

## Short-Term Moderate Energy Restriction Does Not Affect Indicators of Oxidative Stress and Genotoxicity in Humans<sup>1</sup>

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**ABSTRACT** Restriction of energy intake (ER), without malnutrition of essential nutrients, has repeatedly been demonstrated to increase longevity in rodents. In the antioxidant theory of aging the lack of balance between the generation of free radicals and free radical scavenging was thought to be a main causal agent in the aging process. From this point of view the antiaging effect induced by ER might be due to the lower rate of free-radical production and related damage induced by a lower metabolic rate. The antiaging effects of ER might also occur in humans. This study explored the effects of a 10-week moderately energy-restricted diet (80% of habitual) in 24 non-obese middle-aged men (16 ER subjects, 8 controls) on resting metabolic rate (RMR) and indicators of the primary antioxidant defense system, oxidative stress and genotoxicity. RMR decreased significantly in both groups, even when adjustments were made for the change in body composition. The increase in blood vitamin C concentrations correlated with the increase in urinary 8-hydroxydeoxyguanosine (8OHdG) excretion. The change in urinary 8OHdG excretion also correlated with the change in RMR per kg fat-free mass. No differences between groups were found for changes in indicators of genotoxicity, erythrocyte catalase, glutathione peroxidase and superoxide dismutase activity and in plasma vitamin E, A or  $\beta$ -carotene concentrations. We conclude that 10 weeks of moderate ER did not affect indicators of antioxidative capacity, oxidative stress and genotoxicity of humans. Since subjects were not in energy balance at the end of the study, no conclusions can be made with respect to long-term effects. *J. Nutr.* 125: 2631–2639, 1995.

### INDEXING KEY WORDS:

- humans • moderate energy restriction
- oxidative stress • genotoxicity

Energy restriction (ER),<sup>3</sup> without malnutrition of essential nutrients, has repeatedly demonstrated an effective and reproducible increase of longevity in rodents. Although ER retards many of the physiological changes associated with aging, the mechanism(s) responsible for the antiaging effects of ER are not well understood, which is also true for the basic causes of the aging process itself. Among the many theories of aging, the possibility that free radicals, generated by oxygen metabolism, are the main causal agents is receiving particular attention. Harman (1992) formulated his free-radical theory of living as early as 1954, when he postulated that aging might be attributed at least in part to accumulative damage caused by radical generation associated with cellular metabolic activity. According to this theory, free-radical damage can be reduced by lowering metabolic rate. This theory has been supported by studies from Cutler (1984) who showed an inverse relationship between species-specific life span and metabolic rate.

Therefore, one of the causes underlying the antiaging effect induced by ER might be a lower rate of free-radical production and related damage, induced by a lower metabolic rate.

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<sup>3</sup> Abbreviations used: BMI, body mass index; ER, energy restriction; 8OHdG, 8-hydroxy-deoxyguanosine; PUFA, polyunsaturated fatty acids; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; FFM, fat-free mass; SOD, superoxide dismutase; LDL-ox, oxidizability of LDL.

TABLE 1  
 Physiological characteristics of the participants at the start and at the end of the experiment, as well as the change ( $\Delta$ ) observed after 10 wk of moderate energy restriction<sup>1,2</sup>

	Control group (n = 8)			Energy restricted group (n = 16)			P $\Delta$ control vs $\Delta$ ER
	Start	End	$\Delta$	Start	End	$\Delta$	
Body weight, kg	77.7 $\pm$ 6.6	75.7 $\pm$ 6.0	-2.1 $\pm$ 1.5**	78.6 $\pm$ 9.3	71.3 $\pm$ 9.3	-7.4 $\pm$ 1.7***	<0.001
Body mass index, kg/m <sup>2</sup>	24.6 $\pm$ 2.2	23.8 $\pm$ 2.1	-0.8 $\pm$ 0.6	24.9 $\pm$ 1.8	22.6 $\pm$ 1.6	-2.3 $\pm$ 0.8***	<0.001
RMR, MJ/d	7.40 $\pm$ 0.75	6.80 $\pm$ 0.55	-0.60 $\pm$ 0.40**	8.02 $\pm$ 1.26	6.93 $\pm$ 0.95	-1.07 $\pm$ 0.63***	NS
RMR, l/(kg FFM $\cdot$ d)	124.5 $\pm$ 9.4	115.1 $\pm$ 9.2	-9.4 $\pm$ 6.7**	132.7 $\pm$ 16.4	114.9 $\pm$ 13.8	-15.4 $\pm$ 11.1***	NS

<sup>1</sup> Values are means  $\pm$  SD; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  within group.

