

Effect of Increased Intake of Dietary Animal Fat and Fat Energy on Oxidative Damage, Mutation Frequency, DNA Adduct Level and DNA Repair in Rat Colon and Liver

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The effect of high dietary intake of animal fat and an increased fat energy intake on colon and liver genotoxicity and on markers of oxidative damage and antioxidative defence in colon, liver and plasma was investigated in Big Blue rats. The rats were fed ad libitum with semi-synthetic feed supplemented with 0, 3, 10 or 30% w/w lard. After 3 weeks, the mutation frequency, DNA repair gene expression, DNA damage and oxidative markers were determined in liver, colon and plasma.

The mutation frequency of the λ gene *cII* did not increase with increased fat or energy intake in colon or liver. The DNA-adduct level measured by ³²P-postlabelling decreased in both liver and colon with increased fat intake. In liver, this was accompanied by a 2-fold increase of the mRNA level of nucleotide excision repair (NER) gene *ERCC1*. In colon, a non-statistically significant increase in the *ERCC1* mRNA levels was observed. Intake of lard fat resulted in increased ascorbate synthesis and affected markers of oxidative damage to proteins in liver cytosol, but not in plasma. The effect was observed at all lard doses and was not dose-dependent. However, no evidence of increased oxidative DNA damage was found in liver, colon, or urine. Thus, lard intake at the expense of other nutrients and a large increase in the fat energy consumption affects the redox state locally in the liver cytosol, but does not induce DNA-damage, systemic oxidative stress or a

dose-dependent increase in mutation frequency in rat colon or liver.

Keywords: Nucleotide excision repair; Mutation frequency; DNA

Abbreviations: AAS, 2-amino adipic semialdehyde; BER, base excision repair; CAT, catalase; DHAA, dehydroascorbic acid; GGS, gamma-glutamyl semialdehyde; GPX, glutathione peroxidase; GR, glutathione reductase; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; MF, mutation frequency; NER, nucleotide excision repair; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; SB, strand breaks; SOD, superoxide dismutase

INTRODUCTION

Epidemiological studies have identified dietary intake of animal fats as a risk factor for colon cancer.^[1–3] The mechanism is still a subject of discussion^[4] and may be related to saturation, energy content or to other factors.^[5] About 70% of the fat intake in several Northern European countries is of animal origin and 20% of this is lard. Saturated fats have been proposed to have

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a tumour promoting effect although to a lesser extent than poly-unsaturated fat^[6,7] Furthermore, intake of animal fats has been associated with increased lipid peroxidation^[8] and oxidative stress^[9] thereby leading to DNA damage and suggesting a genotoxic mechanism. These findings, however, are controversial and others have not been able to support that intake of saturated fat is of relevance for genotoxicity.^[10,11]

Dietary fat has a high energy content per weight unit. In experimental studies, energy restriction strongly reduces the tumour incidence even with high-fat diets (reviewed by Kritchevsky^[12]). To what extent these findings are relevant for man has not been clarified. Both dietary fat as such and high-energy consumption may be carcinogenic through increased mitochondrial metabolism and thereby increased generation of reactive oxygen species and resulting oxidative damage to DNA.^[13,14] In rodents, energy restriction reduces oxidative modification of both tissue DNA and proteins, which may explain the reduced cancer risk and increased longevity.^[15–17] However, such effects have not been reproduced in normal weight humans subjected to a 20% energy restriction.^[18] So far, the effect of a high fat diet on oxidative DNA damage has been studied in few tissues, including mammary gland, liver, lung and human peripheral leukocytes and those studies provide some support for the association between fat intake and DNA damage.^[16,19–23]

Highly specific mechanisms operate to repair oxidative DNA modifications,^[24] and only a very small fraction of the induced DNA-modifications results in mutations. Nucleotide excision repair (NER) removes large helix-disturbing DNA adducts, whereas small modifications like 8-oxo-guanine are removed by base excision repair (BER) enzymes.^[25] So far, there is only limited data on the transcriptional regulation of these enzymes. Instillation of diesel exhaust particles in the lungs of rats increased the mRNA level of 8-oxo-guanine DNA glycosylase in lung tissue, and exposure to asbestos also increased *OGG1* mRNA levels in a human alveolar cell line.^[26,27]

In designing experiments on the effects of fats most researchers substitute one fat with another in order to tell any relative differences. This is in contrast to humans who may have a high fat intake and a relatively lower intake of other nutrients. The aim of the present study was to investigate whether a total increase of a commonly consumed dietary animal fat, increasing fat energy, is mutagenic in colon and liver, and if a putative increase of the mutation frequency is likely to be caused by oxidative DNA damage. The effect of increased dietary lard is monitored by studying markers of oxidative damage in proteins, lipids and DNA, bulky

DNA adducts, expression of DNA damage repair and mutation frequencies in the colon and liver of transgenic Big Blue rats.

MATERIALS AND METHODS

Chemicals

All chemicals were used as supplied, without further purification. Fluoresceinamine (isomer II), sodium cyanoborohydride, 4-morpholinoethane sulfonic acid (MES) and sodium dodecyl sulfate (SDS) were from Aldrich Chemical Co. Steinheim, Germany. Sucrose and all other chemicals were from Merck (Darmstadt, Germany). Lard was purchased from a local butcher and was prepared by melting abdominal pigs fat at moderate temperature, followed by decanting through a sieve, and cooling to obtain a solid and homogenous white fat. No salt or any other additive was mixed into the product.

