determination by gas–liquid chromatography. The fatty acids were analysed in a Shimadzu GC-14 A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a capillary fused silica Permabond FFAP-DF-0.1 (25 m × 0.25 mm internal diameter) column (Durren, Germany). Fatty acids were expressed as a percentage of total fatty acids or quantified using the internal standard and expressed as μg/mg LDL protein as previously described [2] (intra-assay and interassay variations were 1.8 and 2.6%, respectively).

Cholesteryl ester transfer protein activity

Cholesteryl ester transfer protein (CETP) activity was measured by the method of Groener *et al* [24] using pooled [^{14}C]oleate-labelled LDL and unlabelled HDL from healthy subjects. Patients' plasma was treated with polyethylene glycol (HDL-reagent A, Immuno GMBH Heidleberg, Germany) (1/2 v/v) to precipitate endogenous VLDL and LDL. The supernatant from this procedure was incubated for 4 h at 37°C with [^{14}C]oleate-cholesteryl ester-labelled LDL and unlabelled HDL which act as donor and acceptor of transferred cholesteryl ester. Dithiobis-nitrobenzoic acid (140 mmol/l), was included to inhibit lecithin:cholesteryl acyltransferase. LDL was separated from HDL by dextran sulphate (2 mol/l)-$MgCl_2$ (10 g/l) precipitation and radioactivity measured as the bi-directional transfer of cholesteryl ester between radiolabelled LDL and unlabelled HDL. Results were expressed as nmol of ester transferred/ml plasma/h. Serum samples were stored frozen at –70°C following collection and all assayed in the same batch. The intra-assay variation was 10%.

Cellular cholesterol

Peripheral blood mononuclear cells (PBMC) were isolated from the patients blood at baseline, and at 3 and 6 months by centrifugation on Ficoll/Hypaque gradients using the method of Boyum [25]. Cells were stored frozen at –70°C and cell cholesterol determined in a single batch at the end of the study. Cell cholesterol was measured by a fluorimetric assay [26]. Cholesterol from PBMC was extracted into hexane/isopropanol. Total cell cholesterol was measured after enzymatic hydrolysis of cholesteryl esters, by oxidation of free cholesterol and reaction with *p*-hydroxyphenylacetic acid in the presence of

Table 1 Patient characteristics and diabetic control at baseline and after 6 months treatment with estradiol or estradiol + NETA.

	Baseline	3 month	6 month
Estradiol (n = 19)			
Age	65 (64–66)	–	–
BMI (kg/m^2)	27.7 (26.5–28.9)	27.8 (26.5–29.1)	28.1 (26.8–29.4)
Waist-to-hip	0.9 (0.8–0.9)	–	0.8 (0.7–0.8)
HbA_{1c} (%)	7.7 (7.1–8.2)	7.7 (6.4–8.3)	7.8 (7.0–8.6)
Blood glucose(mmol/l)	8.3 (7.4–9.2)	8.6 (7.5–9.7)	8.8 (7.5–10.1)
Fructosamine (μmol/l)	366 (329–402)	323 (298–347)	332 (308–356)
Fasting serum insulin(μU/ml)	7.4 (5.4–9.4)	8.4 (5.8–11.0)	7.6 (6.3–8.9)
Estradiol + NETA (n = 15)			
Age	62 (58–66)	–	–
BMI (kg/m^2)	27.8 (26.2–29.3)	27.9 (26.3–29.4)	27.7 (23.9–31.5)
Waist-to-hip	0.8 (0.4–1.2)	–	0.9 (0.8–1.0)
HbA_{1c} (%)	7.2 (6.8–7.6)	7.2 (6.8–7.6)	7.5 (6.0–9.0)
Blood glucose (mmol/l)	8.2 (7.3–9.1)	8.5 (7.4–9.6)	8.0 (7.3–8.8)
Fructosamine (μmol/l)	358 (325–391)	338 (369–375)	324 (296–352)
Fasting serum insulin (μU/ml)	9.8 (7.6–12.0)	9.3 (6.1–12.5)	8.8 (6.7–10.9)
	Difference between groups (estradiol – estradiol + NETA)		
BMI	–0.35 (–1.09–0.40)		
waist/hip	0 (–0.05–0.05)		
HbA_{1c} (%)	0.10 (–0.77–0.98)		
Blood glucose (mmol/l)	–0.70 (–2.08–0.67)		
Fructosamine (μmol/l)	–5.52 (–44.0–33.01)		
Fasting serum insulin(μU/ml)	–0.08 (– 0.05–1.89)		