<sup>2</sup> Abbreviations used: RMR, resting metabolic rate; FFM, fat-free mass; NS, not significant.

In addition to the effect on metabolic rate, the beneficial effects of ER on the aging process have been explained by a positive effect of ER on maintaining, or even increasing, the antioxidative defense system with age (Yu 1993). Free radicals are effectively scavenged by both radical-scavenging enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px), as well as by antioxidants such as vitamins E and C and  $\beta$ -carotene.

Thus, the beneficial effects of ER on the aging process may proceed through both effects, that is, by lowering the production rate and by effective scavenging of free radicals, resulting in lowering oxidative stress. An intriguing question remains whether ER might also be beneficial in humans. Because aging is a pleiotropic process, the antiaging effects of ER might also occur in humans. However, it is very difficult, if not impossible, to test this hypothesis in life-long controlled human experiments. The aim of this study was to investigate the effects of a 10-wk moderately energy-restricted diet in nonobese middle-aged men on indicators of the primary antioxidant defense system (antioxidant enzymes as well as antioxidant vitamins), oxidative stress and genotoxicity. The duration of the study was 12 wk because, in our opinion, volunteers would not be willing to participate for a longer period of time under these controlled conditions. It was hypothesized that ER might increase antioxidative capacity or reduce oxidative damage, in parallel with a decrease in resting metabolic rate.

## MATERIALS AND METHODS

**Subjects.** Twenty-four apparently healthy men, aged  $43 \pm 5$  (range, 35–50 y) with a mean body mass index (BMI) of  $24.6 \pm 1.8$  kg/m<sup>2</sup> (range, 20.6–27.2 kg/m<sup>2</sup>) participated in this study. All subjects had a stable weight during the past year, had a normal routine clinical chemistry profile and were nonsmokers. They

had normal dietary patterns and no extreme level of energy intake (9300–13,200 kJ/d). Some physiological characteristics (body weight, BMI, resting metabolic rate) of the subjects are presented in **Table 1**. Each subject was medically screened through a health questionnaire and a medical examination. Informed consent was obtained from each subject, and the research protocol was approved by the institute's external Medical Ethical Committee.

**Experimental design.** A detailed description of the experimental design has been published elsewhere (Velthuis-te Wierik et al. 1994). Briefly, the total experiment lasted 12 wk. Subjects were assigned to two groups: a control group ( $n = 8$ ) and an energy-restricted group (ER group;  $n = 16$ ). Groups were matched on the basis of age and BMI. Habitual food intake was assessed by means of a 7-d dietary record. As calculated with the computerized Netherlands Food Composition Table (UCV/NEVO), habitual intake of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), respectively, was  $17.3 \pm 2.9\%$ ,  $16.0 \pm 2.0\%$  and  $6.6 \pm 1.1\%$  of total energy intake in the control group and  $15.8 \pm 2.2\%$ ,  $15.4 \pm 2.9\%$  and  $6.4 \pm 1.3\%$  of total energy intake in the ER group. The groups did not differ significantly for any of these fatty acid intakes.

During the first 2 wk of the study (run-in period) all subjects received a weight-maintaining test diet based on the outcome of their dietary record. Because energy intake differs among individuals, subjects were divided into six energy groups with a stepwise increase of  $\sim 770$  kJ (that is, 9,304; 10,073; 10,841; 11,610; 12,378 and 13,147 kJ/d, respectively). When subjects lost  $>1$  kg/wk during this run-in period, they were reclassified into a higher energy group.

After these 2 wk the 8 control subjects were kept on their weight-maintaining diet for the next 10 wk, while the other 16 subjects received a test diet that contained 80% of the energy of their habitual (weight-maintaining) diet, with micronutrient levels of at least

their recommended dietary allowance (RDA). The 20% energy restriction was achieved by substituting low-fat and artificially sweetened products for ordinary products, so that the relative contribution of carbohydrates, fat and protein to total energy intake remained approximately the same. During the experimental period intake of SFA, MUFA and PUFA was calculated to be  $13.6 \pm 0.3\%$ ,  $13.2 \pm 0.5\%$  and  $11.1 \pm 0.4\%$  of total energy intake in the control group and  $14.2 \pm 0.1\%$ ,  $13.8 \pm 0.2\%$  and  $8.1 \pm 0.1\%$ , respectively, in the ER group, indicating that PUFA intake was lower in the ER group. Diets with the highest and lowest energy content were analyzed for antioxidant micronutrients. Analyses of these extremes revealed that vitamin E intake was between 7.8 and 18.6 mg/d for the control group and between 9.3 and 10.1 mg/d for the ER group; vitamin C intake was between 97.4 and 136.1 mg/d for the control group and between 117.4 and 135.2 mg/d for the ER group;  $\beta$ -carotene intake was between 1.9 and 2.1 mg/d for the control group and between 1.9 and 2.0 mg/d for the ER group; selenium intake was between 39 and 52  $\mu\text{g}/\text{d}$  for the control group and between 28 and 51  $\mu\text{g}/\text{d}$  for the ER group.