Animals, Exposure Protocol and Preparation of Subcellular Fractions

Thirty male Big Blue[®] (Fischer) rats, approximately 8 weeks of age (weight 180–260 g) from AH Diagnostics (Århus, Denmark), were acclimatized for 7 days while maintained on a semisynthetic diet formulated at the Danish Veterinary and Food Administration.^[28] Animals were randomized into four groups (six animals per group, two per cage). One control group and three lard groups were fed with semi-synthetic diet, 3, 10 or 30% (W/W) (Table I). The fatty acid composition showed mainly saturated and monounsaturated fats in the feed with high lard concentration (Table II). As a positive

TABLE I Composition of the diet per kilogram feed in the four groups

	Control	3% lard	10% lard	30% lard
Protein (casein)	180	174.6	162	126
Fats				
Soya oil	50	48.5	45	35
Carbohydrate				
Potato starch	306	296.82	275.4	214.2
Corn starch	306	296.82	275.4	214.2
Dextrin	34	32.98	30.6	23.8
Sucrose	34	32.98	30.6	23.8
Fibre (cellulose)	50	48.5	45	35
Mineral mixture*	40	38.8	36	28
Lard		30	100	300

* The mixture contains (mg/kg): Ca⁺⁺ (4970), P (phosphate, 3100), K⁺ (3610), S (sulphate, 300), Na⁺ (2530), Cl⁻ (1490), Mg⁺⁺ (600), Fe⁺⁺ (34), Zn⁺⁺ (30), Mn⁺⁺ (10), Cu⁺⁺ (7), I⁻ (200), Mb (molybdate, 0.15), Se (selenite, 0.15), Si (2.5), Cr⁺⁺ (1.0), F⁻ (1.0), Ni⁺⁺ (0.5), B (borate, 0.5), L⁺i (0.1), V (vanadate, 0.1), Co⁺⁺ (0.07), Thiamine (5), riboflavine (6), pyridoxol (8), folate (2), biotin (0.3), cyanocobalamin (0.03), panthotenate (20), choline (hydrogentartrate, 2600), inositol (400), nicotine amide (40), phylloquinone (1000), p-aminobenzoic acid (40), methionine (1000), L-cysteine (2000); (IU/kg): Vitamin A (5000), vitamin D₃ (1000), vitamin E (DL- α -tocopherol, 50).

TABLE II Fatty acid composition of the feed*

	Control [†]	3% lard [‡]	10% lard [‡]	30% Lard [‡]
Fatty acids, g/100g				
C16	0.59	1.26	2.9	7.26
C16:1, n-7	0.01	0.09	0.28	0.81
C18	0.22	0.57	1.45	3.74
C18:1, n-9	1.11	2.20	4.86	12
C18:1, n-7	0.08	0.18	0.42	1.03
C18:2, n-6	2.68	2.86	3.27	4.51
C18:3, n-3	0.37	0.39	0.44	0.58
Total fatty acids*	5.19	7.82	14.22	31.5
Fat content, total	5.9	8.61	14.9	33

*Only fatty acids that constitute more than 0.5g/100g in the highest dose are listed. [†]Analysed. [‡]Calculated based on control and 30% lard feeds.

control group for mutation frequency, six rats were fed with 0.02% IQ in Altromin for comparison with another control group fed with Altromin. Animals were kept in a 12 h light and 12 h dark cycle at an average temperature of 22°C and at 55% humidity. The diet was administered for a total of 21 days. Throughout the study the animals appeared healthy and had free access to the diet and water. Urine was collected for a 24 h period on day 20 and stored at -80°C. At day 21, all rats were anaesthetized with carbondioxide/oxygen, decapitated and blood was collected. Liver and colon were removed, weighed and submerged in liquid nitrogen until storage at -80°C. Heparinized blood samples were separated into plasma, white and red cells by centrifugation on Ficoll Paque®. The washed red blood cells, white blood cells and plasma were also stored at -80°C. In order to evaluate any systemic oxidative damage we also assessed MDA and antioxidant enzyme activities in the blood compartment.

Oxidized Protein

Liver tissue was homogenized, centrifuged at 9000 × g and the supernatant used to obtain the cytosolic fraction by the method of Kim and Lee.^[29] Cytosolic fractions from liver tissue, plasma proteins and erythrocyte proteins were assayed for oxidized lysine residues (2-amino adipic semialdehyde, AAS) and for oxidized proline or arginine residues (gamma-glutamyl semialdehyde, GGS) in proteins as described.^[30] Protein was determined on a Cobas Mira+ analyser using a commercial kit (cat. no. 0736783, Roche, Basel, Switzerland).

Antioxidant Enzymes and MDA Determination

Automated assays for the antioxidant enzymes, glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (GR) in erythrocyte lysate were performed on a Cobas Mira+ analyzer. GPx (Randox, cat. no. RS 505) and hemoglobin (Randox, cat. no. HG 980) were determined using commercially available kits, whereas the activity of GR was

determined by the method of Goldberg and Spooner (1983).^[31] CAT activity was determined as described.^[32] The enzymatic activities in red blood cells were calculated relative to the amount of hemoglobin. Total malondialdehyde (MDA) in plasma was determined by HPLC as described.^[33] Ascorbic acid and dehydroascorbic acid were determined by HPLC.^[34]

Isolation of Cells from Colonic Lining and Liver

The isolation of liver cells was essentially carried out as described previously. The colon was washed with 0.15 M NaCl. It was cut longitudinally into two pieces, frozen in liquid nitrogen and stored at -80°C. The one piece was thawed and washed thoroughly in ice-cold Merchant's medium with 10 mM EDTA (M-EDTA: 0.14 M NaCl, 1.47 mM KH₂PO₄, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 10 mM NaEDTA, pH 7.4) and the epithelial cells were scraped off with a microscope slide.^[35]

Single Cell Gel Electrophoresis Assay

The detection of DNA damage in single cells was carried out as described previously.^[36] The level of endonuclease III sensitive sites was obtained as the difference in scores of parallel slides incubated with and without endonuclease III enzymes at 37°C for 45 min (endonuclease III enzyme was a kind gift from Serge Boiteaux, UMR217 Center National de la Recherche Scientifique et Commissariat à l'Énergie Atomique, France). A total of 50 images were scored for each sample, using Kinetics Imaging Limited version 4, Liverpool, UK software system to determine the amount of DNA migrated from the comet head to the tail.

