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Effect of different contraceptive methods on the oxidative stress status in women aged 40–48 years from the ELAN study in the province of Liège, Belgium

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BACKGROUND: Oxidative stress is associated with the development of several disorders including cardiovascular disease and cancer. Among conditions known to influence oxidative stress, the use of oral contraception (OC) in women has been a matter of ongoing discussion. METHODS: A total of 897 eligible and healthy volunteers were recruited from among the patients of 50 general practitioners participating in the ELAN study (Etude Liégeoise sur les ANtioxydants). A subsample consisting of 209 women aged 40–48 years was studied for a comprehensive oxidative stress status (OSS), including the analysis of antioxidants, trace elements and three markers of oxidative damage to lipids. Among 209 subsample, 49 (23%) were OC users (OCU), 119 (57%) non-contraception users (NCU) and 41 (20%) were intrauterine (hormonal and copper) devices users (IUD). RESULTS: After adjustment for smoking, systolic and diastolic blood pressure and BMI (or waist circumference), a marked and significant increase in lipid peroxides was observed among OCU women when compared with NCU and IUD users. A cut-off value of 660 μM in lipid peroxides allowed the discrimination of OCU from the two other groups. In contrast, no difference was observed in the plasma concentration of both oxidized low-density lipoprotein (LDL) and their related antibodies. The increased level in lipid peroxides was strongly related to higher concentrations of copper ($r < 0.84$; $P < 0.0001$, cut-off value 1.2 mg/l). When compared with NCU and IUD users, plasma antioxidant defences were significantly altered in OCU women as shown by lower levels of β-carotene (decrease of 39%; $P < 0.01$) and γ-tocopherol (decrease by 22%; $P < 0.01$). In contrast, higher concentrations of selenium (increased by 11.8%; $P < 0.01$) were observed in OCU women. Blood concentrations of vitamin C, α-tocopherol and zinc were unaffected by OC use. CONCLUSIONS: The intake of OC significantly increases the lipid peroxidation in women aged 40–48 years. This may represent a potential cardiovascular risk factor for these women.

Keywords: oxidative stress; antioxidants; oral contraception; ELAN study

Introduction

Oxidative stress is defined as an imbalance between antioxidants and reactive oxygen species (ROS) in favour of the latter. ROS, which include free radicals, are continuously produced in the body and play an important physiological role at low concentrations. They act as second messengers capable of regulating apoptosis, activating transcription factors and modulating the expression of various genes involved in immune response. Various conditions, can, however, lead to a non-physiological production of ROS (sun exposure, intense exercise, smoking habits, chronic inflammation, metal poisoning, mitochondrial dysfunction or hyperglycaemia). Because of due to their high reactivity, ROS will cause irreversible cell lesions through oxidative alterations to lipids, DNA and proteins. Alterations of these structures are suspected to be linked to the development of several human pathologies

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including atherosclerosis, cardiovascular disease, cancer, diabetes complications, macular degeneration and arthritis. To limit the harmful effects of ROS, a high-performance antioxidant system consisting of enzymes, proteins, vitamins (A, C and E), carotenoids, flavonoids, trace elements and small molecules, such as glutathione, may interact with ROS and regulate their production down to the physiological range. If these antioxidant defences are overwhelmed by excessive ROS production, or not sufficiently provided by diet or supplements, oxidative stress may consequently take place in the body (Cutler, 2005).

Since the discovery that oral progestational 19-nor steroids could inhibit ovulation (Chang *et al*., 1956), several million women have used different types of synthetic estrogens and progestins to prevent conception. In post-menopausal women, hormone replacement therapy (HRT) is based on the intake of different types of hormones involving estrogens (namely estradiol and conjugated estrogens) and natural progesterone or synthetic progestins in order to replace the failing ovarian secretion.

Ever since the introduction of these drugs, data are scarce about their relationship with oxidative stress and the topic still remains a subject of debate. It has been suggested, but not generally admitted, that estrogens have an antioxidant effect that may contribute to its protective effects on the cardiovascular system through inhibition of lipid oxidation (Brown *et al*., 2000; Ling *et al*., 2006). Studies conducted *in vitro*, but not all (Saha *et al*., 2000; Chiang *et al*., 2004), have shown that estrogens, and more particularly estradiol, were able to reduce significantly the oxidative damage to lipids exposed to several free radical generating systems (Sugioka *et al*., 1987; McManus *et al*., 1996; Markides *et al*., 1998; Miura *et al*., 1998; Mueck *et al*., 2000; Hwang *et al*., 2000; Karbownik *et al*., 2001; Yen *et al*., 2001). According to Thibodeau *et al*. (2002), the apparent discrepancy in the data was largely caused by the chemical heterogeneity in the estrogen family and by their concentration and the environment in which they are found.

