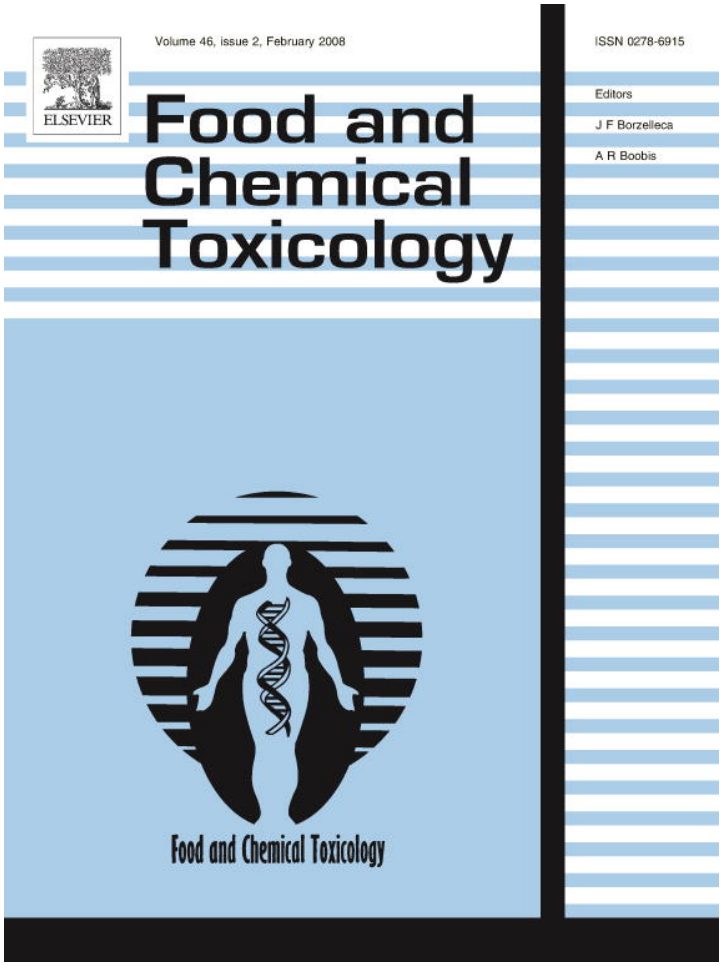

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Sucrose, glucose and fructose have similar genotoxicity in the rat colon and affect the metabolome

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Abstract

We have shown previously that a high sucrose intake increases the background level of somatic mutations and the level of bulky DNA adducts in the colon epithelium of rats. The mechanism may involve either glucose or fructose formed by hydrolysis of sucrose. Male Big Blue® rats were fed 30% sucrose, glucose, fructose or potato starch as part of the diet. Mutation rates and bulky DNA adduct levels were determined in colon and liver. The concentration of short-chain fatty acids and pH were determined in caecum, C-peptide was determined in plasma, biomarkers for oxidative damage and proliferation were determined in colon, and a metabonomic analysis was performed in plasma and urine. The sugars increased the mutation rates in colon and the bulky adduct levels in colon and liver to a similar extent. All sugars decrease the caecal concentration of acetic acid and propionic acid. The metabonomic studies indicated disturbed amino acid metabolism and decrease in plasma and urinary acetate as a common feature for all sugars and confirmed triglyceridemic effects of fructose. In conclusion, the genotoxicity may be related to the altered chemical environment in the caecum and thereby also in the colon but we found no related changes in insulin resistance or oxidative stress.