Detection of 8-Oxo-deoxyguanosine

Levels of 8-oxodG relative to dG were measured in colon mucosa and liver by means of HPLC with electronic detection after isolation and digestion of nuclear DNA as described.^[37] Urinary concentrations

of 8-oxodG were measured by HPLC with tandem mass spectrometry detection as described.^[38]

Quantification of the *rERCC1* and *rOGG1* mRNA Levels in Colon and Liver

Total RNA was purified from 10 mg liver or from 5×10^6 colon cells using a Qiagen total RNA purification kit as recommended by the manufacturer. The RNA was DNase treated as recommended by Qiagen (Kobolab, Denmark). Subsequent quality control showed that all genomic contaminations were removed by the DNase treatment. RNA (200 ng) was used for cDNA synthesis in a reaction volume of 10 μ l using the Taqman Gold RT-PCR kit as recommended by Applied Biosystems (Nærum, Denmark). Quantification of the *rERCC1* and *rOGG1* mRNA levels was performed as previously described.^[39] The mRNA levels were normalized to *18S* as previously described.^[39]

Mutation Analysis

Epithelial colon cells were suspended in 2 ml M-EDTA by pipetting up and down three times. About 20 million cells were filtered through a cell strainer (Falcon, Becton Dickinson, Franklin Lakes, New Jersey, USA) and DNA were purified by the RecoverEase™ DNA isolation kit (Stratagene, La Jolla, USA). DNA from about 60 mg frozen liver was prepared by RecoverEase™ as described (Stratagene, La Jolla, USA).

Eight micro liter DNA preparation was packaged with Transpack packaging extract (Stratagene, La Jolla, USA). If the packaging mixture was viscous after recommended standard packaging time of 180 min, the mixture was incubated for another 60 min. If the mixture was still viscous after this time additional Transpack reagents were added and the mixture was incubated for another 60 min. This phage preparation was used to infect *E. coli* G1250 (*hfl*⁻). Phages with mutations at the *cII* locus were identified by plaque formation under selective growth conditions at 24°C and the total number of infective phages was determined by plaque formation under unselective growth conditions at 37° as described (λ Select-cII™ Mutation Detection System for the Big Blue Rodents, Stratagene, La Jolla, USA).

³²P-postlabelling

DNA was extracted by standard phenol/chloroform extraction procedure, and the ³²P-postlabelling assay was carried out as described previously, using the butanol enrichment procedure.^[40] *In vitro* modified benzo[*a*]pyrene-diol-epoxide-modified calf thymus DNA was used as a standard to correct for day to day assay variation. The relative adduct levels are

expressed as adducts/10⁸ nucleotides, based on the mean of at least two independent assays.

Statistics

All parameters were tested for normality using the Anderson-Darling test. To obtain a normal distributions some parameters were logarithmically transformed. The groups were also tested for homogeneity of variance with Bartlett's test. Differences between groups were determined by one-way ANOVA. If significant differences were observed, the dosed groups were compared to the controls by the *t*-test statistic. For parameters which were not normally distributed or where the groups had heterogeneous variance, groups were compared using Wilcoxon rank scores and Mann-Whitney test. A *p*-value below 0.05 was regarded as significant in these tests. Since fat intake, energy intake and intake of non-fat nutrients (exemplified by protein intake) were not normally distributed they could not be directly included as covariates into an ANOVA model. Spearman rank-sum correlation analysis was performed between these variables and all endpoint parameters. A *p*-value below 0.01 was regarded as significant in this analysis.

RESULTS

Feed Intake and Weight Gain

There was a significant difference in feed consumption between the groups ($p < 0.001$) caused by a lower feed intake among rats in the two higher dose groups (Table III). Total energy intake ($p < 0.05$) and percent energy from fat ($p < 0.01$) also differed significantly between the groups leading to a small but significant difference between groups in absolute ($p < 0.05$) and relative body weight gains ($p < 0.05$). However, terminal weights ($p = 0.60$) and ratio of energy intake to weight gain ($p = 0.21$) in the four groups of rats did not differ. Liver weights were also similar in all four groups, see Table III. Spearman correlation analysis confirmed that total fat intake was proportional to total energy intake ($r^2 = 0.50$, $p < 0.01$) and to the absolute weight gain of the animals ($r^2 = 0.28$, $p < 0.01$) whereas the final weight was unaffected. In contrast, total energy intake was unrelated to body weight and to absolute or relative weight gains ($p > 0.5$).

Oxidative Status in Liver and Plasma

Protein oxidation at both lysine sites (AAS) and at proline or arginine sites (GGs) in liver cytosol was significantly increased in all groups of rats fed with lard ($p < 0.05$, Table IV). However, there was no

TABLE III Feed intakes, body weight gain and final body weights

	Feed intake [†] (g/day)		Energy intake [†] (kJ/kg bw/day)		Body weight gain [‡] (g/day)		Final body weights (g)		Liver weights (g)	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Control group IQ	19.1**	0.68	688	52	2.51*	0.48	298	22	10.7	0.97
Control group lard	16.2	0.83	714	14	1.83	0.45	297	16	10.4	0.75
Low lard group	16.9	0.79	1053*	136	1.91	0.31	287	43	9.16	1.39
Medium lard group	15.0*	0.45	992	122	2.93	1.67	308	54	9.09	0.96
High lard group	13.7**	0.16	1030**	59	2.81**	0.39	313	18	10.2	1.04

[†] Differences between groups significant by ANOVA ($P < 0.0001$). [‡] Differences between groups significant by Wilcoxon rank scores ($P < 0.02$). Significantly different from lard control, * $P < 0.05$, ** $P < 0.01$.

dose-response relationship between the dose of lard and the level of protein oxidation nor was there any correlation with the energy intake. There was no effect of lard intake or energy intake on plasma protein oxidation (Table IV) nor any relationship with lipid oxidation in plasma.

The oxidative index of vitamin C, representing the fraction of total vitamin C in the oxidized form, was increased in the liver of animals fed with lard (Table IV). No relationship was found with increasing lard intake or energy intake. The total concentration of vitamin C in liver was also increased, although the effect was not statistically significant. No effect was found on plasma levels of vitamin C. Total, oxidized and reduced liver vitamin C correlated with total and oxidized plasma vitamin C ($p \leq 0.01$). Hepatic vitamin C also correlated with liver AAS and GGS ($p \leq 0.01$).

The enzymatic activities of CAT, GR and GPx in erythrocytes were not affected by lard (Table IV) or energy intake.