Most of the *in vivo* studies performed on rats and women receiving HRT observed an antioxidant effect of estrogen (Clemente *et al*., 1999; Hernandez *et al*., 2000; Seeger *et al*., 2000; Dantas *et al*., 2002; Telci *et al*., 2002; Wakatsuki *et al*., 2003; Ahotupa *et al*., 2004; Bednarek-Tupikowska *et al*., 2006; Misra *et al*., 2006). This was, however, not confirmed in other reports (McManus *et al*., 1997; Heikkinen *et al*., 1998; Wen *et al*., 2000; Bureau *et al*., 2002; Falkeborn *et al*., 2002). Surprisingly, only a few studies have investigated the relationship between combined oral contraceptives containing estrogens and progestins, and oxidative stress. Reports from the beginning of the nineties demonstrated a significant increase in blood lipid peroxides (Kose *et al*., 1993; Sissan *et al*; 1995) responsible for increased platelet aggregation in rats after oral contraceptive (OC) administration (Ciavatti *et al*., 1989; Ciavetti and Renaud, 1991). In agreement with other studies (Horwitt *et al*., 1975; Prasad *et al*., 1975a; Yeung, 1976; Arab *et al*., 1982; Palan *et al*., 1989; Berg *et al*., 1997a) described a strong decrease in plasma antioxidant β-carotene among OC users in bivariate analysis, more particularly among women above the age of 35 years. In contrast, Capel *et al*. (1981) followed by Massafra *et al*. (1993) found that a 9-cycle course of a combined oral contraceptive (ethinylestradiol 20 μg and desogestrel 150 μg) in young women led to a significant increased activity of antioxidative enzymes, namely catalase and glutathione peroxidase (GPx).

Recently, a large scale study was conducted by our group to assess the oxidative stress status (OSS) of healthy individuals aged 40–60 years living in the Province of Liege, Belgium. Due to the conflicting data described above, we decided to measure, in a subpopulation of women in the age ranged 40–48 years, the effects of contraceptive methods on parameters of oxidative stress, including antioxidant vitamins, trace elements and markers of oxidative damage to lipids.

Material and Methods

Subjects

The ELAN ("Etude Liégeoise sur les ANtioxydants") study was conducted from March through July 2006 as a joint project between the University of Liège, the University Hospital of Liège and the local health services of the Province of Liège (Belgium). A stratified sample of 55 general practitioners working in the Province was selected as follows: 21 (38%) in urban environment, 15 (27%) in semi urban and 19 (35%) in rural environment. Each physician was asked each to recruit in his/her practice 20 presumably healthy volunteers within the age range of 40–60 years. Exclusion criteria for participating in the study were (i) intake of antioxidant supplementation and (ii) any previous history of cardiovascular diseases, diabetes or cancer. A total of 897 eligible volunteers were finally enrolled in the study: 349 (39%) men and 548 (61%) women. The subsample studied in the present work consisted of 209 women aged 40–48 years: 49 (23%) of these women were OC users (OCU), 119 (57%) non-contraception users (NCU) and 41 were (20%) intrauterine (hormonal and copper) devices users (IUD) (Table 1).

Clinical examination

Women were asked to attend their physician's office between 8 and 10 a.m. for taking a 35 ml blood sample. Subjects were fasted for at least 12 h and not allowed to drink fruit juice and to perform physical activity the day before the visit. In the same time, information including age, occupation, height, weight, blood pressure, smoking habits, alcohol and drugs consumption, waist circumference and physical activity was collected. The BMI was calculated from height and weight (kg/m^2). The study protocol was approved by the University Hospital Ethics Committee for medical research. All contacted volunteers received written information about the goal of the study and signed an informed consent form in case of approval prior to enrollment. All participants completed a home-made food questionnaire for evaluating their daily intake in fruits and vegetables. According to tables of diet composition (Souci *et al*., 2000), a score reflecting the daily consumption of both vitamin C and β-carotene was established.

Analytical procedures

antioxidant and trace element determination.

Blood samples were drawn on EDTA or Na-heparin as anticoagulant or clot activating gel according to the investigated parameter. Blood samples were immediately centrifuged on site and plasma or sera were frozen as aliquots on ice packs coming from a −80°C freezer and placed in a refrigerating box. For vitamin C determination, 0.5 ml

Table 1: Contraception methods used among 209 women aged 40–48 years participating in the ELAN study

	n	Ethynilestradiol (mg)	Progestine (mg)
No OC	119		
OC	49		
Monophasic			
	4	0.035	Norgestimate (0.25)
	3	0.035	Cyprotérone (2.00)
	3	0.030	Gestodene (0.075)
	4	0.030	Desosgestrel (0.15)
	5	0.030	Levonorgestrel (0.15)
	8	0.030	Drospirenone (3.00)
	3	0.020	Gestodene (0.075)
	9	0.020	Desogestrel (0.15)
Biphasic			
	1	0.040–0.030	Desogestrel (0.025–0.125)
Triphasic			
	2	0.030–0.040–0.030	Levonorgestrel (0.05–0.075–0.125)
	5	0.030–0.040–0.030	Gestodene (0.05–0.07–0.1)
Without estrogens	2		Desogestrel (0.075)
IUD	41		
Progesterone (Mirena)	32		Levonorgestrel (0.02)
Copper	6		
Unknown	3		