All foods and drinks to be consumed during the 12-wk study were supplied by the institute. The subjects were not allowed to eat or drink (except water) anything but the food supplied.

Resting metabolic rate (RMR) was measured twice before and twice at the end of the experimental period. The mean of the duplicate measurements was used as a variable in the analysis. At the end of the run-in period and at the end of the experimental period body composition was measured, and blood was drawn from fasting subjects for the analysis of variables related to oxidative stress [plasma malondialdehyde (MDA), oxidizability of LDL (LDL-ox) and 8-hydroxydeoxyguanosine (8-OHdG) in both lymphocytes and urine], as well as for the indicators of antioxidative capacity (erythrocyte catalase, GSH-Px and SOD activities as well as plasma vitamins C, E and A and  $\beta$ -carotene concentrations).

**Methods. Resting metabolic rate.** The RMR was measured in the morning after the subjects had stayed overnight in the metabolic ward of the institute.  $\text{O}_2$  consumption (paramagnetic analyzer, Beryl 102; Cosma, Igny, France) and  $\text{CO}_2$  production (AR-400 infrared gas analyzer; Anarad, Santa Barbara, CA) were measured under thermoneutral temperature conditions in fasting subjects on four occasions, twice during the run-in period and twice near the end of the experiment.  $\text{O}_2$  and  $\text{CO}_2$  analyzers were calibrated against standardized gas mixtures every morning. While lying for 30 min in the supine position, the subjects were breathing through a low resistance breathing valve (#2700; Hans Rudolph, Kansas City, MO). Weir's (1949) formula was used for calculating RMR.

**Body composition.** The subjects were weighed to the nearest 0.1 kg every week on a balance (ED-60-T; Berkel, Ridderkerk, The Netherlands) before dinner, while wearing underwear. Body composition, that is, fat mass and fat-free mass (FFM), was estimated from the three-compartment model of Siri (1961) that combines body density with total body water. Body density was measured using the hydrostatic weighing technique of Brozek et al. (1963). Underwater weight was determined to the nearest 0.1 kg with a balance (SK; Berkel) while the residual lung volume was determined simultaneously by helium dilution and a spirometer (Volutest; Mijnhardt, Bunnik, The Netherlands). Total body water was determined by deuterium dilution.

**Plasma malondialdehyde (MDA).** For the analysis of MDA blood was collected in EDTA-containing tubes. Plasma was stored under helium at  $-80^\circ\text{C}$  until analysis. The MDA was analyzed fluorometrically (Perkin-Elmer spectrophotometer 204-A, Gouda, The Netherlands) after formation of an adduct with thiobarbituric acid (TBA) as reported by Hendriks and Assman (1988).

**Low density lipoprotein resistance of ex-vivo copper-mediated oxidation (LDL-ox)** was analyzed according to the method of Princen et al. (1992). The lag-time (min) was used as a measure of LDL-ox.

**8-Hydroxydeoxyguanosine (8OHdG) in lymphocytes.** Lymphocytes were isolated using Ficoll. From these lymphocytes DNA was isolated. The number of 8OHdG adducts was measured by HPLC with electrochemical detection, essentially according to Richter et al. (1988) and was expressed as mol 8OHdG per  $10^5$  mol deoxyguanosine. Analysis of lymphocyte 8OHdG was completed in 19 subjects at the start of the intervention (ER group  $n = 13$ ; control group  $n = 6$ ), and in 22 subjects at the end of the intervention (ER group  $n = 15$ ; control group  $n = 7$ ).

**Urinary 8OHdG.** The 24-h urine samples were collected at the end of the run-in period and at the end of the experimental period and stored at  $-20^\circ\text{C}$  until analysis of 8OHdG by HPLC with electrochemical detection (Loft et al. 1992). The second urine collection was not completed in one subject from the ER group.