DNA Damage and Mutation Frequency

Lard had no effect on the level of 8-oxodG in the urine or in nuclear DNA from the liver or colon

(Table V). The level of DNA adducts measured by the ³²P-postlabelling assay in both liver and colon was lower in all the groups fed with lard than in the control group in both liver and colon (Fig. 1). The adduct level in liver had a stronger inverse relationship with total fat intake ($r^2 = 0.52$, $p < 0.0001$) than with total energy intake ($r^2 = 0.32$, $p = 0.005$). Adducts in the colon were correlated with energy intake and with fat intake to about the same extent ($p \leq 0.01$). No effect was detected on the steady-state levels of DNA strand breaks and oxidized pyrimidines as measured by endonuclease III sensitive sites in liver or colon (Table V).

The lowest dose of lard significantly increased the mutation frequency in the reporter gene *cII* in colon compared to control ($p < 0.01$) (Fig. 2 and Table V), whereas the mutation frequency was unchanged in liver. The positive control group fed with Altromin and 0.02% IQ had significantly higher mutation frequency than the control groups (Fig. 2) in agreement with previous reports.^[41]

Induction of DNA Repair Gene Expression

The mRNA transcription levels of the NER gene *rERCC1*^[42] and the BER gene *rOGG1*^[43] were

TABLE IV Antioxidants and markers of oxidative stress. Mean values are given

	Control diet [†]		3% lard		10% lard		30% lard	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Plasma								
AAS (pmol/mg protein)	117.00	(10.28)	120.33	(11.06)	114.33	(9.05)	121.33	(10.98)
MDA (μ M)	7.10	(1.46)	8.13	(2.14)	6.55	(1.80)	7.93	(1.10)
GR (U/g Hb)	8.94	(4.79)	7.36	(1.54)	7.80	(2.10)	7.34	(2.29)
GPx (U/g Hb)	2,066	(934)	2,194	(596)	2,363	(432)	2,325	(623)
CAT (U/g Hb)	76.7	(39.1)	64.3	(17.0)	80.4	(18.9)	90.5	(43.0)
Total vit C (μ M)	12.0	(13.3)	23.0	(10.7)	27.4	(16.3)	20.2	(15.0)
Ascorbate (μ M)	10.6	(12.1)	19.8	(8.9)	22.6	(14.6)	14.9	(11.7)
DHAA (μ M)	1.48	(1.44)	3.23	(2.9)	4.75	(3.91)	5.75	(4.47)
Liver								
AAS (pmol/mg protein)	76.50	(10.13)	95.40	(12.88)*	108.80	(9.20)**	93.67	(11.22)*
GGS (pmol/mg protein)	84.33	(8.04)	121.80	(21.90)**	122.40	(22.10)**	100.33	(11.52)*
Total vit C (mM)	1.89	(0.88)	2.69	(0.46)	2.65	(0.46)	2.80	(0.31)
Ascorbate (mM)	1.90	(0.88)	2.54	(0.43)	2.53	(0.37)	2.65	(0.28)
DHAA (mM)	-0.01	(0.10)	0.15	(0.03)*	0.13	(0.12)	0.15	(0.04)*
Oxidative index	-0.005	(0.04)	0.05	(0.01)**	0.04	(0.04)	0.05	(0.01)*

[†] The values for the control group has been previously published.^[39] Significantly different from the control group for lard, * $p < 0.05$, ** $p < 0.01$.

TABLE V DNA repair, DNA adduct and mutation frequency. Mean values are given

	Control diet*	(SD)	3% lard	(SD)	10% lard	(SD)	30% lard	(SD)
Colon								
SB (% DNA in tail)	8.0	(4.7)	11.7	(6.5)	15.8	(17.8)	9.0	(3.7)
EndIII sites- (% DNA in tail)	5.3	(3.2)	2.4	(3.2)	3.5	(3.5)	3.1	(1.7)
Relative <i>rERCC1</i> level ($\times 10^6$)	14	(6)	15	(7.9)	20	(7)	29	(20)
Relative <i>rOGG1</i> level ($\times 10^6$)	76	(28)	57	(45)	105	(85)	94	(48)
8-oxo-dG per 10^6 dG	4.0	(2.8)	2.4	(0.7)	2.6	(1.2)	4.0	(4.0)
Bulky DNA adducts pr. 10^8 nucleotides	0.031	(0.012)	0.013	(0.008)**	0.016	(0.004)*	0.016	(0.01)*
Mutation frequency pr 10^6 phages	24.6	(3.5)	47.3	(16.0)**	29.7	(13.5)	33.9	(11.6)
Liver								
SB (% DNA in tail)	3.9	(0.9)	5.6	(2.4)	8.0	(6.6)	5.8	(2.8)
EndIII sites (% DNA in tail)	1.1	(1.7)	-0.3	(1.4)	1.2	(1.1)	1.5	(1.5)
Relative <i>rERCC1</i> level ($\times 10^6$)	6.2	(2)	15	(4.7)*	11	(4)*	13	(6)*
Relative <i>rOGG1</i> level ($\times 10^6$)	7.8	(4.0)	8.7	(6.1)	6.4	(1.6)	17.1	(18)
8-oxo-dG per 10^6 dG	0.38	0.11	0.49	0.33	0.39	0.14	0.33	0.06
Bulky DNA adducts per 10^8 nucleotides	0.027	(0.018)	0.016	(0.003)	0.007	(0.008)**	0.005	(0.006)**
Mutation frequency per 10^6 phages	26.9	(16.6)	32.8	(8.2)	23.4	(14.9)	24.7	(9.1)
Urine								
8-oxo-dG (pmol/24h)	166.6	(54.3)	117.9	(32.2)	163.3	(73.2)	128.8	(30.7)

*The values for the control group have been previously published.^[39] Significantly different from the lard control group. * $p < 0.05$, ** $p < 0.01$.