plasma was immediately transferred to ice-cold tubes containing 0.5 ml of 10% metaphosphoric acid. The whole mixture was frozen on ice packs. Analyses were performed on the day of blood collection by a spectrophotometric method using the reduction of 2,6-dichlorophenolindophenol (Perkin Elmer Lambda 40 Norwalk, USA) (Omaye *et al*., 1979) (sensitivity: 2 μg/ml, inter and intra CV: 4 and 6%). Plasma vitamin E (α and γ-tocopherols) (α-tocopherol: sensitivity 0.2 μg/ml, inter and intra CV: 2.93 and 3.53%; γ-tocopherol: sensitivity 0.24 μg/ml, inter and intra CV: 9.01 and 14.34%) and β-carotene (sensitivity 0,022 mg/l, inter and intra CV: 5.35 and 10.73%) were determined simultaneously by HPLC procedure (Alliance Waters, USA) coupled with a diode array detector (PDA 2996, Waters, USA) (Zhao *et al*., 2004). Blood levels of vitamin E were normalized to a reference lipid, namely cholesterol, which was determined by an enzymatic method with cholesterol oxidase. Thiol proteins were detected according to the Ellman's method (Butterworth *et al*., 1967) (sensitivity 50 μM, inter and intra assay CV: 10%). The plasma levels of selenium (sensitivity 23.4 μg/l, inter and intra CV: 4.28 and 3.81%), copper (sensitivity 0.12 mg/l, inter and intra assay CV: 3.62 and 5.64%) and zinc (sensitivity 0.10 mg/l, inter and intra CV: 2.74 and 6.51%) were determined by inductively coupled plasma-mass spectroscopy (Sturup *et al*., 2005). The ratio of vitamin C/α-tocopherol was used as a potential risk factor for cardiovascular disease (Gey, 1998). The γ/α-tocopherol ratio was determined as a sensitive index of α-tocopherol ingestion (Baker *et al*., 1986).

markers of lipid peroxidation.

The analysis of lipid peroxides as markers of oxidative damage to lipids was performed with the commercial kit (Oxystat, Biomedica Gruppe, Austria). Briefly, the peroxide (-OOH) concentration was determined spectrophotometrically by reaction of the biological peroxides with peroxidase and a subsequent colour reaction using 3,3′,5,5′-tetramethylbenzidine as substrate (sensitivity: 11 μM, inter and intra CV: 5.4 and 10%).

Oxidized low-density lipoprotein (LDL) in plasma samples was determined spectrophotometrically with a competitive enzyme-linked immunosorbent assay (ELISA) kit (Immunodiagnostik, Germany) (sensitivity: 9.66 ng/ml, inter and intra CV: 6.17 and 7%). The titre in free antibodies (IgG) against oxidized LDL (Ab-Ox-LDL) was assessed with a commercial enzyme immunoassay (Biomedica Gruppe, Austria) using Cu^{2+} oxidized LDL as antigen (sensitivity: 37 IU/ml, inter and intra CV: 10.5%).

Statistical analysis

Results were expressed as mean ± standard deviation (SD) for quantitative variables, while frequencies and proportions (%) were used for categorical variables. Mean values were compared by one-way analysis of variance followed by multiple comparisons. Proportions were compared by the chi-squared test for contingency tables. Non-parametric Kruskal–Wallis and Wilcoxon rank sum tests were also used for comparing samples from different groups when normality assumptions could not be fulfilled. Correlation coefficients (classical or non-parametric Spearman's test) were calculated for measuring the association between two quantitative variables. The relationship between biochemical parameters and other variables was assessed by multiple regression analysis, while for binary variables the logistic regression method was used. The latter approach also allows to determination of cut-off points in the covariates. Data were also displayed graphically; in particular probability densities were fitted by a non-parametric fitting method. Calculations were always carried out on the maximum number of data available. Missing data were not replaced. Results were considered to be significant at the 5% critical level ($P < 0.05$). Data analysis was carried out using SAS (version 9.1 for Windows) and S-Plus (version 9.0) statistical packages.

Results

Effect of the type of contraceptive method on clinical and biochemical parameters

Demographic, biometric and clinical characteristics of the studied population are given in Table 2. The groups differed with respect to smoking. Women in the NCU group presented a higher BMI than those in the IUD group (25.8 ± 5.2 versus 23.1 ± 3.8 kg/m^2, $P < 0.01$). This result was confirmed by the measure of the waist circumference. Patients in the IUD group had a lower systolic blood pressure than those of the other two groups ($P < 0.01$), while diastolic blood pressure was similar ($P = 0.14$). For all other variables, the three groups were comparable. With respect to the daily intake of β-carotene, no significant changes were observed between all three groups. Respective scoring for this parameter was 4.7 ± 2.8 mg/day (NCU), 4.9 ± 2.9 mg/day (OCU) and 4.0 ± 2.7 mg/day (IUD). For vitamin C, scoring was as follows: 143.3 ± 75.8 mg/day (NCU), 152.8 ± 61 mg/day (OCU) and 108.7 ± 58.4 mg/day (IUD). Statistical analysis revealed that only IUD scoring was significantly different ($P < 0.02$) than those of the two other groups.