Keywords: Simple carbohydrates; Metabonomic; Colon; Mutation; Short-chain fatty acids

1. Introduction

Colon cancer is associated with diet and other lifestyle factors typical for the Western countries, such as low intake of fruits and vegetables, sedentary lifestyle, obesity, and probably high intake of dietary energy, cooked meat, and sugar (Bruce et al., 2000; Merrill et al., 1999). Epidemiological studies show some association between colon cancer and high intake of sucrose. In a recent review 16 of 18 studies indicate that a high intake of sucrose is associated with increased risk of colon cancer (Giovannucci, 2001). In three-week exposure studies in rats sucrose increased the mutation frequency in colon (Dragsted et al., 2002; Hansen et al., 2004) and the level of bulky DNA adducts in liver and colon compared to control rats given cornstarch (Hansen et al., 2004; Moller et al., 2003). In humans,

Abbreviations: AAS, 2-amino adipic semialdehyde; O-PLS-D, orthogonal projections to latent structures discriminant; O-PLS-DA, orthogonal projections to latent structures discriminant analysis; PCA, principal components analysis; PCNA, proliferating cell nuclear antigen; SCFA, short-chain fatty acid; VLDL, very low density lipoprotein.

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one study indicated that a high intake of sucrose may increase the proliferation rate in the colorectal epithelium, and expand the proliferative zone from less than 60% of the colonic crypt to the entire crypt (Bostick et al., 1997). Increased colon cell proliferation has also been observed in rodents, especially when sucrose is given as a bolus dose (Luceri et al., 1996). Increased cell proliferation may be a risk factor for colon cancer and it has been suggested that increased fasting insulin levels and decreased insulin sensitivity are important links between Western-type diets and colon cancer risk (Bruce et al., 2000). In a two-stage colon carcinogenesis model insulin acted as a tumour promoter when given i.v. to rats initiated with azoxymethane (Tran et al., 1996). Although sucrose is hydrolysed to the monosaccarides, fructose and glucose, shortly after ingestion, sucrose increased colon cell proliferation more than fructose and glucose in a feeding study in rats (Caderni et al., 1996). It was suggested that the increased proliferation was caused either by a rapid absorption of the fructose or glucose after ingestion of sucrose or by an increase in the insulin level caused by a rapid absorption of glucose (Caderni et al., 1996). In humans it is well known that a Western-type diet (high saturated fat and high sucrose) induces the metabolic changes associated with insulin resistance (Daly, 2003). However, insulin resistance may be caused by both fat and sucrose which are known to influence insulin sensitivity by different mechanisms (Thresher et al., 2000). The impact of intake of sucrose and fructose on markers for insulin resistance has been studied in rodents and to a lesser extent in humans. In rodents, a high intake of sucrose (>60% of total energy) or fructose (>35% of total energy), has consistently decreased insulin sensitivity, and increased fasting plasma insulin levels (Daly, 2003; Huang et al., 2004; Thresher et al., 2000), whereas glucose did not impair insulin sensitivity (Thorburn et al., 1989). In humans, the link between intake of sucrose or fructose and markers of insulin resistance is conflicting (Daly, 2003).

Other metabolic effects of simple carbohydrates have been observed. In humans, fructose is considered the most hypertriglyceridemic sugar and is thought to account for the hypertriglyceridemic effect of sucrose (Fried and Rao, 2003). Rodent feeds having high contents of sucrose and/or fructose increased the levels of triglycerides and of very low density lipoprotein (VLDL) in plasma (Farombi et al., 2004; Taghibiglou et al., 2000; Yoshino et al., 1997).

A high intake of simple sugars may also alter the colonic microenvironment, leading to changes in pH and in the formation of fermentation products such as short-chain fatty acids (SCFA), which are an important energy source for the colonic epithelium (Zambell et al., 2003). In rats, a feed with non-resistant starches or simple carbohydrates has consistently decreased caecal production of SCFA and increased pH compared to feeds rich in resistant starch (Caderni et al., 1996; Henningsson et al., 2003; Le Leu et al., 2003; Nakanishi et al., 2003).

Sugar has also been suggested to alter oxidative stress through glycoxidation processes (Miyata et al., 1997). Previous studies indicate that the genotoxicity of sucrose may not be related to oxidative DNA damage or altered DNA repair (Dragsted et al., 2002; Hansen et al., 2004; Moller et al., 2003) but increased oxidative damage may still take place in other macromolecules leading to indirect effects on DNA.