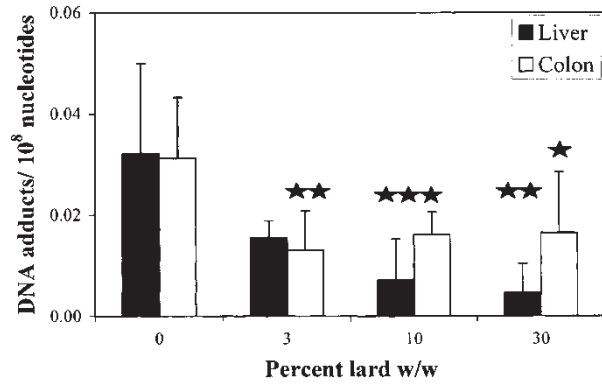


FIGURE 1 DNA adduct levels measured by ^{32}P -labeling as function of lard intake. Colon: white bars, liver: grey bars. Bars represent means plus one standard deviation. One star indicate $p < 0.05$, two stars indicate $p < 0.01$ as compared with controls. Each group consisted of six animals.

quantified as markers of the DNA repair capacity. The *rERCC1* mRNA level was increased 2-fold in liver at all lard doses ($p < 0.05$) (Fig. 3 and Table V). In colon cells a 2-fold dose-dependent increase in the *rERCC1* mRNA level was found. The effect was marginally statistically significant ($p = 0.056$), but became statistically significant (10% lard: $p = 0.018$, 30% lard: $p = 0.005$) if both control groups were combined. No changes were observed in *rOGG1* expression in either liver or colon. None of the mRNA levels correlated with total energy intake.

DISCUSSION

In the present experiment, Big Blue rats were fed at four different levels of animal fat for three weeks and sacrificed immediately afterwards. The study was designed to address the possible genotoxic effects of a relative increase in total animal fat and fat energy caused by increased oxidative stress locally or systemically.

We found that the amount of ^{32}P -labelled DNA adducts decrease with increasing animal fat intake in agreement with previous results.^[44] The mutation frequency was only increased in colon and only at the lowest dose of lard. This is in agreement with a previous study of the mutation frequency in colon and intestine of transgenic mice,^[45] where the mutation frequency was found to be unaffected by a high dietary intake (40%) of either unsaturated fat or saturated fats. Likewise, it has previously been shown that a diet of high corn oil content did not induce DNA damage measured by the comet assay.^[46] Our results indicate that short-term exposure to a high-fat diet or an increased energy intake does not cause genotoxic effects in colon or liver.

The lowest lard dose increased the mutation rate in colon. It is likely that this is a chance finding since

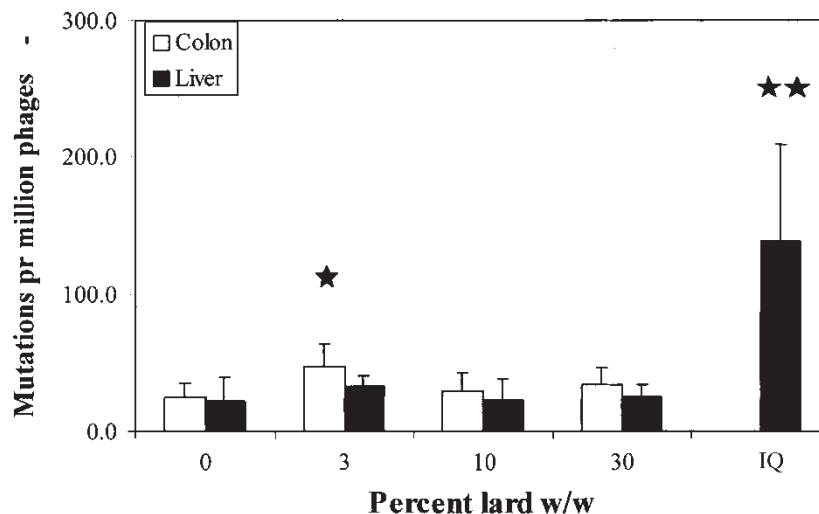


FIGURE 2 Mutation frequency of the *cII* gene as function of percent lard intake. The data are given as means plus one standard deviation. One star indicates $p < 0.05$, two stars indicate $p < 0.01$ as compared with controls. Each group consisted of six animals.

there was no signs of increase at the higher dose levels. On the other hand, the observed increase of the NER gene *ERCC1* mRNA levels might indicate that animal fat induced DNA damage, since only DNA damaging agents have been found to induce DNA repair.^[26,27,47–49] The fat-induced change in oxidative status in liver may result in formation of reactive unsaturated aldehydes, which are able to form cyclic propano adducts with deoxyguanosine.^[50] These adducts are repaired by NER.^[51] It is thus conceivable that *ERCC1* expression is increased in response to the formation of these fat-induced DNA adducts. The expression level of *ERCC1* has previously been shown to correlate with the DNA repair capacity in human lymphocytes.^[42,52] An increased DNA repair capacity might explain the lowered level of DNA adducts detected in liver and colon by ³²P-postlabelling.

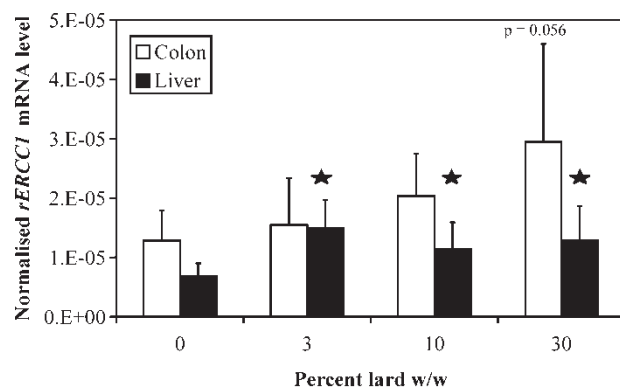


FIGURE 3 The relative *rERCC1* mRNA level as a function of percent lard intake. *rERCC1* mRNA and 18S were quantified by RT-PCR, and the *rERCC1* mRNA level was normalized to the 18S level. Data are represented as means plus one standard deviation. One star indicates $0.01 < p < 0.05$ as compared with controls. Each group consisted of six animals.

We did not detect any changes in the number of strand breaks by the comet assay.