As shown in Table 3, the contraceptive groups compared with NCU were similar concerning levels of vitamin C, α-tocopherol, vitamin C/α-tocopherol ratio, α-tocopherol/cholesterol ratio, zinc, oxidized LDL and Ab-Ox-LDL. From a multivariate point of view, when adjusting the levels of biochemical parameters for smoking, systolic and diastolic blood

Table 2: Characteristics of the women ($n = 209$, age ranged 40–48 years) categorized as NCU, OCU and IUD. In OCU group, the estrogen contents currently used was lower or equal to 50 μg. Progestin compounds were classified according to their chemical structure into preparations with desogestrel, levonorgestrel, lynestrenol/northisterone or antiandrogen. IUD includes both hormonal (Mirena) and Cu IUD. Data were expressed as means ± SD and were statistically analysed from an univariate point of view

Variable	Contraception			*P*-value
	NCU ($n = 119$)	OCU ($n = 49$)	IUD ($n = 41$)	
Age (years)	44.6 ± 2.5	44.4 ± 2.7	44.0 ± 2.1	0.48
Smoking habits				
No smoker	76 (64)	41 (84)	31 (76)	0.034
Smoker	42 (36)	8 (16)	10 (24)	
Height (cm)	1.64 ± 0.06	1.65 ± 0.07	1.66 ± 0.06	0.15
Weight (kg)	69.5 ± 14.7	66.2 ± 11.9	63.9 ± 10.6	0.046
BMI (kg/m^2)	25.8 ± 5.2*	24.3 ± 4.8	23.1 ± 3.8*	0.0079
Waist size (cm)	84.7 ± 13.2*	80.4 ± 11.2	76.9 ± 10.8*	0.0017
Systolic blood pressure (mmHg)	121 ± 13	121 ± 12	114 ± 14	0.004**
Diastolic blood pressure (mmHg)	76 ± 0.84	75 ± 0.94	72 ± 0.98	0.14
Intestinal disorder				
Yes	28 (24)	13 (27)	4 (9.8)	0.11
No	91 (76)	35 (73)	37 (90)	
Physical activity				
Yes	36 (30)	23 (48)	15 (37)	0.096
No	83 (70)	25 (52)	26 (63)	
Environment				
Rural	38 (32)	20 (41)	21 (51)	0.21
Semi urban	29 (24)	9 (18)	9 (22)	
Urban	52 (44)	20 (41)	11 (27)	

*significantly different; **IUD significantly different of OCU and NCU.

pressures and BMI (or waist circumference), there was a small though significant effect on the γ-tocopherol level ($P < 0.05$) according to the type of contraception, as this level was lower in the OCU and IUD than in the NCU group. This was confirmed on the ratio of α-tocopherol/γ-tocopherol ($P < 0.05$). Women taking OC, when compared with IUD or NCU, had a significantly lower β-carotene level ($P < 0.0001$) and higher selenium ($P < 0.0001$), lipid peroxides ($P < 0.0001$) and copper ($P < 0.0001$) levels as well as a higher copper/zinc ratio ($P < 0.0001$). The level of thiol proteins was higher for women with IUD than for women who were NCU or OCU ($P < 0.01$).

As shown in Table 3, in the OCU group, the values of copper, the copper/zinc ratio and lipid peroxides reached

Table 3: Effects of the use of a contraceptive method or not on plasma parameters of OSS among 209 women aged from 40–48 years participation to the ELAN study. Data were expressed as means ± SD and were statistically analysed from an univariate point of view. References values were those established by CHU, Liège, Belgium (Laboratory of Clinical Biology)

Variable	Contraception				Reference values[e]
	NCU ($0 = 119$)	OCU ($n = 49$)	IUD ($n = 41$)	*P*-value	
Vitamin C (μg/ml)	9.8 ± 3.9	10.8 ± 4.2	10.0 ± 2.7	0.26	8.60–18.83
α-tocopherol (μg/ml)	13.2 ± 2.8	13.1 ± 2.7	12.2 ± 2.3	0.11	8.60–19.24
Vitamin C/α-tocopherol	0.77 ± 0.35	0.87 ± 0.39	0.86 ± 0.30	0.17	0.59–1.19
Cholesterol (g/l)	1.9 ± 0.3[a]	1.9 ± 0.3	1.8 ± 0.27[a]	0.03	1.5–2.0
γ-tocopherol (mg/l)	1.1 ± 0.52	0.86 ± 0.32	0.82 ± 0.27	0.0024[b]	0.28–2.42
α-tocopherol/cholesterol (mg/g)	6.9 ± 1.5	6.9 ± 1.1	6.8 ± 0.91	0.94	4.40–7.00
γ/α-tocopherol	0.08 ± 0.03[e]	0.07 ± 0.02[e]	0.07 ± 0.02	0.0047	0.055–0.213
β-carotene (mg/l)	0.36 ± 0.27	0.22 ± 0.18	0.44 ± 0.39	0.0010[c]	0.05–0.68
Thiol proteins (μM)	408 ± 64	387 ± 69	448 ± 51	<0.0001[d]	310–523
Selenium (μg/l)	85 ± 13	95 ± 17	85 ± 16	0.0003[c]	94–130
Copper (mg/l)	0.98 ± 0.23	1.5 ± 0.45	0.86 ± 0.21	<0.0001[c]	0.80–1.55
Zinc (mg/l)	0.57 ± 0.11	0.58 ± 0.10	0.57 ± 0.12	0.82	0.70–1.20
Copper/zinc	1.8 ± 0.45	2.7 ± 0.92	1.6 ± 0.45	<0.0001[c]	1.00–1.17
Lipid peroxides (μM)	493 ± 196[a]	878 ± 274[a]	377 ± 192[a]	<0.0001	48–400
Oxidized LDL (ng/ml)	333 ± 509	280 ± 268	331 ± 551	0.79	0–500
Ab-Ox-LDL (IU/l)	376 ± 383	442 ± 425	431 ± 388	0.53	200–600

[a]are significantly different;
[b]NCU significantly different of OCU and IUD;
[c]OCU significantly different of NCU and IUD;
[d]IUD significantly different of OCU and NCU;
[e]CHU Liège, Belgium.