For the analysis of *catalase*, *GSH-Px* and *SOD* in erythrocytes, heparinized blood was collected. Erythrocytes were washed three times and stored at  $-80^\circ\text{C}$  until analysis.

**Catalase activity (EC 1.11.1.6)** was measured by monitoring substrate ( $\text{H}_2\text{O}_2$ ) disappearance spectrophotometrically (LKB, Cambridge, UK) at 240 nm according to the method of Aebi (1984).

The *GSH-Px* activity (EC 1.11.1.9) was measured according to a FLAIR-standardized method (Belsten and Wright, unpublished results). This FLAIR assay is based on Paglia and Valentine's method (1967), including an enzyme stabilization step and using *t*-butyl hydroperoxide rather than hydrogen peroxide.

The SOD activity (EC 1.15.1.1) was measured spectrophotometrically (LKB, Cambridge, UK) using the RANSOD kit (Randox Laboratories, Ireland) according to the kit's protocol.

**Total vitamin C.** Vitamin C concentration in whole blood was measured by HPLC with fluorometric detection [Speek et al. 1984] immediately after blood sampling [between-assay variability: 5%].

**Vitamin E, vitamin A and  $\beta$ -carotene.** Heparinized blood was collected. Plasma was separated immediately after collection by centrifugation (2000 $\times$  g for 10 min) and stored at  $-80^{\circ}\text{C}$  until analysis. Vitamin E, vitamin A and  $\beta$ -carotene were analyzed by HPLC as described by van Vliet et al. (1991).

**Total cholesterol.** For the analysis of total cholesterol concentration a commercially available kit was used [(CHOD-PAP) Boehringer Mannheim Diagnostica, Amsterdam, The Netherlands].

**Hemoglobin (Hb) concentration** was determined by using a semiautomatic computer equipped hematology analyzer (Sysmex K1000, Japan; DA1000, Japan).

Near the end of the experimental period (after 10 wk of moderate energy restriction) after an overnight fast, blood was collected by venipuncture into heparinized tubes. Lymphocyte cultures were set up using whole blood and phytohemagglutinin stimulation for the assessment of a variety of cytogenetic biomarkers by the following routine methods: 1) sister chromatid exchanges (SCE) [WHO 1985, Carrano and Natarajan 1988]; 2) chromosomal aberrations [WHO 1985, Carrano and Natarajan 1988]; and 3) micronuclei in binucleated cells [cytokinesis block method; Fenech and Morley 1985, Fenech 1993].

**Statistics.** BMDP statistical software (Los Angeles, CA; version 1990 VAX/VMS) was used to detect changes within and between the groups and to calculate correlations between variables. Changes within the groups were tested for significance with the paired Student's *t* test. Differences in changes between the two groups were tested with the unpaired Student's *t* test. Relationship between two variables was calculated with Pearson linear correlations. Statistical significance was considered at  $P < 0.05$ .

## RESULTS

Table 1 presents the physiological characteristics, including body weight and RMR before and after the intervention as well as the change ( $\Delta$ ) that had occurred at the end of the intervention. As expected, there was a considerable weight loss in the ER group that was significantly greater than the relatively small weight loss in the control group. At the end of the intervention period subjects were still losing weight, and there was no tendency to stabilize. As a consequence of the weight loss, BMI decreased significantly in the ER

group. The RMR significantly decreased in both groups, but RMR tended ( $P = 0.06$ ) to decrease more in the ER group than in the control group. Even after adjusting for body composition, RMR still significantly decreased in both groups, suggesting an increase in metabolic efficiency in subjects in negative energy balance.

The effects of a moderately energy-restricted diet on indicators of oxidative stress and antioxidative capacity are summarized in **Table 2**. No effects could be demonstrated for any of the indicators of oxidative stress (MDA, LDL-ox and 8OHdG) measured. The 8OHdG was measured in both lymphocytes and urine. Lymphocyte 8OHdG is considered to reflect the balance between damage and repair at the time of sampling, whereas urinary 8OHdG is interpreted as the rate of oxidative DNA modification. Neither indicator of oxidative DNA modification was affected significantly by ER. However, the change in 8OHdG excretion, that is, the change in the rate of rate of oxidative DNA modification, correlated positively with the change in RMR ( $r = 0.516$ ,  $P = 0.017$ ,  $n = 21$ ) when both indicators were expressed per kg fat-free mass (FFM). Of the free-radical-scavenging enzymes measured, catalase significantly decreased in both groups, with no difference between groups. The activities of the two other free-radical-scavenging enzymes, GSH Px and SOD, did not change. Of the antioxidant vitamins analyzed, vitamin C concentrations increased significantly in the ER group but not in the control group. The change in whole blood vitamin C concentration correlated with the change in 8OHdG excretion when all subjects were included in the analysis ( $r = 0.478$ ,  $P = 0.021$ ) (**Fig. 1**). Plasma vitamin E concentration decreased in the ER group. However, when expressed relative to plasma total cholesterol concentration, this decrease was not significant. No changes were observed in plasma vitamin A concentrations.  $\beta$ -Carotene concentration increased significantly in the ER group, but this increase was not significantly different from the nonsignificant increase in the control group.

