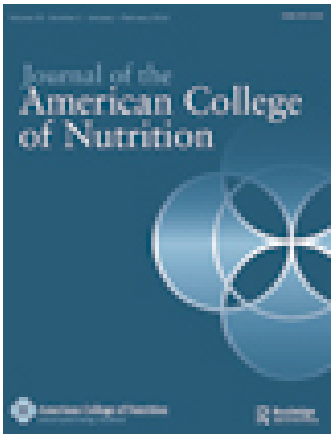

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No Antioxidant Effect of Combined HRT on LDL Oxidizability and Oxidative Stress Biomarkers in Treated Post-Menopausal Women

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Original Research

No Antioxidant Effect of Combined HRT on LDL Oxidizability and Oxidative Stress Biomarkers in Treated Post-Menopausal Women

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Key words: Combined HRT, LDL oxidizability, oxidative stress biomarkers

Objective: To compare oxidative stress and LDL oxidizability in postmenopausal women with and without HRT.

Methods: In a cross sectional study, two groups of women, with or without combined per os HRT (1.5–2 mg estrogen associated with 10 mg dydrogesteron), were age and duration of menopause matched. Women were recruited after medical examination at LBSO (Oxidative Stress Laboratory), Joseph Fourier University, Grenoble, and Department of Gynecology, Grenoble University Hospital, France. Main outcome measures included determination of lipid profile and oxidative stress biomarkers (TBARS, LDL oxidizability, auto-antibodies against oxidized-LDL). Measurement of circulating levels of vitamin C, E, β-carotene, lycopene and total antioxidant plasma capacity.

Results: HRT led to decreased plasma total and LDL cholesterol ($p < 0.05$), but did not affect oxidizability and oxidation of LDL. Circulating levels of antioxidant vitamins (β-carotene, vitamin C, vitamin E/triglycerides) and total antioxidant capacity of plasma and lipid peroxidation, assessed by plasma TBARs, were not different from controls in postmenopausal women receiving HRT.

Conclusion: This study suggests that even if combined HRT modifies the blood lipid profile, it does not appear to influence oxidative status.

INTRODUCTION

Women after menopause experience an increased incidence of cardiovascular disease [1]. In contrast, women receiving hormonal replacement therapy (HRT) seem to be protected [2]. The mechanism of HRT-related cardiovascular protection is yet unclear and seems mediated by several factors [3,4]. It may be related to favorable changes in several important cardiovascular risk factors, such as circulating blood low density lipoproteins (LDL), high density lipoproteins (HDL) and lipoprotein(a) [5], coagulation factors, blood pressure and insulin tolerance [6] [7]. Oxidative stress has been also implicated as a risk factor in the pathogenesis of cardiovascular disease [8]. It has been suggested that increased oxidative stress, related to an unbalanced pro-oxidant/antioxidant equilibrium, is a potential inducer of cardiovascular risk [9]. Oxidative modifications of low-density lipoproteins (LDL) are thought to play a major role in the process of atherosclerosis [10,11]. The atheroprotective effect of estrogen might also be partly due to its antioxidant action [12], resulting in a decrease of LDL oxidation [13]. In postmenopausal women, hormonal replacement therapy might either counteract the effect of a possible increased oxidative stress or improve antioxidant status.

During aging, a decrease of antioxidant enzyme [14] as well as a lower antioxidant nutriture has been reported [15,16]. In addition, middle age is often associated with environmental

Abbreviations: LDL = Low Density Lipoprotein, HDL = High Density Lipoprotein, HPLC = High Performance Liquid Chromatogratography, TAS = Total Antioxidant Plasma Capacity, HRT = Hormonal Replacement Therapy.

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modifications resulting in decreased intakes of micronutrient [17], which are directly related to antioxidant protective mechanisms. Despite the key role of antioxidant micronutrients in preventing accelerated aging, data related to relationship between oxidative stress and antioxidant status in menopausal women are scarce.