Increased dietary lard enhanced carbonyl formation in liver cytosol proteins. This was accompanied by an increased biosynthesis of vitamin C and an increased formation of dehydroascorbate. It is well known that a high-fat load in rats can increase hepatic glucose levels and glucose availability^[53–55] and may thereby increase its availability for ascorbate synthesis. There was no changes in the 8-oxo-guanine content of either DNA, or urine, nor was the expression level of *OGG1* affected. In blood, no indications of dose-related systemic oxidative stress were found on protein oxidation, lipid oxidation, vitamin C levels or enzyme activities of CAT, GR and GPx. A slight increase in plasma vitamin C correlated with hepatic levels and probably reflects increased biosynthesis (Table IV). Since gulonolactone oxidase activity is maintained in rats this is compatible with an increased synthesis of vitamin C and a following increased formation of hydrogen peroxide may result in an increased formation of dehydroascorbic acid.^[56,57]

The evidence of a DNA damaging effect of a high-fat diet is conflicting. Increased dietary fat increases energy in the diet leading to increasing plasma thyroid hormones and consequently an increased rate of metabolism in rats.^[58] In rats fed a diet with 26% fat based on corn oil or coconut oil for 15 weeks the oxidative damage to deoxyguanosine estimated from the urinary excretion of 8-oxodG was enhanced compared to the control group and the level of 8-oxodG correlated with the energy intake.^[59] In mice fed a high fat diet ad lib the 8-oxodG levels in lung DNA was 7-fold increased compared to the control group^[21] although this was not reproduced in a later similar study.^[22] In rats fed diets with up to 20%

corn oil but the same amount of energy as the control group for only two weeks the oxidative base lesion, 5-hydroxyuracil, was not increased in mammary gland epithelium or the liver.^[16] The lack of effect of up to 30% fat in the present study may have been due to the relatively short feeding period.

When macromolecular components of the diet are studied it is difficult to make major variations in one component without affecting the overall composition of the diet. Thus, the diets containing animal fat used in this study contained relatively less dietary fiber, less vitamins and minerals etc., and had a higher energy density than the control diet. This experimental design does not allow us to distinguish between effects of increased dietary fat and the decreased intake of other feed components, including protein, fiber, carbohydrates, vitamins and minerals. However, correlation analysis allows us to evaluate whether the dietary change to increased fat or the increased energy intake may best explain the observed effects. No biomarker correlated more strongly with energy intake than with fat intake, indicating that the increased lard or the decrease in other nutrients caused the effects. In an identical experimental set-up where the intakes of other nutrients were replaced by sucrose we also observed a decrease in colon DNA adducts and an increase in hepatic ascorbate.^[39] Increased intake of sucrose did not induce oxidative modification of liver proteins. This indicates that most of the observed effects in the present study are caused by the increased consumption of animal fat rather than total energy but that the decreased colon adducts and the increased hepatic ascorbate may be caused by a decrease in another nutrient.

In summary oral administration of animal fat and increased fat-energy was not mutagenic in liver and only the lowest dose was mutagenic in colon under the conditions used. Increased carbonyl formation was induced in liver proteins, but no oxidative damage was observed in liver DNA or in plasma proteins. No effect on markers of oxidative DNA damage or repair was observed. Feeding high-fat diets was accompanied by an increased *rERCC1* mRNA level and a dose-dependent decrease in the background level of bulky DNA-adducts in the liver.

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References

- [1] Boutron-Ruault, M.C., Senesse, P., Faivre, J., Chatelain, N., Belghiti, C. and Meance, S. (1999) "Foods as risk factors for colorectal cancer: a case-control study in Burgundy (France)", *Eur. J. Cancer Prev.* **8**, 229–235.
- [2] Byers, T. (2000) "Diet, colorectal adenomas and colorectal cancer", *N. Engl. J. Med.* **342**, 1206–1207.
- [3] Sugimura, T. (2000) "Nutrition and dietary carcinogens", *Carcinogenesis* **21**, 387–395.
- [4] Bruce, W.R., Giacca, A. and Medline, A. (2000) "Possible mechanisms relating diet and risk of colon cancer", *Cancer Epidemiol. Biomark. Prev.* **9**, 1271–1279.
- [5] Bruce, W.R., Wolever, T.M. and Giacca, A. (2000) "Mechanisms linking diet and colorectal cancer: the possible role of insulin resistance", *Nutr. Cancer- An Int. J.* **37**, 19–26.
- [6] Reddy, B.S. (1992) "Dietary fat and colon cancer: animal model studies", *Lipids* **27**, 807–813.
- [7] Carroll, K.K., Braden, L.M., Bell, J.A. and Kalamegham, R. (1986) "Fat and cancer", *Cancer* **58**, 1818–1825.
- [8] Hietanen, E., Bartsch, H., Berezziat, J.C., Ahotupa, M., Camus, A.M., Cabral, J.R. and Laitinen, M. (1990) "Quantity and saturation degree of dietary fats as modulators of oxidative stress and chemically-induced liver tumors in rats", *Int. J. Cancer* **46**, 640–647.
- [9] Slim, R.M., Toborek, M., Watkins, B.A., Boissonneault, G.A. and Hennig, B. (1996) "Susceptibility to hepatic oxidative stress in rabbits fed different animal and plant fats", *J. Am. Coll. Nutr.* **15**, 289–294.
- [10] Parnaud, G., Peiffer, G., Tache, S. and Corpet, D.E. (1998) "Effect of meat (beef, chicken and bacon) on rat colon carcinogenesis", *Nutr. Cancer- An Int. J.* **32**, 165–173.
- [11] Sesink, A.L., Termont, D.S., Kleibeuker, J.H. and Van Der, M.R. (2000) "Red meat and colon cancer: dietary haem, but not fat, has cytotoxic and hyperproliferative effects on rat colonic epithelium", *Carcinogenesis* **21**, 1909–1915.
- [12] Kritchevsky, D. (1993) "Colorectal cancer: the role of dietary fat and caloric restriction", *Mutation Res.* **290**, 63–70.
- [13] Ames, B.N. (1983) "Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases", *Science* **221**, 1256–1264.
- [14] Loft, S. and Poulsen, H.E. (1996) "Cancer risk and oxidative DNA damage in man", *J. Mol. Med.* **74**, 297–312.
- [15] Chung, M.H., Kasai, H., Nishimura, S. and Yu, B.P. (1992) "Protection of DNA damage by dietary restriction", *Free Radic. Biol. Med.* **12**, 523–525.
- [16] Djuric, Z., Lu, M.H., Lewis, S.M., Luongo, D.A., Chen, X.W., Heilbrun, L.K., Reading, B.A., Duffy, P.H. and Hart, R.W. (1992) "Oxidative DNA damage levels in rats fed low-fat, high-fat or calorie-restricted diets", *Toxicol. Appl. Pharmacol.* **115**, 156–160.
- [17] Youngman, L.D., Park, J.Y. and Ames, B.N. (1992) "Protein oxidation associated with aging is reduced by dietary restriction of protein or calories", *Proc. Natl Acad. Sci. USA* **89**, 9112–9116.
- [18] Loft, S., Velthuis-te Wierik, E.J., van den, B.H. and Poulsen, H.E. (1995) "Energy restriction and oxidative DNA damage in humans", *Cancer Epidemiol. Biomark. Prev.* **4**, 515–519.
- [19] Djuric, Z., Heilbrun, L.K., Reading, B.A., Boomer, A., Valeriote, F.A. and Martino, S. (1991) "Effects of a low-fat diet on levels of oxidative damage to DNA to human peripheral nucleated blood cells", *J. Natl Cancer Inst.* **83**, 766–769.
- [20] Haegele, A.D., Briggs, S.P. and Thompson, H.J. (1994) "Antioxidant status and dietary lipid unsaturation modulate oxidative DNA damage", *Free Radic. Biol. Med.* **16**, 111–115.
- [21] Nagashima, M., Kasai, H., Yokota, J., Nagamachi, Y., Ichinose, T. and Sagai, M. (1995) "Formation of an oxidative DNA damage, 8-hydroxydeoxyguanosine, in mouse lung DNA