No differences between the two groups were observed for any of the indicators of genotoxicity measured after 10 wk of moderate energy restriction only (**Table 3**).

## DISCUSSION

One of the hypotheses that has been put forward to explain the aging process is that aging is related to a deregulation in the balance between prooxidants and antioxidants leading to oxidative stress. The large body of literature on animal studies indicates the following: 1) the levels of antioxidant defense are positively correlated with species life span; 2) the rate of production

TABLE 2  
 Indicators of oxidative stress and antioxidant capacity in humans at the start and at the end of the experiment,  
 as well as the change ( $\Delta$ ) observed after 10 wk of moderate energy restriction<sup>1,2</sup>

	Tissue	Control group (n = 8)			Energy restricted group (n = 16)			P $\Delta$ control vs. $\Delta$ ER
		Start	End	$\Delta$	Start	End	$\Delta$	
<b>Oxidative stress</b>								
MDA, $\mu\text{mol/L}$	P	1.04 $\pm$ 0.21	1.22 $\pm$ 0.21	0.19 $\pm$ 0.28	1.09 $\pm$ 0.19	1.17 $\pm$ 0.39	0.13 $\pm$ 0.35	NS
LDL-ox, lag time, min	P	68.7 $\pm$ 15.7	74.6 $\pm$ 6.4	5.9 $\pm$ 16.2	67.3 $\pm$ 25.9	73.3 $\pm$ 10.6	5.9 $\pm$ 31.0	NS
8-OHdG/ $10^5$ dG, mol/mol	L	3.45 $\pm$ 2.00 <sup>3</sup>	3.09 $\pm$ 2.15 <sup>4</sup>	1.46 $\pm$ 2.37 <sup>5</sup>	2.88 $\pm$ 2.11 <sup>6</sup>	2.81 $\pm$ 2.49 <sup>7</sup>	-0.02 $\pm$ 2.84 <sup>8</sup>	NS
8-OHdG, nmol/24 h	U	39.5 $\pm$ 12.5	36.7 $\pm$ 10.9	-2.85 $\pm$ 8.86	37.8 $\pm$ 13.2	41.3 $\pm$ 12.5	3.51 $\pm$ 11.6	NS
8-OHdG, nmol/(kg FFM $\cdot$ d)	U	0.67 $\pm$ 0.20	0.62 $\pm$ 0.18	-0.04 $\pm$ 0.16	0.63 $\pm$ 0.22	0.69 $\pm$ 0.24	0.06 $\pm$ 0.19	NS
<b>Antioxidative capacity</b>								
Catalase, k/g Hb	E	123.2 $\pm$ 22.2	109.9 $\pm$ 15.0	13.3 $\pm$ 12.5*	125.0 $\pm$ 20.7	114.9 $\pm$ 17.4	10.1 $\pm$ 17.9*	NS
GSH-Px, U/g Hb	E	72.7 $\pm$ 13.2	73.0 $\pm$ 12.1	0.3 $\pm$ 7.3	73.6 $\pm$ 11.9	73.0 $\pm$ 9.4	0.7 $\pm$ 8.7	NS
SOD, U/g Hb	E	680 $\pm$ 137	663 $\pm$ 143	16 $\pm$ 55	719 $\pm$ 115	704 $\pm$ 118	-15 $\pm$ 69	NS
Vitamin C, $\mu\text{mol/L}$	B	54.3 $\pm$ 7.1	52.3 $\pm$ 15.4	-2.0 $\pm$ 20.7	51.6 $\pm$ 13.7	64.9 $\pm$ 11.4	13.4 $\pm$ 14.0**	0.04
Vitamin E, $\mu\text{mol/L}$	P	28.6 $\pm$ 6.6	28.8 $\pm$ 5.3	0.2 $\pm$ 10.3	30.8 $\pm$ 6.3	27.4 $\pm$ 4.5	-3.4 $\pm$ 5.3*	NS
Vitamin E/total cholesterol, $\mu\text{mol/mol}$	P	5.89 $\pm$ 0.71	6.59 $\pm$ 2.92	0.69 $\pm$ 2.64	6.11 $\pm$ 0.79	5.76 $\pm$ 0.59	-0.35 $\pm$ 0.72	NS
Vitamin A, $\mu\text{mol/L}$	P	2.01 $\pm$ 0.48	2.06 $\pm$ 0.32	0.05 $\pm$ 0.26	1.99 $\pm$ 0.32	1.94 $\pm$ 0.24	-0.05 $\pm$ 0.29	NS
$\beta$ -Carotene, $\mu\text{mol/L}$	P	0.29 $\pm$ 0.08	0.37 $\pm$ 0.16	0.09 $\pm$ 0.12	0.30 $\pm$ 0.10	0.40 $\pm$ 0.14	0.09 $\pm$ 0.09***	NS