Table 4: Comparison of OSS markers among women (n = 38, age 40–48 years) using hormonal or copper (Cu) IUD. Data were expressed as means +/− SD. The sample size was too small for an adequate statistical analysis

Variable	IUD type		
	Copper ($n = 6$)	Levonorgestrel ($n = 32$)	P-value
Vitamin C (μg/ml)	9.4 ± 1.4	10.3 ± 2.9	0.28
α-tocopherol (μg/ml)	12.8 ± 1.8	11.9 ± 2.4	0.21
Vitamin C/ α−tocopherol	0.73 ± 0.16	0.90 ± 0.31	0.18
Cholesterol (g/l)	1.81 ± 0.23	1.77 ± 0.27	0.56
γ-tocopherol (mg/l)	0.91 ± 0.26	0.78 ± 0.25	0.25
α-tocopherol/cholesterol (mg/g)	7.1 ± 1.1	6.7 ± 0.9	0.38
γ/α-tocopherol	0.07 ± 0.02	0.07 ± 0.02	0.67
β-carotene (mg/l)	0.44 ± 0.17	0.45 ± 0.43	0.46
Thiol proteins (μM)	446 ± 42	448 ± 55	0.79
Selenium (μg/l)	86 ± 7.2	86 ± 17	0.75
Copper (mg/l)	0.98 ± 0.32	0.84 ± 0.19	0.34
Zinc (mg/l)	0.54 ± 0.09	0.58 ± 0.12	0.36
Copper/zinc	1.87 ± 0.80	1.48 ± 0.37	0.36
Lipid peroxides (μM)	321 ± 154	372 ± 198	0.63
Oxidized LDL (ng/ml)	272 ± 313	356 ± 607	0.75
Ab-Ox-LDL (IU/l)	444 ± 397	454 ± 400	0.99

values that largely exceeded the normal range established for these three parameters (i.e. 0.70–1.40 mg/l, 1.00–1.17 and 48–400 μmol/l for copper, copper/zinc ratio and lipid peroxides, respectively). However, despite modifications, the levels in selenium, γ-tocopherol and β-carotene remained within the reference values established by the Clinical Laboratories of the University Hospital of Liège.

Table 4 compares OSS among women using IUD containing either copper or levonorgestrel. No significant difference could be observed between both groups.

Prediction of the type of contraception oxidative stress

Figure 1 exhibits a strong, positive correlation ($r = 0.84$; $P < 0.0001$) between the concentration of plasma copper and lipid peroxides for all women involved in the ELAN study. Similar findings were observed with the copper/zinc ratio (r = 0.78; $P < 0.0001$). Statistical analysis allowed us to measure the potential effect of OC on the values of these parameters. Since lipid peroxides and copper levels were similar in the NCU and IUD groups, the two groups were merged (NCU + IUD). As seen in Fig. 2, the concentration of lipid peroxides clearly shifted to high values in OCU when comparison with NCU + IUD. By logistic regression analysis, a plasma level of lipid peroxides of 660 μM yielded sensitivity of 80% (proportion of OCU women with level >660 μM) and a specificity of 84% (proportion of NCU with level <660 μM). Figure 3A and B display the level of plasma copper and the copper/zinc ratio in groups of OCU versus NCU + IUD. For these parameters, the cut-off values were 1.2 mg/l (sensitivity 82%; specificity 85%) and 2.1 (sensitivity 78%; specificity 81%), respectively.

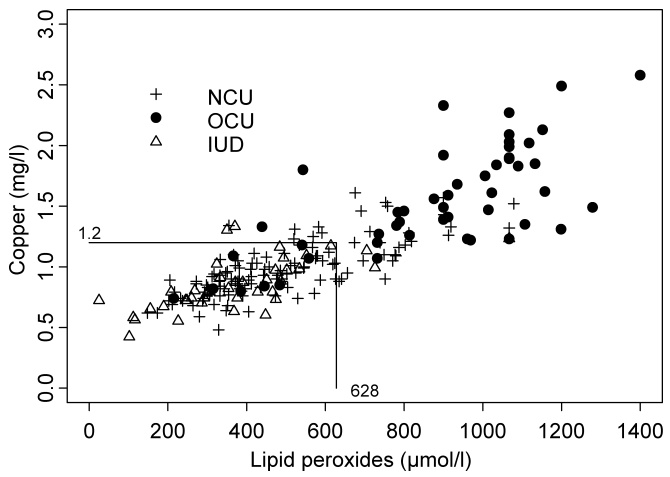

Figure 1: Relationship between plasma copper and lipid peroxides levels among the whole group of women ($n = 209$; age ragned 40–48 years) participating in the ELAN study ($r = 0.84$; $P < 0.0001$). Linear regression reveals that the majority of women taking OC were situated outside the rectangle defined by 1.2 mg/l in copper and 628 μM in lipid peroxides. OCU: Oral contraception Users; NCU: Non-Contraception Users; IUD: Intrauterine Devise Users