The aim of the present study was to investigate whether the colon genotoxicity of sucrose can be ascribed specifically to either fructose or glucose using Big Blue® rats and whether the mechanisms behind this effect are related to the endogenous metabolism of the sugars, changes in colonic fermentation, colon cell turnover, colon protein oxidation or to insulin sensitivity.

2. Materials and methods

Chemicals were used as supplied, without further purification. Fluoresceinamine (isomer II), sodium cyanoborohydride, 4-morpholinoethane sulfonic acid, and SDS were from Aldrich Chemical Co. (Steinheim, Germany). Sucrose, fructose and glucose were from Applichem (Darmstadt, Germany). If not otherwise stated, all other chemicals were from Merck (Darmstadt, Germany).

2.1. Animals and exposure

Forty-nine male Big Blue® (Fischer) rats (9–12 weeks of age; 180–270 g) from AH Diagnostics (Aarhus, Denmark) were acclimatized for 10 days while being maintained on a purified diet without sucrose. The diet was modified from the standard diet formulated at the National Food Institute according to Meyer et al. (Meyer et al., 1982) with some modification (see Table 1). Animals were stratified by weight and randomly assigned into four groups: control (0% w/w sucrose, $n = 13$), 30% sucrose (w/w, $n = 11$), 30% fructose (w/w, $n = 12$) and 30% glucose (w/w, $n = 12$). Animals were kept two in each cage as described previously (Dragsted et al., 2002). The diet was administered for a total of 35 days and feed consumption was monitored. A 24-h urine sample was collected on day 34 and stored at −80 °C. No disinfectant was used in the cage. At day 35 each

Table 1
Composition of animal diets

Ingredient (g/kg feed)	Control	30% Sucrose	30% Fructose	30% Glucose
Protein (casein)	180	180	180	180
Fats (soya oil)	50	50	50	50
Carbohydrate				
Potato starch	340	40	40	40
Sucrose	0	300	0	0
Fructose	0	0	300	0
Glucose	0	0	0	300
Cornstarch	306	306	306	306
Dextrin	34	34	34	34
Cellulose	50	50	50	50
Mineral/vitamin mixture[a]	40	40	40	40

[a] The mixture contains (mg/kg) Ca^{2+} (4970), P (phosphate, 3100), K^{+} (3610), (sulphate, 300), Na^{+} (2530), Cl^{-} (1490), Mg^{2+} (600), Fe^{2+} (34), Zn^{2+} (30), Mn^{2+} (10), Cu^{2+} (7), I^{-} (200), Mb (molybdate, 0.15), Se (selenite, 0.15), Si (2.5), Cr^{4+} (1.0), F^{-} (1.0), Ni^{2+} (0.5), B (borate, 0.5), Li^{+} (0.1), V (vanadate, 0.1), Co^{2+} (0.07), thiamine (5), riboflavine (6), pyridoxol (8), folate (2), biotin (0.3), cyanocobalamine (0.03), panthotenate (20), choline (hydrogentartrate, 2600), inositol (400), nicotine amide (40), phylloquinine (1000), *p*-aminobenzoic acid (40), methionine (1000), L-cysteine (2000) and (IU/kg) vitamin A (5000), vitamin D3 (1000), and vitamin E (DL-α-tocopherol, 50).

rat was weighed, anaesthetized in a mixture of 40% carbon dioxide and 60% oxygen, and decapitated. Blood was collected through a polyethylene funnel into EDTA-coated tubes and separated into plasma and erythrocytes by centrifugation (3000*g*, 10 min at 4 °C). Erythrocytes were mixed 1:1 with water and plasma and erythrocytes were stored at –80 °C. Liver weight was recorded. Tissues were handled as previously described, except that liver tissue was sampled from the left and right lateral lobe 1 cm from the ventral edge (Dragsted et al., 2002). Liver and colon tissue were fixed in 4% buffered formaldehyde for 48 h. The remaining liver tissue was frozen in liquid nitrogen and stored at −80 °C. Housing and treatments of the rats followed procedures approved by the Danish animal experiment inspectorate.