- after intratracheal instillation of diesel exhaust particles and effects of high dietary fat and beta-carotene on this process", *Carcinogenesis* **16**, 1441–1445.
- [22] Ichinose, T., Yajima, Y., Nagashima, M., Takenoshita, S., Nagamachi, Y. and Sagai, M. (1997) "Lung carcinogenesis and formation of 8-hydroxy-deoxyguanosine in mice by diesel exhaust particles", *Carcinogenesis* **18**, 185–192.
- [23] Ip, C., Briggs, S.P., Haegele, A.D., Thompson, H.J., Storkson, J. and Scimeca, J.A. (1996) "The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet", *Carcinogenesis* **17**, 1045–1050.
- [24] Lindahl, T. and Wood, R.D. (1999) "Quality control by DNA repair", *Science* **286**, 1897–1905.
- [25] Wood, R. (1996) "DNA repair in eukaryotes", *Annu. Rev. Biochem.* **65**, 135–167.
- [26] Tsurudome, Y., Hirano, T., Yamato, H., Tanaka, I., Sagai, M., Hirano, H., Nagata, N., Itoh, H. and Kasai, H. (1999) "Changes in levels of 8-hydroxyguanine in DNA, its repair and OGG1 mRNA in rat lungs after intratracheal administration of diesel exhaust particles", *Carcinogenesis* **20**, 1573–1576.
- [27] Kim, H.-N., Morimoto, Y., Tsuda, T., Ootsuyama, Y., Hirohashi, M., Hirano, T., Tanaka, I., Lim, Y., Yun, I.-G. and Kasai, H. (2001) "Changes in DNA 8-hydroxyguanine levels, 8-hydroxyguanine repair activity and hOGG1 and hMTH1 mRNA expression in human lung alveolar epithelial cells induced by crocidolite asbestos", *Carcinogenesis* **22**, 265–269.
- [28] Meyer, O., Blom, L. and Sondergaard, D. (1982) "The influence of minerals and protein on the nephrocalcinosis potential for rats of semisynthetic diets", *Lab. Anim.* **16**, 271–273.
- [29] Kim, K.B. and Lee, B.M. (1997) "Oxidative stress to DNA, protein and antioxidant enzymes (superoxide dismutase and catalase) in rats treated with benzo(a)pyrene", *Cancer Lett.* **113**, 205–212.
- [30] Daneshvar, B., Frandsen, H., Dragsted, L.O., Knudsen, L.E. and Autrup, H. (1997) "Analysis of native human plasma proteins and haemoglobin for the presence of bityrosine by high-performance liquid chromatography", *Pharmacol. Toxicol.* **81**, 205–208.
- [31] Goldberg, D. and Spooner, R.J. (1983) "Glutathione reductase", In: Bergmeyer, H.U., ed, *Methods of Enzymatic Analysis*, 3rd Ed. (Verlag Chemie, Deerfield Beach, FL), pp 258–265.
- [32] Wheeler, C.R., Salzman, J.A., Elsayed, N.M., Omaye, S.T. and Korte, D.W. Jr. (1990) "Automated assays for superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase activity", *Anal. Biochem.* **184**, 193–199.
- [33] Lauridsen, S.T. and Mortensen, A. (1999) "Probuco selectively increases oxidation of atherogenic lipoproteins in cholesterol-fed mice and in Watanabe heritable hyperlipidemic rabbits", *Atherosclerosis* **142**, 169–178.
- [34] Lykkesfeldt, J., Loft, S., Nielsen, J.B. and Poulsen, H.E. (1997) "Ascorbic acid and dehydroascorbic acid as biomarkers of oxidative stress caused by smoking", *Am. J. Clin. Nutr.* **65**, 959–963.
- [35] Brunborg, G., Holme, J.A., Soderlund, E.J., Hongslo, J.K., Vartiainen, T., Lotjonen, S. and Becher, G. (1991) "Genotoxic effects of the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) in mammalian cells *in vitro* and in rats *in vivo*", *Mutation Res.* **260**, 55–64.
- [36] Thein, N., Moller, P., Amtoft, H., Vogel, U., Korsholm, B., Autrup, H. and Wallin, H. (2000) "A strong genotoxic effect in mouse skin of a single painting of coal tar in hairless mice and in MutaMouse", *Mutation Res.* **468**, 117–124.
- [37] Wellejus, A. and Loft, S. (2001) "Receptor-mediated 17-ethynylestradiol-induced oxidative DNA damage in rat testicular cells", *FASEB J.: off. publ. Fed. Am. Soc. Exp. Biol.* **16**, 195–200.
- [38] Weimann, A., Belling, D. and Poulsen, H.E. (2001) "Measurement of 8-oxo-2'-deoxyguanosine and 8-oxo-2'-deoxyadenosine in DNA and human urine by high performance liquid chromatography-electrospray tandem mass spectrometry", *Free Radic. Biol. Med.* **30**, 757–764.
- [39] Dragsted, L.O., Daneshvar, B., Vogel, U., Autrup, H.N., Wallin, H., Risom, L., Moller, P., Molck, A.M., Hansen, M., Poulsen, H.E. and Loft, S. (2002) "A Sucrose-rich diet induces mutations in the rat colon", *Cancer Res.* **62**, 4339–4345.
- [40] Autrup, H., Daneshvar, B., Dragsted, L.O., Gamborg, M., Hansen, M., Loft, S., Okkels, H., Nielsen, F., Nielsen, P.S., Raffn, E., Wallin, H. and Knudsen, L.E. (1999) "Biomarkers for exposure to ambient air pollution—comparison of carcinogen-DNA adduct levels with other exposure markers and markers for oxidative stress", *Environ. Health Perspect.* **107**, 233–238.