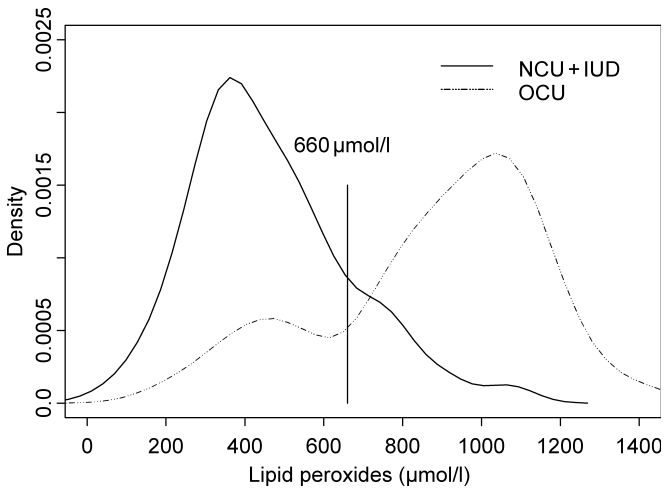

Figure 2: Determination of a cut-off point in plasma lipid peroxides concentration that discriminates women taking OC (OCU) or not (NCU+IUD)

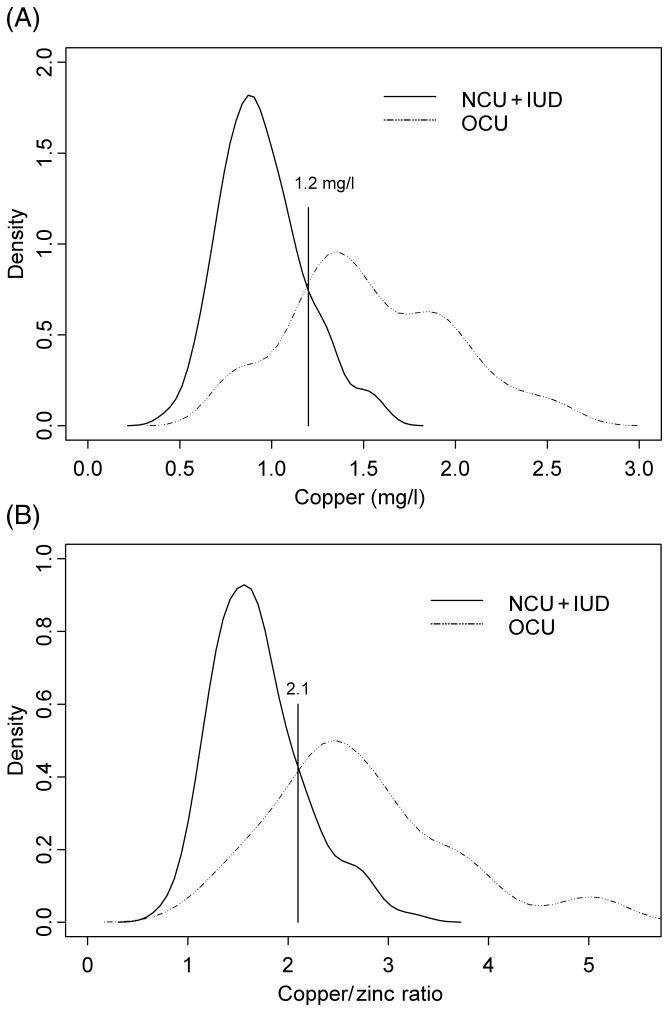

Figure 3: Determination of a cut-off point in plasma copper concentration and copper/zinc ratio that discriminates women taking OC (OCU) or not (NCU+IUD)

Discussion

The initial goal of the ELAN study was to document the OSS of the population living in the Province of Liège, Belgium. To the best of our knowledge, this is the first study performed in this country on such a large scale (897 subjects), using the analysis of 16 parameters including antioxidants, trace elements and markers of lipid peroxidation. The study only addressed men and women aged 40–60 years. This choice was based on the fact that this group is associated with increasing risk for developing several pathologies including cancer and cardiovascular diseases.

Among conditions known to influence OSS, the use of OC in women has been a matter of ongoing discussion. Although this method of contraception is estimated to be used by 50–100 million women worldwide, data about the relationship between steroid contraception and oxidative stress are scarce and, overall, conflicting. As discussed earlier, data obtained from both *in vitro* and *in vivo* studies have pointed out pro- and antioxidant properties of estrogens but also an absence of effect. Our study shows that OC significantly alters the OSS of women aged 40–48 years.