3.1. Mutation analysis

The mutation frequencies of the *E. coli* lambda bacteriophage cII gene packaged in liver and colon epithelium tissue extracts were determined as described previously (Dragsted et al., 2002).

3.2. Oxidative parameters and DNA damage

Protein oxidation (presence of specific protein aldehydes), was determined in plasma and liver as described previously and the product of protein oxidation, 2-amino adipic semialdehyde was determined by HPLC (Daneshvar et al., 1997). DNA strand breaks in colon mucosa cells were determined by the comet assay (Moller et al., 2002).

3.3. DNA adducts and cell proliferation

The level of bulky DNA adducts in colon and liver were determined by a general ^{32}P-postlabelling procedure using standard conditions similar to those used for PAH adducts (Autrup et al., 1999). Cell proliferation was assayed in liver and colon by immunohistochemistry using proliferating cell nuclear antigen (PCNA) staining (Lindecrona et al., 2004). Data on cell proliferation in liver is given as the mean of the labelling index of the left and right lateral lobes. The colon samples were taken from the mid part of the colon and were treated like the liver samples with a few exceptions: the monoclonal mouse anti-PCNA (DAKO, M0879, clone PC10, Denmark) was diluted 1:1600 and EnVision + (DAKO, K4000, Denmark) was used as secondary antibody. The labelling index was evaluated as previously described (Poulsen et al., 2001).

3.4. C-peptide

Plasma C-peptide was determined using a commercial kit from (Cat. #RCP-21K; Linco research, St. Charles, USA).

3.5. SCFA

Acetic acid, propionic acid and butyric acid in caecal contents were analysed using capillary electrophoresis and indirect UV detection by a method modified from Westergaard et al. (Westergaard et al., 1998). Briefly, 1 part of caecum contents (approximately 0.1 g) were suspended in 9 parts of alkaline buffer (0.1 M tris, pH 9.6, and 5 mg/ml trichloroacetic acid, as internal standard), centrifuged (14,000*g*, 10 min, 4 °C) and filtrated using a sterile 0.2 μm filter (minisart). Samples were kept at –80 °C until analysis. Prior to analysis the samples were diluted 60 times by running buffer (0.2 mM 1,2,4-benzenetricarboxylic acid (chromophor), 8 mM TRIS, 0.3 mM tetradecyltrimethylammonium bromide and 0.15 mM heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin), pH 7.6. The fused silica capillary (0.75 μm, 80.5 cm (72 cm to detector window) from Agilent, Germany) was rinsed with 1 M NaOH before each sequence and pre-treated with water for 0.5 min, 0.1 M NaOH for 1 min and running buffer for 5 min before each run. Samples were injected by pressure (35 mbar, 2 sec.) and run at –30 kV for 12 min on a G1600A ^{3D}Capillary electrophoresis Instrument (Hewlett-Packard, Waldbronn, Germany).

3.6. ^{1}H NMR spectroscopy

The samples were stored at approximately –80 °C until NMR analysis. Urine samples were prepared for NMR measurement by mixing 400 μl of urine with 200 μl of buffer (0.2 M Na_2HPO_4). After centrifugation an aliquot of 500 μl was mixed with 50 μl of D_2O with the internal standard TSP (3-(Trimethylsilyl)propionic acid-d_4, 1 mg/ml). The plasma samples were made up from 100 μl of plasma and 350 μl of saline (0.9% NaCl). Fifty microliter of a solution of formic acid in D_2O (27.9 mM) with formic acid as the internal standard was added to the centrifuged solution. ^{1}H NMR spectra were recorded at 600.13 MHz on a Bruker DRX-600 spectrometer at ambient probe temperature (298 K) using a flow-inject system (Bruker Biospin, Rheinstetten, Germany). The 1D spectra of plasma and urine were acquired using a standard 1D pulse sequence for water suppression using the first increment of the NOESY pulse sequence (Nicholson et al., 1995), with irradiation at the water frequency during the relaxation delay of 3 s and the pulse sequence mixing time of 100 ms. In addition, a water-suppressed spin-echo Carr–Purcell–Meiboom–Gill experiment (Meiboom and Gill, 1958) was used with a pulse sequence which acts as a T2 filter to suppress signals from macromolecules and other substances with short T2 values in the plasma samples. A spin–spin relaxation delay 2 nτ of 300 ms was used ($\tau = 1$ ms), and water suppression irradiation was applied during the relaxation delay of 2 s. For all sample measurements, 128 transients were acquired into 32 K data points with a spectral width of 12019.230 Hz and an acquisition time of ~1.36 s. The FIDs were zero-filled to 64 K prior to Fourier transformation with no window function applied. Identification of endogenous metabolites was made with reference to the literature data and ^{1}H NMR spectra of reference standards.