Mean (95% confidence intervals)

peroxidase to form a fluorescent product. Results were expressed as mg cholesterol/mg cell protein. Cells were assayed in duplicate in the same assay batch and the intra-assay variation was 7.5%.

Statistical analyses

Results were expressed as mean with confidence intervals. Statistical analysis was performed using Student's t-tests for between group analysis and ANOVA analysis of variance for repeated measures, with time included as a factor, using SAS (release:11.6 for Unix; SPS Institute, Cary, NC). Correlation coefficients were determined by linear and multiple regression analysis using Statworks software (Computer Associates International, Islandia, NY) on an Apple Macintosh computer. Data were analysed using an analysis of covariance approach. Six-month values were modelled as a function of treatment (E or E + NETA) with baseline values included as a covariate. This approach estimates the difference between those receiving oestrogen alone and those receiving oestrogen and NETA corrected for differences between the groups in initial values of the parameter being examined. Specifically, the estimate of the difference between the groups is an estimate of the effect of treatment at the mean level of the pretreatment values. Data were analysed using Stata Release 6 (Stata Corporation, College Station, TX). Lp(a) was log-transformed before analysis, which improved its fit to a normal distribution. The resulting distribution was narrower than a true normal distribution, but symmetrical, and correlated 0.950 with the values expected from a normal distribution. A P-value of < 0.05 was considered to be statistically significant

Results

Subjects characteristics at baseline, and 6 months are given in Table 1. The patients were of similar age. There was no significant difference in BMI between the groups at the beginning of the study and no difference between the groups during the study. There was no difference in waist-to-hip ratio between the groups and no change with time. The other parameters also remained unchanged during the study and in particular there was no change in diabetic control or fasting serum insulin. No subject had clinical evidence of endometrial hypertrophy at the end of the study. In the estradiol alone group, one subject had simple hyperplasia without atypia, the others were classified as being normal. In the estradiol + NETA one subject had an endometrial polyp at the end of the study and the rest were normal.

Estradiol and follicle stimulating hormone (FSH) levels showed no difference in the two groups at the beginning (estradiol 16.8 (9.9–21.9) and estradiol + NETA 19.9 (14.1–25.7) pg/ml) and end of the study 96.0 (57.3–134.7) and 102 (71.7–133.3)pg/ml). FSH decreased in both groups from 56.2 (49.4 to 63.0) to 30.7 (24.4–37.0)

Table 2 Effect of 6 months' treatment with estradiol or estradiol + NETA on plasma lipoproteins