<sup>1</sup> Values are means  $\pm$  SD; \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  within group.

<sup>2</sup> Abbreviations used: MDA, malondialdehyde; LDL-ox, LDL resistance of ex-vivo copper-mediated oxidation; 8-OHdG, 8-hydroxydeoxyguanosine; dG, deoxyguanosine; FFM, fat free mass; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; P, plasma; L, lymphocytes; U, urine; E, erythrocyte; B, whole blood; NS, not significant.

<sup>3</sup> n = 6.

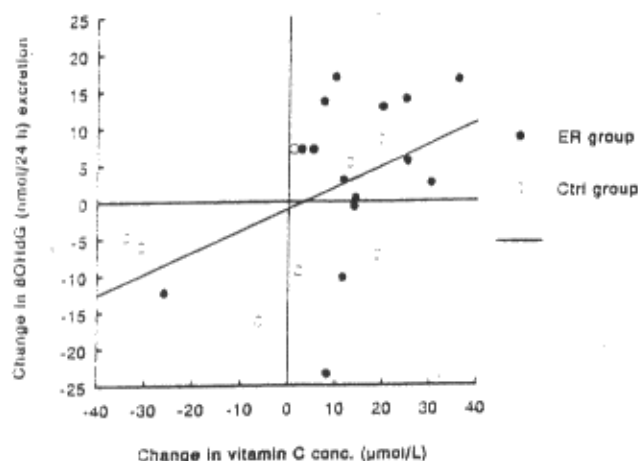
<sup>4</sup> n = 7.

<sup>5</sup> n = 5.

<sup>6</sup> n = 13.

<sup>7</sup> n = 15.

<sup>8</sup> n = 12.



**FIGURE 1** Relationship between the change in the rate of oxidative DNA damage (8OHdG excretion) and the change in whole blood vitamin C concentration after 10 wk of consuming a weight-maintaining diet or a moderately energy-restricted diet.

of free radicals correlates inversely with species life span; 3) antioxidant defense declines with increasing age; 4) the rate of production of free radicals increases with age and that, as a result, 5) oxidative damage increases with age [Warner 1993]. However, results are not always comparable because of differences in study protocol and type of experiment (matrix, type of marker, species, sex, age groups, etc.). Nevertheless, results obtained in lifelong studies in animals strongly suggest that the maintenance of an appropriate antioxidant/prooxidant balance does have an important role in maintaining health in aging [Barja de Quiroga et al. 1992]. One of the best known age-related signs of lipid peroxidation in both animals and humans is an accumulation of the lipoidal fluorescent called lipofuscin or age pigment. Lipofuscin is formed in non-dividing cells, such as neurons and skeletal and cardiac muscle cells, from the auto-oxidation of lipids, which involves free-radical chain reactions [Sohal and Brunk 1989]. An age-related increase in another lipid peroxidation product, MDA, has also been observed in human subjects aged between 10 and 80 y [Rodríguez-Martínez and Ruiz-Torres 1992] and when comparing young people (21–43 y) with elderly (>65 y) [Vericel et al. 1992]. In addition, lower platelet vitamin E levels [Vericel et al. 1992] and lower platelet GSH-Px activities [Vericel et al. 1992] were found in the elderly humans. Niwa et al. [1990] reported that the inducibility of lymphocyte SOD in health subjects decreases with age. Moreover, low inducibility at 67–73 y appeared to be predictive of malignancy or fatal cardiovascular events for the next 5 y. Recently, Orr and Sohal [1994] reported that simultaneous overexpression of SOD and catalase in *Drosophila melanogaster* resulted in a one-third extension of lifespan and a longer mortality rate doubling time, which supports the free-radical hypothesis of aging.