Antioxidants

Our data indicate that OC has no effect on the concentration of vitamin C, α-tocopherol, vitamin C/α-tocopherol ratio, α-tocopherol/cholesterol ratio when compared with both NCU and IUD (Table 3). In contrast, our study points to significant disturbances in the level of β-carotene in the plasma of women taking contraceptive pills. Our original food frequency questionnaire clearly indicates that such modifications in plasma β-carotene cannot be attributed to diet changes between all three groups. From a univariate point of view, the concentration of β-carotene in OCU decreased by ∼39% ($P < 0.01$) and 50% ($P < 0.001$) when compared, respectively, with the groups of NCU and IUD. This is in agreement with the study of Palan *et al*., (2006), and, more specifically, the study of Berg *et al*. in 1997 on a sample of 610 women aged between 18 and 44 years. It has been speculated that estrogens induce an activation of the retinol binding protein (Mooij *et al*., 1991), thus possibly increasing the conversion of β-carotene into retinol. Whatever the mechanism, it is important to highlight how this decrease in β-carotene concentration must be interpreted in OCU's. The reference values for β-carotene (0.08–0.60 mg/l) established in our hospital are in good agreement with those generally listed and admitted in the literature (Galan *et al*., 2005). With respect to our laboratory values, a woman taking an oral hormonal contraception and having a mean concentration of 0.22 mg/l as shown in our study should be, therefore, considered as belonging to a normal population. In terms of disease prevention, this is, however, not a normal value since various epidemiological studies including the important WHO Monica Vitamin Substudy (Gey, 1994) have clearly determined a cut-off point of 0.215 mg/l associated with an increased risk of developing a cancer or a cardiovascular disease. Using a multivariate analysis, we also observed a cumulative effect of cigarette smoking (another factor of cardiovascular risk) and oral contraceptive usage on the decrease in plasma β-carotene (data not shown), which is in agreement with Palan *et al*., (1989).

Trace elements

Although being in the range of normal reference values, selenium concentration appeared to be significantly higher (11.8%; $P < 0.01$) in OCU than those of NCU and IUD (Table 2). This could be explained by the fact that the use of combined OC (20 μg ethinylestradiol and 150 μg desogestrel) led to a significant increase of GPx, a seleno-dependent enzyme assuming the elimination of lipid peroxides (Massafra *et al*., 1993). Such a difference in selenium status could be, however, misinterpreted in our study since plasma selenium (as well GPx activity) in women of reproductive age were fluctuating in synchrony with variations in circulating estrogen concentrations. Indeed, Ha and Smith (2003) demonstrated that selenium level were significantly greater during the periovulatory phase by 14.2 and 11.3% than during the early follicular and mid-luteal phases, respectively. Interestingly, the peak of selenium concentrations strictly coincided with those of 17-β-estradiol ($P < 0.0001$). In the current study, we had no information at which phase blood sampling was performed in women still having a menstrual cycle.

As early as the discovery of the role of estrogens in the reproductive processes, Russ and Raymund (1956) described that the use of estrogens resulted in an increase of plasma copper level that run parallel to that of ceruloplasmin. Further studies have well confirmed this observation (Prasad *et al*., 1975b; Sher 1977; Crews *et al*., 1980; Prema *et al*., 1980; Chilvers *et al*., 1985; Berg *et al*., 1998b). In our study, the mean plasma copper level reached a higher value (1.5 mg/l), i.e. in agreement with the recent study of Benes *et al*. (2003). A report by Kluft *et al*. (2002), it has shown that estrogen-containing preparations caused an increase in a specific set of acute-phase proteins elaborated by the liver. Therefore, the increase in plasma copper in OCU is generally explained by the over expression of ceruloplasmin, a copper carrier protein (Limpongsanurak *et al*., 1981). In contrast, it is interesting to note in our study that women having a plastic T-shaped frame, i.e. wrapped with copper and/or has a copper band presented a plasma copper value that remained within the normal range (0.98 ± 0.32 mg/l). With respect to the correlation between plasma copper and lipid peroxides (Fig. 1), we did not observe in IUD users, plasma levels of lipid peroxides as high as in OCU (Table 3).

As shown in Fig. 2, a cut-off value of 1.2 mg/l in plasma concentration of copper was found to discriminate all the OCU from the others (NCU + IUD). From a biological point of view, it seems to us, however, more relevant to evaluate the copper/zinc ratio since these two trace elements have opposite effects on the oxidative stress. Similarly to iron, copper becomes at high concentration a potent catalyst of free radical production through a Fenton-like reaction (Gaetke and Chow, 2003). In contrast, increased levels of zinc result in the over-expression of antioxidant metallothioneins and in the inhibition of copper-induced free radical species. In our study, the cut-off point for discriminating OCU from all NCU + IUD) was found to be 2.1 for copper/zinc (Fig. 3). From a clinical point of view, it should be highlighted that recent epidemiological studies have described an increased mortality from cardiovascular diseases in subjects with higher serum copper levels (Wu *et al*., 2004; Leone *et al*., 2006).

Markers of lipid peroxidation

One of the major findings of the present study was the dramatic and significant increase in lipid peroxides observed in the group of women taking a steroid contraception (Table 3). Oxidative lipid damage was on average 1.77-fold higher in OCU than in NCU. As shown in Fig. 2, a cut-off value of 660 μM for plasma lipid peroxides was found to discriminate all the OCU from the others. As the ELAN women female cohort exhibited a too large diversity in the intake of progestins and steroids (Table 1), we were, however, unable to delineate a different response in intensity of lipid peroxide increase according to the type of OC used (mono, bi and tri-phasic pills). Further investigations should be are necessary to appreciate whether one specific formulation of oral contraceptives could be more responsible for lipid peroxidation than another one. In contrast to lipid peroxides, no modifications were observed at the level of oxidized LDL and Ab-Ox-LDL between all investigated groups.