	Baseline	3 month	6 month	Change 0–6 months
Estradiol (n = 19)				
Cholesterol (mmol/l)	6.8 (6.2–7.4)	6.3 (5.7–6.8)	6.1 (5.7–6.5)	–0.65 (0.2–1.1)
LDL cholesterol (mmol/l)	4.8 (4.3–5.2)	4.1 (3.6–4.6)	3.9 (3.6–4.2	–0.8 (0.5–1.3)
HDL cholesterol (mmol/l)	1.3 (1.1–1.5)	1.3 (1.1–1.5)	1.3 (1.1–1.5)	0.05 (–0.1–0.2)
VLDL cholesterol (mmol/l)	0.7 (0.5–0.9)	0.8 (0.6–1.0)	0.8 (0.6–1.0)	–0.1 (–0.6–0.2)
Triglyceride (mmol/l)	1.7 (0.4–2.1)	1.8 (1.6–2.3)	1.7 (1.3–2.0)	0.0 (–0.2–0.3)
Lp(a) (log mg/dl)	1.2 (0.9–1.5)	1.2 (0.9–1.5)	1.2 (0.9–1.5)	–0.1 (0.1–0.15)
Range (median)	1–144 (16)	1–168 (11)	1–168 (18)	
Estradiol + NETA (n = 15)				
Cholesterol (mmol/l)	6.1 (5.6–6.6)	5.6 (5.0–6.2)	5.6 (5.0–6.2)	–0.5 (0.2–0.8)
LDL cholesterol(mmol/l)	4.3 (3.8–4.8)	3.7 (3.1–4.3)	3.5 (3.0–4.0)	–0.7 (0.3–1.2)
HDL cholesterol(mmol/l)	1.1 (0.9–1.2)	1.1 (1.0–1.2)	1.2 (1.0–1.3)	0 (–0.05 to –0.1)
VLDL cholesterol(mmol/l)	0.8 (0.7–0.9)	0.8 (0.6–1.0	0.8 (0.5–1.1)	0 (–0.1 to –0.2)
Triglyceride (mmol/l)	1.8 (1.4–2.2)	1.8 (1.4–2.2)	1.9 (1.4–2.4)	0.1 (–0.3–0.4)
Lp(a) (log mg/dL)	1.4 (1.0–1.8)	1.2 (0.8–1.6)	1.2 (0.8–1.6)	–0.1 (0.0–0.2)
Range (median)	1–176 (35)	1–132 (28)	1–110 (31)	
	Difference between groups (estradiol – estradiol + NETA)			
Cholesterol (mmol/l)	–0.16 (–0.62–0.31)			
LDL cholesterol (mmol/l)	–0.11 (–0.57–0.34)			
HDL cholesterol (mmol/l)	0.06 (–0.22–0.10)			
VLDL cholesterol (mmol/l)	–0.03 (–0.25–1.20)			
Triglyceride (mmol/l)	0.08 (–0.35–0.51)			
*Lp(a) (log mg/dl)	–0.05 (–0.17–0.07)			

Mean (95% confidence intervals). *A difference of –0.051 in the means of the log values is equivalent to a ratio of 1:(1–0.051) between the geometric means of the two groups.

mU/ml in the estradiol group and from 61.1 (32.9 to 70.7) to 22.5 (17.0–28.0) mU/ml in the estradiol + NETA group. There was no significant difference between the groups at the beginning or end of the study.

Plasma lipoproteins and lipoprotein composition

Lipoprotein levels are shown in Table 2. During the trial there was a reduction in total plasma cholesterol from 6.8 to 6.1 mmol/l in the estradiol group and from 6.1 to 5.6 mmol/l in the estradiol + NETA group but no significant difference in the change between the groups. There was a 17% decrease between 0 and 6 months in LDL cholesterol in both groups. The 17-β-estradiol group change of 0.8 (0.5–1.3) mmol/l was not significantly different from the 0.7 (0.3–1.2) mmol/l for the 17-β-estradiol + NETA group. Lp(a) decreased by 29% in the estradiol + NETA group but there was no significant difference in the change between the groups. There was no significant change in plasma triglycerides or VLDL cholesterol and no change in plasma HDL cholesterol during the study. VLDL composition was not significantly different in the two groups at baseline and there was no change in VLDL composition over the course of the study with regards to triglyceride, cholesterol phospholipid and protein. LDL and HDL compositions were similar in the two groups and neither changed with either treatment during the study.

LDL fatty acid composition and oxidizability

Neither total nor polyunsaturated fatty acids were significantly different at baseline or at the end of the study between the groups (Table 3). There was no significant difference in the change in LDL oxidizability between the groups (Table 4).

Cholesteryl ester transfer protein (CETP) and cell cholesterol

There was a 9% increase in CETP in the estradiol group and a 17% increase in the estradiol + NETA group over time. The increase was not significantly different between the groups (Table 5). Cell cholesterol was similar in the estradiol and estradiol + NETA groups at the beginning of the study (29.2 (25.9–32.5) and 29.3 (25.9–32.7) μg/mg cell protein) and at 6 months (30.6 (28.0–33.2) and 28.2 (25.1–31.3) μg/mg cell protein). There was no difference between the groups during the study.