Thus, in both animals and humans an age-related increase in lipid peroxidation is apparent. Two years of ER in rodents seems to prevent this age-related increase in lipid peroxide levels and decrease in antioxidant defense [Velthuis-te Wierik and van den Berg 1994]. The ER might therefore have a similar beneficial effect in humans.

The aim of our intervention was to study the short-term effects of moderate energy restriction on indicators of oxidative stress and antioxidative capacity as well as on resting metabolic rate in humans. Because, to our knowledge, no previous studies have addressed this question, this appears to be the first attempt to study the relevance of findings from animal studies to humans. An interesting long-term experiment involving ER in humans is Biosphere 2. The diet of the eight subjects in this ecosystem is low in energy (7500 kJ/d) and fat (10% of energy intake). Body weight and clinical chemistry data were published [Walford et al. 1992] but not data concerning pro-oxidant/antioxidant balance.

Our results are hard to compare with data obtained from animals because data on the effects of short-term ER in animals are scarce and unequivocal. Yu [1993] found that the antilipid peroxidative action of ER (40% of ad libitum), initiated at 6 wk of age, was discernable after 6 mo of ER as indicated by suppressed MDA formation, whereas Rao et al. [1990] did not demonstrate an effect of ER (60% of ad libitum), also initiated at 6 wk of age, on MDA formation after 4 mo. Although these studies are classified as short-term, the duration of ER is approximately one-fourth of the life span of the rodent, which might be compared with ~15–20 y in humans. Another difficulty in comparing our data with data from animal studies is the age of the subjects used. Although ER initiated in adult rodents extends longevity [Weindruch and Walford 1988], it remains to

**TABLE 3**  
*Indicators of genotoxicity after 10 wk of moderate energy restriction in humans<sup>1</sup>*

	Control group (n = 8)	Energy restricted (n = 16)
Sister chromatid exchanges <sup>2</sup>	4.2 ± 0.3	4.3 ± 0.2
Chromosomal aberrations, gaps incl. <sup>3</sup>	0.7 ± 0.5	0.7 ± 0.7
Chromosomal aberrations, gaps excl. <sup>3</sup>	0.3 ± 0.5	0.3 ± 0.6
Micronuclei/1000 binucleates <sup>4</sup>	10.0 ± 3.7	10.4 ± 4.5

<sup>1</sup> Values are means ± SD.

<sup>2</sup> Number of sister-chromatid exchanges per cell; 50 cells scored per individual from two slides.

<sup>3</sup> Number of chromosomal aberrations per 100 cells; 100 cells scored per individual from four slides.

<sup>4</sup> Number of micronucleated cells per 1000 binucleated cells; 2000 binucleated cells scored per individual from two slides.

be established whether ER imposed in full-grown animals acts through similar physiological mechanisms as when imposed soon after weaning.

Considerably more data are available concerning the effects of long-term (24 mo) ER in animal models. Yu (1993) noted that MDA generation in rats freely fed increased with age. The antilipid peroxidative action of 60% ER was demonstrated by suppressed MDA formation. Rao et al. (1990) observed that the 40% ER-induced retardation of the age-dependent increase in MDA levels was apparent at 12 mo of age. With respect to *ex vivo* LDL oxidizability, to our knowledge, no studies have reported the effects of ER on LDL oxidizability. Oxidative DNA damage as measured by 8OHdG was attenuated by 40% ER in livers of 24-month-old rats (Chung et al. 1992). The products of DNA damage (including nonoxidative DNA damage) accumulate with age in mice (Holmes et al. 1992) and may therefore contribute to the aging process. We therefore hypothesized that the beneficial effects of ER might be reflected in fewer cytogenetic biomarkers for genotoxicity. Although cytogenetic biomarkers were not measured at the start of the study, there is no reason to assume that they differed between the groups at the start of the intervention. Pieri et al. (1990) studied antioxidant enzymes activities in erythrocytes from young (6 mo), old (24 mo) and energy-restricted (fed every other day) old rats and found an age-associated decrease in activities of catalase and GSH-Px, which was prevented by ER. Most of the data concerning ER and aging are generated by lifelong animal studies. Therefore, it was uncertain whether we could expect a beneficial effect of ER on the indicators presented in this paper. In addition, a complicating factor in comparing human with animal data is that most of the evidence in animal studies is based on findings obtained from inaccessible organ tissues, especially liver. Studies in humans are limited with respect to the substrates for analysis because only blood and urine samples are readily available.