This study was designed to compare oxidative stress and LDL oxidizability in postmenopausal women receiving or not hormonal replacement therapy. Oxidative process was evaluated in plasma by lipid peroxidation (TBARs), autoantibodies against oxidized LDL, LDL oxidizability, antioxidant vitamin status (vitamin C, vitamin E, β-carotene, lycopene) and total antioxidant plasma capacity to monitor the occurrence of oxidative stress taking place *in vivo*. Since antioxidant status is partly dependent on antioxidant micronutrient intakes, we also assess the dietary intakes of the subjects.

METHODS

Experimental Design

In a cross-sectional study, two groups of women aged 50−60 years old were constituted and compared for their oxidant/antioxidant status. One group was taking orally combined hormonal replacement therapy (HRT) for at least six months (group I, n = 18), the other (group II, n = 25) not. Used orally combined HRT was 1.5–2 mg estradiol associated with 10 mg of dydrogesteron. We checked that women in group I had not been on HRT prior to the study. Key exclusion criteria included natural menopause before age 40 years or <2 years or >15 years at time of enrollment, hysterectomy or any surgical interventions within six months, body mass index >35 kg/m2, supplementation with micronutrients within three months and during the study, current medication that is known to influence lipoprotein metabolism, abnormal routine biochemical tests and a medical history (e.g., stroke, endometrial or breast cancer, diabetes, liver or renal diseases). We checked that untreated and treated women did not receive antioxidant supplementation within three months and during the study.

The Ethical Committee of Grenoble University Hospital approved the study, and each volunteer was given a written informed consent.

Blood Sampling and Analysis

Sampling: all blood samples were collected after a 12-hour overnight fast. Tubes were placed on ice and centrifuged at 4000 × *g* for 10 minutes at 4°C. Plasma and erythrocytes were immediately isolated, aliquoted and stored at −80°C.

Plasma Lipid Profile and Serum Estradiol

Plasma levels of total cholesterol, low density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c) and triglycerides (Tg) were measured by enzymatic colorimetric methods with a Cobas analyzer according to the manufacturer instructions. The concentration of serum LDL-c was calculated according to the Dahlen equation [18], in the form as LDL-c = Chol − (HDL-c + 0.20 Tg + 0.3 × Lp(a)) (g/L).

Apolipoproteins A1 and B and lipoprotein a (Lpa) were measured by turbidimetric immunoassays.

HDL-c and LDL-c subfractions were separated by electrophoresis in a non-denaturing polyacrylamide gradient gel [19]. LDL size was estimated as the apparent diameter of the major peak. HDL_2 and HDL_3 were quantified by scanning the gel with a CD60 densitometer [20].

Estradiol (E2) serum level was measured by radioimmunoassay method.

Plasma Lipoperoxidation and LDL Oxidizability

Lipid peroxidation was measured by thiobarbituric reactive substance (TBARS) concentration in plasma [21].

LDL particles were isolated by density gradient ultracentrifugation using a Beckman TL-100 ultracentrifuge with a fixed angle rotor (100.2). The susceptibility of LDL to oxidation was estimated by incubating isolated LDL at 37°C in PBS and the reaction was initiated by adding 3 μM $CuCl_2$ [22]. The rate of oxidation and the lag time were determined for each subjects. The lag time was defined as the intercept between the baseline at t_0 and the slope of the propagation phase.

Auto-antibodies of the IgG class to malondialdehyde (MDA)-modified LDL were determined by ELISA method according to Maggi with the following modifications: MDA-modified LDL were prepared according to Purunen and used as antigen [23,24]. The assay was calibrated with serial dilutions of a pool of plasma with high antibody levels. The antibody titer was calculated after subtraction of the absorbency of a reagent blank and expressed as the dilution of the standard corresponding to the absorbency of the sample, taking into account the dilution (arbitrary unit: AU). Coefficient of variation is 15.2% (mean = 550UA and n = 30).

Antioxidant Plasma Vitamins and Antioxidant Plasma Capacity

Plasma concentrations of β-carotene, lycopene and vitamin E were quantified using the High Performance Liquid Chromatography (HPLC) [25]. Coefficient of variation for vitamin E, lycopene, β carotene are, respectively: 5.1%, 13.6%, 7.1%.