Discussion

Diabetes carries with it an increased risk of cardiovascular disease. The dyslipidaemia of diabetes is thought to be an important reason for this increased risk. Most studies have

Table 3 Low density lipoprotein (LDL) fatty acids (μg/mg LDL protein) before and during treatment with estradiol or estradiol + NETA

	Palmitic 16 : 0	Palmitoleic 16 : 1	Stearic 18 : 0	Oleic 18 : 1	Linoleic 18 : 2	Arachidonic 20 : 4	Total
Estradiol (n = 19)							
Baseline	197 (124–270)	19 (10–28)	65(51–79)	119(80–158)	225(157–293)	35(14–56)	661(495–827)
3 month	223 (184–262)	25 (19–31)	58(45–71)	127(91–163)	236(178–294)	36(15–57)	703(543–863)
6 month	220 (166–274)	22 (16–28)	57(44–70)	121(90–152)	236(176–367)	30(13–47)	682(536–828)
Estradiol + NETA (n = 15)							
Baseline	219 (183–255)	28 (17–38)	73(54–92)	146(105 487)	275(200–350)	34(20–48)	777 (614–940)
3 month	229 (165–262)	28 (18–38)	60(50–70)	148(109–186)	282(198–366)	36(20–51)	782(616–948)
6 month	242 (210–273)	31 (20–42)	64(50–78)	173(128–260)	287(209–366)	44(26–62)	841(672–1010)
Difference between groups (estradiol – estradiol + NETA)							
Baseline	–22 (–318–274)	–9 (–38–20)	–8 (–92–76)	–27 (–417–383)	–50 (–1376–1276)	1 (–106–104)	–116(–6329–6097)
3 month	–6 (–262–250)	–3 (–29–23)	–2 (–34–30)	–21 (–373–331)	–46 (–1719–1627)	0 (–108–108)	–79(–6531–6373)
6 month	–22 (–254–210)	–9 (–35–17)	–7 (–59–45)	–52 (–524–420)	–51 (–1488–1386)	14 (–128–100)	–159(–6812–6494)
Difference between groups	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	20 : 4	Total
	5 (–31–42)	3 (–6.8–13.7)	3 (–13–18)	22.3 (–10–55)	8 (–55–71)	16 (1–31)	53 (–82–189)

Mean (95% confidence intervals)

Table 4 Copper-stimulated low density lipoprotein (LDL) oxidation at 4 h (mmol MDA/mg LDL protein)

	Baseline	3 month	6 month
Estradiol (n = 19)	48.5 (45.7–51.3)	51.4 (47.4–55.4)	46.9 (43.1–50.1)
Estradiol + NETA (n = 15)	44.3 (40.0–49.0)	43.7 (40.5–46.9)	46.2 (41.4–51.0)
Difference between groups	8.0 (–11.8–27.8)		

Mean (95% confidence intervals)

Table 5 Cholesterol ester transfer protein (nmol.ml plasma^{-1}.h^{-1}) during 6 months treatment with estradiol (n = 19) or estradiol + NETA (n = 15)

	Baseline	3 month	6month
Estradiol	122 (103–141	122 (102–142)	133 (115–151)
Estradiol + NETA	119 (106–132)	132 (115–149)	140 (124–156)
Difference between groups	3.07 (–1.37–7.5)		

Mean (95% confidence intervals)

shown that post-menopausal oestrogen replacement therapy confers cardiovascular protection although this has recently been challenged in a large prospective secondary prevention trial in older patients [27]. The purpose of the present study was to examine the effect of the addition of continuous NETA to estradiol on the efficacy of estradiol treatment in diabetic patients and a control group of subjects was therefore not used. Four patients withdrew from the study. These patients were found to all be in the estradiol + NETA group. It was to be expected that if withdrawls were to occur they would have been mostly in the estradiol + NETA group since the addition of progesterone is known to diminish patient acceptability. The study has demonstrated that 1 mg of continuous estradiol treatment reduced total and LDL cholesterol with no change in serum triglycerides or HDL cholesterol. The addition of low-dose continuous NETA did not effect these changes. The reduction in total and LDL cholesterol is similar to that seen with the usual 2.0 mg estradiol/1 mg NETA studies in non-diabetic patients with average serum cholesterol levels [5,6] and thus unlikely to be a result of a study effect.