Several studies in both rats and women have already mentioned an increased level of plasma lipid peroxides due to the use of estrogen (Plaa and Wittchi, 1976; Wynn *et al*., 1979; Behall *et al*., 1980; Ciavatti *et al*., 1989; Subakir *et al*., 2000). In their animal studies, Ciavatti *et al*. (1989, 1991) noted that the elevation of lipid peroxides observed in OC-treated rats was able to increase in the plasma the aggregability of platelets exposed both to thrombin and ADP. Such an effect, i.e. potentially involved in the development of venous thromboembolism, may be blocked by antioxidants such as γ-tocopherol (Wagner *et al*., 2004), the concentration of which is significantly lower in OCU than in NCU (Table 3).

The change in the copper concentration is certainly the major event leading to the increased level of lipid peroxides. As shown in Fig. 1, a strong and positive correlation was observed between the concentration of plasma copper and lipid peroxides for all women confounded ($r = 0.84$; $P < 0.0001$). A similar observation was made concerning the copper/zinc ratio ($r = 0.78$; $P < 0.0001$) but with a lower value for the correlation. Relationship between copper (as prooxidant)/zinc (as antioxidant) ratio and circulating lipid peroxides was already reported by Mezzetti *et al*. (1998) for both men and women. Since the correlation coefficient between the lipid peroxides and the plasma level of copper was 0.84 ($P < 0.0001$), we used a linear regression to predict the level of lipid peroxides as a function of the level of copper. The expected value of lipid peroxides corresponding to the cut-off value of 1.2 mg/l in copper (see above) was 628 μM. As shown in Fig. 1, the majority of women taking OC were situated outside the area defined by these two points. It should be noted that this value of lipid peroxides was close to those obtained (660 μM) with the predicted model used for discriminating OC from NOC (Fig. 2).

The question is to know if such an increased level of lipid peroxides could have an impact on the health of OCU. Actually, it is well accepted that oxidative damage to lipids and lipoproteins is a process potentially involved in the development of atherosclerosis. In addition to lipid peroxides, we analysed in the ELAN study the concentration of two other markers of lipid peroxidation associated with a higher incidence of cardiovascular diseases: oxidized LDL and their respective antibodies (Holvoet *et al*., 1998). In contrast to lipid peroxides, no difference was observed between OCU and NCU for both parameters, suggesting that oral contraceptives containing <50 μg of estrogen and second- or third-generation progestins may be reasonably safe from a cardiovascular point of view (Carr and Ory, 1997; Rosenberg *et al*., 1997). Nevertheless, the increase in lipid peroxides due to OC should be taken into consideration. Further research is necessary to elucidate its real impact as cardiovascular risk factor in OCU.

Conclusions

The relationship between estrogens and oxidative stress remains a matter of debate. Estrogens are recognized to be beneficial in the prevention of atherosclerosis although they are capable of inducing oxidative stress, which is involved in the development of the same atherosclerosis. The results of the

present study indicate that the intake of estrogens is associated with a significantly altered OSS among women aged 40–48 years. Increase in the concentration of copper, selenium and lipid peroxides and decrease in γ-tocopherol and β-carotene strongly support this theory. It clearly appears that the plasma copper increase in OCU plays a central role in enhancing lipid peroxidation as evidenced by the strong correlation existing between these two parameters. In our hands, plasma values of copper, lipid peroxides and copper/zinc ratio, respectively, >1.2 mg/l, 660 μM and 2.1, respectively, should be considered in OCU as a potential indication for considering antioxidant supplementation. Checking these parameters in young and older women taking OC should be, therefore, of interest with respect to potential increased risk of developing venous thromboembolism (Ageno *et al*., 2006) and cardiovascular disorders (Nema *et al*., 2006). Additional systematic studies, however, are needed to clarify the precise role of oxidative stress mediated by estrogens.

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Authorship and Contributorship

The ELAN ("Etude Liégeoise sur les ANtioxydants") study was conducted from March through July 2006 as a joint project between the University of Liège, the University Hospital of Liège and the local health services of the Province of Liège (Belgium). Pr JO Defraigne and Dr Sc J Pincemail (CREDEC and Dept of Cardiovascular Surgery, Pr R Limet) were the main coordinators of the ELAN study. They collected all data and wrote the manuscript in the present form with the inestimable help of Pr U Gaspard (Dept of Gynaecology). Pr C Charlier, Pr JP Chapelle and Dr J Haleng (Laboratories of Clinical Biology and Toxicology) allowed the analysis of all biological parameters investigated in the study, except for vitamin C that was performed by Mr JP Cheramy – Bien (CREDEC). Pr D Giet and Dr G Collette (Dept of General Medicine) allowed the recruitment of all general practitioners around the Province of Liège, Belgium. Pr A Albert and Mrs S Vanbelle (Dpt of Medical Informatics and Biostatistics) were involved in the statistical analysis of all data.

All investigators critically revised the manuscript for the intellectual content and gave their final approval of the version to be published.

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