3.7. NMR spectral processing and multivariate analysis

Spectral processing was performed using an in-house routine developed in MATLAB (The Mathworks, Inc., Natick, MA). The spectra were all phase-corrected and corrected for baseline offset errors. The urine spectra were referenced to TSP at 0.0 ppm and the plasma spectra were referenced to formate at 8.46 ppm, respectively. The region 4.5–5.0 ppm surrounding the water resonance was excluded in all measured spectra to remove the effects of variations in the presaturation of the water resonance. The plasma spectra had the regions 2.52–2.6, 2.68–2.72, 3.07–3.25 and 3.6–3.66 excluded due to EDTA peaks, before the spectra were divided into segments of 0.01 ppm and the integral of each region was calculated. The urine spectra had the area 5.98–5.20 ppm excluded because of cross-relaxation alterations to the urea signal via solvent exchanging protons, and the regions 2.50–2.58 and 2.66–2.74 ppm were merged to compensate for pH dependent shifts in the citrate resonance's. The NMR spectra of urine were divided into segments of 0.04 ppm and the integral of each region was calculated. The reduced urine spectra were normalised to a total area of one in order to standardise samples and partially remove concentration differences between urine samples.

The plasma and urine data matrices were transferred to the chemometrics software package SIMCA-P + ver. 11 (Umetrics A/S, Umeå, Sweden) and principal components analysis (PCA) (Wold et al., 1987) and orthogonal projections to latent structures (O-PLS) (Trygg and Wold, 2002) were used to model the data. The data was mean centred by subtracting from each column its average, so that all results were interpretable in terms of variation round the mean. Pareto scaled data was used in all models to weigh down the dominating variables by applying a weight of $1/\sqrt{\delta}$, where δ is the standard deviation of the variable. PCA were used as an unsupervised model to check the data for outliers and general trends in the spectral data. The principal components (PCs) are calculated to explain the variance in the data with subsequent orthogonal PCs explaining progressively less variance. For each principal component, a loading (or weight) containing the influence of each of the original

variables common for all the samples, is extracted from the data, where score values (coefficients of the principal component) reflect the contribution of that loading in each sample.

O-PLS was used as a supervised model to extract the metabolite changes in the spectral data. O-PLS is a linear regression method with the same predictive ability as PLS (projections to latent structures also known as partial least squares regression) (Martens and Næs, 1993). The O-PLS method has the advantage that the structured noise is modelled separately from the variation common to Y (the descriptive variable) and X (the NMR data). We used the O-PLS model as an O-PLS-DA model (O-PLS discriminant analysis) with Y defined as a class variable consisting of dummy variables, e.g. 1, 2, 3, and 4, for each group to be modelled (control, 30% sucrose, 30% fructose and 30% glucose). Full cross validation was used, i.e., one samples was predicted at a time from calibration model consisting of the rest of the samples.

3.8. Statistics

Biomarker endpoints were tested for homogeneity of variance using Levene's test and for normal distribution using the Kolmogorov–Smirnov test. Log-transformations were performed for data, which did not meet these criteria. The nonparametric Kruskal–Wallis test was used for datasets, which were not normally distributed or did not have homogeneity of variance even after log-transformation other data was analysed by ANOVA. These statistical analyses were performed using the SAS Statistical Package, ver. 8.02 (SAS Institute Inc., Cary, NC).