Total vitamin C: 400 μL of plasma was added to 3600 μL aqueous solution of metaphosphoric acid (5% w/v) and stored at −80°C until analysis. The determination was done fluorometrically by HPLC after derivatization [26]. Coefficient of variation for vitamin C is 4.16%.

Total antioxidant plasma capacity (TAS) was measured using a Randox kit (Randox 1115 rue H. Boucher, BP 82, Montpellier Frejorgues, 34131 Mauguio cedex, France).

Nutritional Assessment

Seven-day dietary records were used to assess dietary intakes. Estimation of macronutrients (proteins, carbohydrates, lipids), alcohol consumption, fiber, vitamins C, E, A, carotenoids and folates was determined using the REGAL WINDOW software (CNEVA, Ciqual, Paris, France).

Statistics

Data are expressed as means ± S.D. The distribution of all variables was first checked for normality using the Kolmogorov-Smirnov test. Depending upon the distribution of the population, data were analyzed either using parametric methods differences where statistical significance was tested using the Student *t* test or using non-parametric methods differences where statistical significance was tested using the Mann-Whitney U test.

Statistical evaluations were performed with the analysis program Statistica (statistical software, Paris, France). Statistical differences by either Student *t* test or Mann-Whitney U test were significant at $p \leq 0.05$.

RESULTS

Study Population

Basic characteristics of the subjects are reported in Table 1. BMI, alcohol and tobacco consumption, physical activity habits were not significantly different in the two groups, whereas mean age and duration of menopause were similar. Women receiving hormonal replacement therapy exhibited a plasma estradiol level significantly higher than untreated women.

Lipid Profile

Plasma lipids are reported in Table 2. In untreated postmenopausal women, significantly higher in plasma cholesterol levels were observed. This effect was partly due to a higher LDL-c. Similarly, lipoprotein (a) tended to be lower in HRT-treated women, but the standard deviation was too high to achieve a significant difference. HDL-c was non-significantly raised in the group receiving hormonal replacement therapy, but the ratio HDL_2/HDL_3 remained unchanged. Triglyceride levels were in the normal range.

Table 1. Baseline Characteristics of the Subjects

	Postmenopausal Women HRT (n = 18)	Postmenopausal Women non-HRT (n = 26)
Age (years)	54.1 ± 2.2	55.4 ± 3
Menopause duration (years)	4.5 ± 2.3	4.8 ± 3.3
BMI (kg/m^2)	23 ± 3	24 ± 4
Smokers[a]	1	1
Physically active person[b]	3	3
Alcohol (absolute ethanol, g/week)	56 ± 87	38 ± 48
Estradiol (nmol/L)	574 ± 399	72 ± 48*

Values are mean ± SD.

* $p < 0.005$.

[a] Number of smokers in each group, (between 10 and 20 cigarettes/day).

[b] Number of physically active persons, (three hours or more [maximum six hours] of regular physical activity/week).

HRT = Hormonal Replacement Therapy.

Table 2. Plasma Lipids in Postmenopausal Women

	Postmenopausal Women (HRT) (n = 18)	Postmenopausal Women (non-HRT) (n = 26)
Cholesterol total (mmol/L)	6.1 ± 1.0	6.7 ± 0.9*
LDL-Cholesterol (mmol/L)	3.5 ± 0.8	4.4 ± 0.7**
Apolipoprotein B (g/L)	0.82 ± 0.14	0.96 ± 0.16**
Triglycerides (mmol/L)	1.0 ± 0.2	0.9 ± 0.3
HDL-cholesterol (mmol/L)	2.1 ± 0.5	1.8 ± 0.4
Apolipoprotein A1 (g/L)	1.75 ± 0.34	1.56 ± 0.23*
HDL2/HDL3 (%)	1.16 ± 0.64	1.02 ± 0.42
Lipoprotein (a) (mg/L)		
Mean:	246 ± 219	465 ± 574
Median:	182 (112–393)	279 (42–633)

Values are mean ± SD.