Increase in LDL fatty acids is one of the major abnormalities of the lipoproteins in diabetes [1,2]. Since there is good correlation between the oxidizability of LDL and total fatty acids and, in particular, those fatty acids with two or more double bonds [2,28–30], the fatty acid composition of LDL was examined in the presents subjects

but no significant difference was found between the treatment groups. There was also no difference between the groups in 18:2 and 20:4 suggesting that the susceptibility to oxidation of LDL would not be altered. Shwary *et al* [31] demonstrated that 17-β-estradiol, but not estrone or estriol, exhibited antioxidant activity *in vitro*. They suggested that plasma-derived estradiol esters which they found attached to the LDL and which were not found using estrone or estriol were the reason for the protection. McManus *et al* [14], in an examination of non-diabetic women, found a small but significant decrease in plasma hydroperoxide concentration 4 weeks after insertion of an estradiol implant. Oral estradiol + NETA did not alter plasma hydroperoxides and neither treatment altered lag time to oxidation or the propagation phase. The study also did not support the role of oestrogens as antioxidants *in vivo*.

The reduction in Lp(a) which was demonstrated is similar to the changes found in non-diabetic subjects where Lp(a) was reduced by estradiol/progestogen regimes [5,32,33]. The hypothesis remains that the reduction in Lp(a) may confer health benefit.

No changes were found in HDL cholesterol after treatment in the present diabetic patients. This compares favourably with the significant decrease in HDL found in studies of non-diabetic women using 17-β-estradiol + NETA [5,6]. It also supports the finding in the ARIC study [34] which demonstrated in a large cross-sectional population study that the HDL response to hormone replacement therapy may be blunted in Type 2 diabetes. Brussaard *et al* [10] in a short 6 week study did show a small but significant HDL increase with oestrogen therapy in patients with Type 2 diabetes from 1.2 ± 0.47 to 1.47 ± 0.56 mmol/l ($P < 0.0002$). However the effects of HRT on HDL may wain with time in non-diabetic subjects [31] and may be dose-dependent.

CETP is raised in diabetes [35,36], and raised CETP is often associated with low HDL [37]. A recent study, however, has suggested that among hypertriglyceridaemic men, low CETP, associated with high HDL, is possibly atherogenic [38]. Although HDL did not alter in the present study, the increase in CETP in both groups during treatment may cause a beneficial increase in turnover of HDL cholesterol. Esterification of cholesterol in HDL is the first step in reverse cholesterol transport. Ulloa *et al* [39] have demonstrated in non-diabetic subjects an increase in LCAT following treatment with conjugated equine oestrogen/medroxyprogestin acetate therapy. This study together with the present results adds further support to a role of oestrogen/progestogen in the increase in cellular cholesterol mobilization.

In conclusion, the present study demonstrates that the addition of NETA to estradiol does not alter the potentially beneficial effects of estradiol on lipoproteins in Type 2 diabetic subjects. This may be relevant to risk reduction for coronary artery disease.

Acknowledgements

The study was supported by a grant from Novo Nordisk. The authors would like to thank Dr Bowman for gynacological assessment and biopsies. The authors wish to thank Mr Soren Larsen, Novo Nordisk and Dr Ronan Conroy Royal College of Surgeons in Ireland for their help with the statistical analysis.