Ten days of ER (5000 kJ/d) in one human subject is shown to decrease urinary 8OHdG excretion (Simic and Bergtold 1991). In our study we found no support for our hypothesis, that is, no reduction in measures of lipid peroxidation, antioxidative capacity, DNA modification or genotoxicity were observed after moderate ER for 10 wk. No effects were observed on plasma antioxidant vitamins, except for vitamin C concentrations, which increased in the ER group. Because vitamin C intake did not differ between groups (see Experimental Design), differences in intake cannot explain the increase in whole blood vitamin C concentrations. Surprisingly, there was a positive correlation between the change in the rate of oxidative DNA damage, 8OHdG excretion, and the change in whole blood vitamin C concentrations when all subjects were considered. Because vitamin C is a free-radical scavenger, we expected a negative correlation, that is, an

increase in vitamin C level should reduce the rate of oxidative DNA damage. The explanation, presumably, is related to an interplay between vitamin C and other antioxidants. An additional factor could be a changed distribution between intra- and extracellular vitamin C. Whether there is a relationship between oxidative DNA damage and vitamin C concentration in the habitual, or in the vitamin C-supplemented state, and whether this correlation is physiologically relevant is presently unknown.

In the ER group the PUFA/SFA ratio (P/S ratio) appeared to be higher in the food supplied ( $0.82 \pm 0.04$  in the control group;  $0.57 \pm 0.03$  in the ER group) than in the subjects' habitual diet ( $0.39 \pm 0.09$  in the control group;  $0.42 \pm 0.10$  in the ER group). The increase in P/S ratio ( $0.43 \pm 0.12$  in the control group;  $0.15 \pm 0.10$  in the ER group) could have strengthened a potential effect because this increase was most apparent in the control group. However, because no effects could be demonstrated, the change in dietary fatty acid profile was of no apparent importance.

Because free radicals are thought to be derived from  $O_2$  during oxidative metabolism, lower metabolic rate may partly explain the beneficial effects of ER. Although the rate of oxidative DNA damage as evaluated by a paired *t* test did not change after moderate energy restriction, we observed that the change in RMR per kg fat-free mass (FFM) correlated with the change in 8OHdG excretion per kg FFM. Detailed analysis of this finding will be published elsewhere (Loft S, Velthuis-te Wierik E. J. M., van den Berg H. & Poulsen H. E., unpublished results). The subjects did not reach a new energy balance because they were still losing weight at the end of the experimental period. It is not certain whether RMR would still be lower after a new weight equilibrium had been reached. It was reported that obese subjects have a higher RMR/kg FFM (Hoffmans et al. 1979), whereas girls suffering from anorexia nervosa have a lower RMR/kg FFM (Vaisman et al. 1991) than normal weight subjects. Despite the fact that we studied lean instead of obese or anorectic subjects and moderate energy restriction instead of semistarvation, on the basis of these reports it could be expected that RMR/kg FFM would remain lower in the ER subjects studied in this experiment. Several explanations for not finding any changes in the indicators measured can be suggested as follows: 1) because humans have a low metabolic rate, they may suffer relatively less from damage due to free radicals; therefore, they might benefit less from ER than species with a high metabolic rate; 2) the small ranges of the variables measured may be the cause of not finding any correlations between variables; 3) the period studied might have been too short; however, under controlled conditions as in this study, 10 wk is the longest period one can expect a subject to participate in; 4) antioxidative capacity might be rather stable in the age category used in this study; and 5) as already mentioned,

another reason for not finding changes in the indicators of oxidative stress and antioxidative capacity might be the tissue used. Finding no effects on a peripheral level does not exclude changes on a cellular level.

The imposed level of ER (20% of reported intake) was probably too high to be acceptable for the long term because the subjects kept losing weight and showed no tendency to stabilize. In this study we aimed at 20% ER, but the real imposed ER may have been higher because of underreporting of habitual intake (as indicated by weight loss in the control group). A milder level of energy restriction would probably be more feasible, but whether this level of ER beneficially affects the aging process in humans remains to be studied.

To summarize, in this study ER did not affect indicators of antioxidative capacity, oxidative stress or genotoxicity in humans. A correlation between the change in RMR/kg of FFM and the change in urinary 8OHdG/kg of FFM was found. However, because subjects were not stabilized at a new energy balance, no conclusions can be drawn with respect to the long-term effect. Not finding an effect on the variables measured in this study does not necessarily mean that ER does not affect oxidative stress and/or the aging process in humans. More studies using better markers for oxidative stress than presently available are needed before a definitive conclusion can be drawn.

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