Statistical differences between groups were significant for $p < 0.05$ (* $p < 0.05$, ** $p < 0.005$).

HRT = Hormonal Replacement Therapy.

Table 3. Lipoperoxidation and LDL Oxidazibility of the Subjects

	Postmenopausal Women (HRT)	Postmenopausal Women (non-HRT)
TBARs (μmol/l)	2.6 ± 0.3 (n = 18)	2.4 ± 0.2* (n = 25)
TBARs/Cholesterol (μmol/mmol)	0.44 ± 0.08 (n = 18)	0.37 ± 0.05** (n = 25)
Oxidation rate of LDL (μmol diene/minutes/g apo B)	10.8 ± 1.3 (n = 16)	10.7 ± 1.3 (n = 26)
Lag time (minutes)	48.8 ± 4.5 (n = 16)	51.6 ± 5.3 (n = 26)
LDL size (nm)	27.2 ± 0.4 (n = 18)	27.2 ± 0.4 (n = 25)
Auto-antibodies anti oxLDL (AU)	533 ± 360 (n = 18)	677 ± 575 (n = 26)

Values are means ± SD.

Statistical differences between groups were significant for $p < 0.05$ (* $p < 0.05$, ** $p < 0.005$).

HRT = Hormonal Replacement Therapy.

Table 4. Antioxidant Vitamins and Total Antioxidant Capacity of Plasma (TAS)

	Postmenopausal Women (HRT) (n = 18)	Postmenopausal Women (non-HRT) (n = 25)
Vitamin C (μmol/L)	57 ± 13	72 ± 15***
β carotene (μmol/L)	0.78 ± 0.32	1.28 ± 0.92*
Lycopene (μmol/L)	0.90 ± 0.49	1.13 ± 0.67
Vitamin E/Triglycerides (μmol/mmol)	33 ± 10	44 ± 16**
TAS (μmol/L)	1.35 ± 0.06 (n = 16)	1.40 ± 0.04** (n = 21)

Values are means ± SD.

Statistical differences between groups were significant for $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

HRT = Hormonal Replacement Therapy.

Lipoperoxidation and LDL Oxidizability

Lipoperoxidation and LDL oxidizability are reported in Table 3. Oxidizability of LDL, estimated by the lag time and the rate of oxidation, was not different between groups. Mean LDL size was in the middle size range in the two groups. This type of LDL is associated with a low level of oxidation and, therefore, a low cardiovascular risk [27]. Plasma levels of auto-antibodies against oxidized LDL were not modified by hormonal replacement therapy.

Antioxidant Vitamins and Total Antioxidant Capacity of Plasma

Antioxidant vitamins and total antioxidant capacity of plasma of all subjects are reported in Table 4. Plasma concentration of ascorbic acid, β-carotene, lycopene and the ratio vitamin E/Triglycerides were significantly different between the two groups.

Nutritional Assessment

As reported in Table 5, mean dietary intakes were adequate in the two groups according to the Recommended French Dietary Allowances (ANC) [28]. Intake of energy, fiber, vitamin C, E, equivalent β-carotene, retinol and folates were not significantly different between groups.

DISCUSSION

The higher prevalence of cardiovascular diseases in postmenopausal women could be related to an increased oxidative stress. Estrogen has been reported to have antioxidant capabilities and HRT could protect against cardiovascular diseases throughout this mechanism. The objective of this study was to investigate LDL oxidizability, plasma lipoperoxidation and plasma antioxidant capacity in treated and untreated women. Our data confirmed that hormonal replacement therapy resulted in beneficial changes in the conventional lipid risk estimators. However, LDL oxidation and oxidizability were not affected by this treatment.