References

1 Dimitriadis E, Griffin M, Owens D, Johnson A, Collins P, Tomkin GH. Oxidation of low density lipoprotein in NIDDM. its relationship to fatty acid composition. *Diabetologia:* 1995; **38:** 1300–1306.
2 Dimitriadis E, Griffin M, Owens D, Johnson A, Collins P, Tomkin GH. Lipoprotein composition in NIDDM. The effect of dietary oleic acid on composition and oxidisability and function of low and high density lipoproteins. *Diabetologia:* 1996; **39:** 667–676.
3 Barrett-Connor E. Heart disease in women. *Fert Ster* 1994; **62:** 1069–1075.
4 The Writing Group for the PEPI Trial. Effects of estradiol and estradiol/progestin regimes on heart disease risk factors in postmenopausal women. The Postmenopausal Estradiol/Progestin Intervention (PEPI) Trial. *JAMA* 1995; **273:** 199–208.
5 Taskinen M-R, Poulakka J, Pyorala J, Luotola H, Bjorn M *et al.* Hormone replacement therapy lowers plasma Lp (a) concentrations. Comparison of cyclic transdermal and continuous estradiol/progestin regimens *Arterioscler Thromb Vasc Biol* 1996; **16:** 1215–1221.
6 Stadberg E, Mattsson L-A, Uverbrant M. Low doses of 17-β-estradiol and norethisterone acetate as continuous combined replacement therapy in postmenopausal women: lipid metabolic effects. *Menopause* 1996; **3:** 90–96,.
7 Etinger B, Genant HK, Steiger P, Madvig P. Low-dosage micronised 17-β-estradiol prevents bone loss in postmenopausal women. *Am J Obstet Gynecol* 1992; **166:** 479–488.
8 Stadberg E, Mattsson L-A, Uverbrant M. 17-β-estradiol and norethisterone acetate in low doses as continuous combined hormone replacement therapy. *Maturitas* 1996; **23:** 31–39.
9 Kaplan RC, Heckbert SR, Weiss NS, Wahl PW, Smith NL, Newton KM *et al.* Postmenopausal estrogens and risk of myocardial infarction in diabetic women. *Diabetes Care* 1998; **21:** 1117–1121.
10 Brussaard HE, Gevers Leuven JA, Frolich M, Kluft C, Krans HMJ. Short-term oestradiol replacement therapy improves insulin resistance, lipids and fibrinolysis in postmenopausal women with NIDDM. *Diabetologia* 1997; **40:** 843–849.
11 Ravi Subbiah MT, Kessel B, Agrawal M, Rajan R, Abplan W, Rymaszewski Z. Antioxidant potential of specific oestradiols on lipid peroxidation. *J Clin Endocrinol Metabol* 1993; **77:** 1095–1100.
12 Rifici VA, Khachadurian AK. The inhibition of low density lipoprotein oxidation by 17-β-estradiol. *Metabolism* 1992; **41:** 1110–1114.
13 McManus J, McEneny J, Young IS, Thompson W. The effect of various estradiols and progestogens on the susceptibility of low density lipoproteins to oxidation *in vitro*. *Maturitis* 1996; **25:** 125–131.
14 McManus J, McEneny J, Thompson W, Young IS. The effect of hormone replacement therapy on the oxidation of low density lipoprotein in postmenopausal women. *Atherosclerosis* 1997; **135:** 73–81,.
15 Nabulsi AA, Folsom AR, White A, Patsch W, Heiss G *et al.* Association of hormone replacement therapy with various cardiovascular risk factors in post-menopausal women. *N Engl J Med* 1993; **328:** 1065–1075,.
16 Marsh M, Crook D, Whitcroft S, Worthington M, Whitehead M,