- [41] Bol, S.A., Horlbeck, J., Markovic, J., de Boer, J.G., Turesky, R.J. and Constable, A. (2000) "Mutational analysis of the liver, colon and kidney of big Blue(R) rats treated with 2-amino-3-methylimidazo[4,5-f]quinoline", *Carcinogenesis* **21**, 1–6.
- [42] Vogel, U., Dybdahl, M., Frentz, G. and Nexø, B.A. (2000) "DNA repair capacity: inconsistency between effect of over-expression of five NER genes and the correlation to mRNA levels in primary lymphocytes", *Mutation Res.* **461**, 197–210.
- [43] Kondo, S., Toyokuni, S., Tanaka, T., Hiai, H., Onodera, H., Kasai, H. and Imamura, M. (2000) "Overexpression of the hOGG1 gene and high 8-hydroxy-2'-deoxyguanosine (8-OHdG) lyase activity in human colorectal carcinoma: regulation mechanism of the 8-OHdG level in DNA", *Clin. Cancer Res.: off. J. Am. Assoc. Cancer Res.* **6**, 1394–1400.
- [44] Li, D. and Randerath, K. (1992) "Modulation of DNA modification (I-compound) levels in rat liver and kidney by dietary carbohydrate, protein, fat, vitamin and mineral content", *Mutation Res.* **275**, 47–56.
- [45] Zhang, X.B., Tao, K., Orlando, C., Shaver-Walker, P. and Heddle, J.A. (1996) "Mutagenicity of high fat diets in the colon and small intestine of transgenic mice", *Mutagenesis* **11**, 43–48.
- [46] Giovannelli, L., Testa, G., De Filippo, C., Cheynier, V., Clifford, M.N. and Dolara, P. (2000) "Effect of complex polyphenols and tannins from red wine on DNA oxidative damage of rat colon mucosa *in vivo*", *Eur. J. Nutr.* **39**, 207–212.
- [47] Li, Q., Ding, L., Yu, J.J., Mu, C., Tsang, B., Bostick-Bruton, F. and Reed, E. (1998) "Cisplatin and phorbol ester independently induce ERCC-1 protein in human ovarian carcinoma cells", *Int. J. Oncol.* **13**, 987–992.
- [48] Li, Q., Gardner, K., Zhang, L., Tsang, B., Bostick-Bruton, F. and Reed, E. (1998) "Cisplatin induction of ERCC-1 mRNA expression in A2780/CP70 human ovarian cancer cells", *J. Biol. Chem.* **36**, 23419–23425.
- [49] Holt, S., Roy, G., Mitra, S., Upton, P.B., Bogdanffy, M.S. and Swenberg, J.A. (2000) "Deficiency of N-methylpurine-DNA-glycosylase expression in nonparenchymal cells, the target cell for vinyl chloride and vinyl fluoride", *Mutation Res.* **460**, 105–115.
- [50] Chung, F.L., Nath, R.G., Ocando, J., Nishikawa, A. and Zhang, L. (2000) "Deoxyguanosine adducts of t-4-hydroxy-2-nonenal are endogenous DNA lesions in rodents and humans: detection and potential sources", *Cancer Res.* **60**, 1507–1511.
- [51] Johnson, K.A., Fink, S.P. and Marnett, L.J. (1997) "Repair of propanodeoxyguanosine by nucleotide excision repair *in vivo* and *in vitro*", *J. Biol. Chem.* **272**, 11434–11438.
- [52] Cheng, L., Guan, Y., Li, L., Legerski, R.J., Einspahr, J., Bangert, J., Alberts, D.S. and Wei, Q. (1999) "Expression in normal human tissues of five nucleotide excision repair genes measured simultaneously by multiplex reverse transcription-polymerase chain reaction", *Cancer Epidemiol. Biomark. Prev.* **8**, 801–807.
- [53] Bach, A., Phan, T. and Metais, P. (1975) "Influence of a long or medium chain triglyceride diet on intermediary hepatic metabolism of the rat", *Nutr. Metabol.* **19**, 103–110.
- [54] Ramirez, R., Lopez, J.M., Bedoya, F.J. and Goberna, R. (1990) "Effects of high-carbohydrate or high-fat diet on carbohydrate metabolism and insulin secretion in the normal rat", *Diabetes Res.* **15**, 179–183.
- [55] Akiyama, T., Tachibana, I., Shirohara, H., Watanabe, N. and Otsuki, M. (1996) "High-fat hypercaloric diet induces obesity, glucose intolerance and hyperlipidemia in normal adult male Wistar rat", *Diabetes Res. Clin. Pract.* **31**, 27–35.

- [56] Braun, L., Puskas, F., Csala, M., Meszaros, G., Mandl, J. and Banhegyi, G. (1997) "Ascorbate as a substrate for glycolysis or gluconeogenesis: evidence for an interorgan ascorbate cycle", *Free Radic. Biol. Med.* **23**, 804–808.
- [57] Banhegyi, G., Braun, L., Csala, M., Puskas, F. and Mandl, J. (1997) "Ascorbate metabolism and its regulation in animals", *Free Radic. Biol. Med.* **23**, 793–803.
- [58] Rabolli, D. and Martin, R.J. (1977) "Effects of diet composition on serum levels of insulin, thyroxine, triiodothyronine, growth hormone, and corticosterone in rats", *J. Nutr.* **107**, 1068–1074.
- [59] Loft, S., Thorling, E.B. and Poulsen, H.E. (1998) "High fat diet induced oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2-deoxyguanosine excretion in rats", *Free Radic. Res.* **29**, 595–600.