The higher incidence of cardiovascular diseases after menopause is well-documented [29,30]. The lipid modifications are mainly involved in atherogenic risk [31]. Our results confirm the beneficial effects of hormonal replacement therapy in regard to blood lipid levels. Others have previously reported these observations [32]. Risks of cardiovascular diseases are due to several factors including those related to aging and those specifically linked to hormonal estrogenic deficiency [33]. It has been reported that estradiol possesses antioxidant properties [34,35]. However, in our study, the lipoprotein susceptibility to oxidation, assessed by *ex vivo* analysis, was not affected by hormonal replacement therapy. Our data corroborate with those of Mc Manus *et al.* and Wen *et al.*, who showed that oral equine estrogen therapy, transdermal estradiol or combined HRT did not protect LDL against oxidation [36,37]. Similarly, Nenseter *et al.* demonstrated that non-smoking, hypercholesterolemic postmenopausal women were not protected by HRT in regard to LDL oxidation [38]. More recently, interest has focused on other plasmatic biomarkers of lipid peroxidation such as levels of autoantibodies against oxidized-LDL. In the present study, autoantibodies against oxidized LDL were not affected by hormonal replacement therapy. Heikkinen has also

Table 5. Nutritional Assessment

	Postmenopausal Women (HRT) (n = 14)	Postmenopausal Women (non-HRT) (n = 19)	ANC (>50 years)
Caloric Intake (KJ/d)	7759 ± 1515	7470 ± 1590	7114
(Kcal/d)	(1854 ± 362)	(1785 ± 380)	(1700)
Fiber (mg/d)	17.4 ± 4.1	18.7 ± 6	20
Vitamin C (mg/d)	97 ± 36	99 ± 44	80
Vitamin E (mg/d)	10.6 ± 3.5	11.1 ± 6.1	12
Eq. β carotene (μg/d)	4079 ± 1761	3971 ± 2764	2880
Retinol (μg/d)	403 ± 328	328 ± 148	320
Folates (μg/d)	266 ± 66	283 ± 91	300

Values are means ± SD.

HRT = Hormonal Replacement Therapy.

observed that one-year HRT did not influence oxLDL antibody titer [39]. Controversially, it has been shown that HRT decreased the level of oxLDL antibody in postmenopausal women with coronary heart disease [40]. We observed large size LDL particles although it has been described that postmenopausal women had a preponderance of small dense LDL particles [41]. Since the large size of LDL is less susceptible to oxidative modification, it is therefore not expected to be associated with a higher cardiovascular risk [27].

Plasma lipoperoxidation, monitored by TBARS and TBARS/cholesterol was different in untreated and treated women. Inal *et al.* have reported that malondialdehyde (MDA) levels decreased in postmenopausal women receiving hormonal replacement therapy only when associated with a daily supplementation of vitamin E [43]. This observation points out the fundamental role of the antioxidant component in the maintenance of pro-oxidant/antioxidant equilibrium. Furthermore, it has been shown that intake of dietary vitamin E is inversely associated with the risk of death from cardiovascular disease in postmenopausal women [44].

In non-treated women, the lower lipoperoxidation observed could be due to a protection against oxidative stress related to optimal level of plasma antioxidants. All the women exhibited an adequate antioxidant status.

Our data do not agree with studies reporting a risk of deficit in antioxidants after menopause [17]. The plasma concentrations for vitamins E, C and carotenoids in the non-HRT group appears likely to be related to an optimal diet in this group. The recruited women were at an early stage of menopause and their antioxidant status was optimal. These volunteers were health conscious as shown by body mass index (BMI) and had healthy environmental habits (few smokers, no alcohol drinking, low triglyceridemia regarding aging).

The role of estrogen as antioxidant *in vivo* is matter of debate [36,45]. Controversies still exist regarding the beneficial protecting effect of HRT. In some trials estrogen replacement therapy had a beneficial effect in prevention of coronary artery disease, morbidity and mortality [46,47]. However, pooled data from clinical trials do not support the notion that HRT prevents cardiovascular events [48]. Moreover, the only randomized data available to date do not support any beneficial effect in postmenopausal women with coronary heart disease [49].

In conclusion, the antioxidant effect of combined hormonal replacement therapy remains controversial. In untreated postmenopausal women, optimal antioxidant micronutrient intakes could be a powerful tool in counteracting the effect of hormonal modifications in terms of oxidative stress. Under these conditions, nutrition could offer an interesting alternative way in preventing cardiovascular risks.

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