Stevenson J. Effects of continuous combined estradiol and desogesterol hormone replacement on serum lipids and lipoproteins. *Obstet Gynecol* 1994; **83:** 19–23.
17 Lahdenpera S, Sane T, Vourinen-Markkola H, Knudsen P, Taskinen M-R. LDL particle in mildly hypertriglyceridaemic subjects: no relation to insulin resistance or diabetes. *Atherosclerosis* 1995; **113:** 227–236.
18 Havel RA, Eder HM, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955; **34:** 1345–1353.
19 Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978; **87:** 206–210.
20 Bowie A, Owens D, Collins P, Johnson A, Tomkin GH. Glycosylated low density lipoprotein is more sensitive to oxidation: implications for the diabetic patient? *Atherosclerosis* 1993; **102:** 63–67.
21 Breug JA, Aust SD. Microsomal lipid peroxidation. *Meth Enzymol* 1978; **52:** 302–310.
22 Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957; **226:** 497–509.
23 Sattler W, Puhl H, Kostner GM, Esterbauer H. Determination of fatty acids in the main lipoprotein classes by capillary gas chromatography: BF3/Methanol transesterification of lyophilised samples instead of Folch extraction gives higher yields. *Anal Biochem* 1991; **198:** 184–190.
24 Groener JEM, Pelton RW, Kostner GM. Improved estimation of cholesteryl ester transfer/exchange activity in serum or plasma. *Clin Chem* 1986; **322:** 283–286.
25 Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *J Clin Invest* 1968; **97:** 77–89.
26 Heider JG, Boyett RL. The picomole determination of free and total cholesterol in cells in culture. *J Lipid Res* 1978; **19:** 513–518.
27 Hulley S, Grady D, Bush T, Furlong C, Herrington D, Riggs B *et al.* Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. *JAMA* 1998; **280:** 605–613.
28 Griffin M, Dimitriadis E, Lenehan K, Collins P, Johnson A, Owens D, Tomkin GH *et al.* Dietary monounsaturated fatty acids alter low density lipoprotein composition and function in Type 2 diabetes mellitus. *Q J Med* 1996; **89:** 211–216.
29 Griffin M, McInerny D, Collins P, Johnson A, Owens D, Tomkin GH. Autoantibodies to oxidised LDL are related to LDL fatty acid composition in diabetes. *Diabetic Med* 1997; **14:** 741–747.
30 Callow J, Samra JS, Frayn KN. Effect of infusion of a triacylglycerol emulsion on low density lipoprotein composition and oxidisability. *Atherosclerosis* 1998; **137:** 115–123.
31 Shwaery GT, Vita JA, Keaney JF. Antioxidant protection of LDL by physiological concentrations of estradiols is specific for 17-β-estradiol. *Atherosclerosis* 1998; **138:** 255–262.
32 Espeland MA, Marcovina SM, Miller V, Wood PD, Wasilauskas C, Sherwin R *et al.* Effect of postmenopausal hormone therapy on lipoprotein (a) concentration. *Circulation* 1998; **97:** 979–986.
33 Mijatovic V, Kenemans P, Netelenbos CJ, Peters-Muller ERA, vanKamp GJ, Voetberg GA *et al.* Oral 17-β-estradiol continuously combined with dydrogesterone lowers serum lipoprotein (a) concentrations in healthy postmenopausal women. *J Clin Endocrinol Metab* 1997; **82:** 3543–3547.
34 Robinson JG, Folsom AR, Nabulsi AA, Watson R, Brancati FL, Cai J. For the Atherosclerosis Risk in Communities Study Investigators. Can postmenopausal hormone replacement improve plasma lipids in women with diabetes. *Diabetes Care* 1996; **19:** 480–482.
35 Owens D, Cox M, Caird J, Gilligan S, Collins PB, Johnson AH *et al.* Altered regulation of cholesterol metabolism in type 1 diabetic women during the menstrual cycle. *Diabetic Med* 1993; **10:** 647–653.
36 Jones RJ, Owens D, Brennan C, Collins PB, Johnson AH, Tomkin GH. Increased esterification of cholesterol and transfer of cholesteryl ester to apo B-containing lipoproteins in Type 2 diabetes: relationship to serum lipoproteins A-1 and A-11. *Atherosclerosis* 1996; **119:** 151–157.
37 Bagdade JD, Lane JT, Subbaiah PV, Otto ME, Ritter MC. Accelerated cholesteryl ester transfer in non insulin-dependent diabetes mellitus. *Atherosclerosis* 1993; **104:** 69–75.
38 Bruce C, Sharp DS, Tall AR. Relationship of HDL and coronary heart disease to a common amino acid polymorphism in the cholesteryl ester transfer protein in men with and without hypertriglyceridaemia. *J Lipid Res* 1998; **39:** 1071–1078.
39 Ulloa N, Vurdgo C, Rios M, Sepulveda J, Sepulveda S, Naveas R *et al.* Increased activity of lecithin: cholesterol acyltransferase during short-term oral estradiol progestin replacement therapy in postmenopausal women. *Metabolism* 1998; **47:** 297–300.