4. Results

There was an increase in energy intake in all dosed groups, which resulted in a higher body weight gain during the experiment (control 73 ± 17 g, sucrose 89 ± 12 g, fructose 91 ± 7 g and glucose 94 ± 16 g (mean ± SD)). The mutation frequency in the colon epithelium was increased 1.5 fold in animals given simple carbohydrates compared to animals given potato starch ($p = 0.027$). This increase was similar in each of the individual sugar groups although it did not achieve statistical significance in any of them (see Table 2). No change was observed on the mutation frequency in the liver (see Table 2). The level of bulky DNA adducts in colon and liver was increased by all sugars (see Table 2). In colon no effects were seen on DNA strand breaks, 2-amino-adipic-semialdehyde (AAS, marker for protein oxidation) or cell proliferation (see Table 2). An increase in caecal pH was observed in rats given simple carbohydrates (see Fig. 1a) reflecting a decrease in the concentrations of acetic acid and propionic acid (see Fig. 1b). The caecal concentration of butyric acid was not affected. Liver weights were significantly increased in sucrose and fructose

Table 2
Oxidative damage in colon mucosa, proliferation in colon crypts, plasma C-peptide concentration, and markers related to DNA damage

	Control	Sucrose	Fructose	Glucose
Mutation frequency in colon epithelium (per 10^6 phages)	22 ± 11	29 ± 11	31 ± 16	35 ± 13
Level of bulky DNA adducts in colon (per 10^8 nucleotides)	24 ± 1	40 ± 1**	34 ± 2**	38 ± 3**
Mutation frequency in liver (per 10^6 phages)	22 ± 9	29 ± 11	29 ± 17	25 ± 11
Level of bulky DNA adducts in liver (per 10^8 nucleotides)	22 ± 1	38 ± 3**	33 ± 2**	41 ± 3**
AAS in colon (pmol/mg protein)	51 ± 27	45 ± 35	52 ± 50	45 ± 28
PCNA in colon[a]	4.6 ± 4,8	2.9 ± 2.3	4.0 ± 4.0	1.4 ± 1.7
PCNA in colon[b]	2.6 ± 2.5	1.4 ± 1.3	1.8 ± 1.6	0.7 ± 0.8
C-Peptide in plasma pmol/l	495 ± 235	588 ± 340	630 ± 322	677 ± 345
Strand breaks in colon (%DNA in tail)	10.5 ± 4.0	9.9 ± 5.5	9.1 ± 3.2	9.2 ± 4.0

Values are means ± SD. **$P < 0.01$.
[a] Percentage of cells staining positive in the lower third of the coloncrypt.
[b] Percentage of cells staining positive in whole coloncrypt.

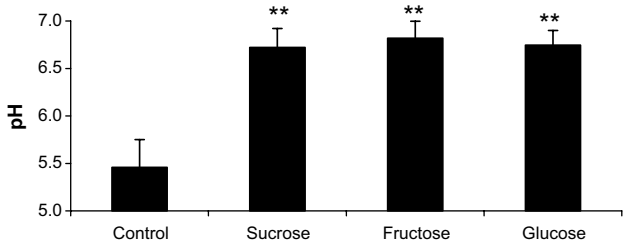

Fig. 1a. pH in caecum content. Values represent means ± SD. **$P < 0.01$ compared to control.

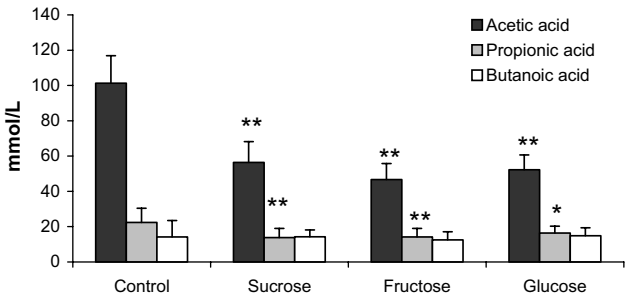

Fig. 1b. Concentration of SCFA in caecum contents. Values are means ± SD. *$P < 0.05$ compared to control. **$P < 0.01$ compared to control.

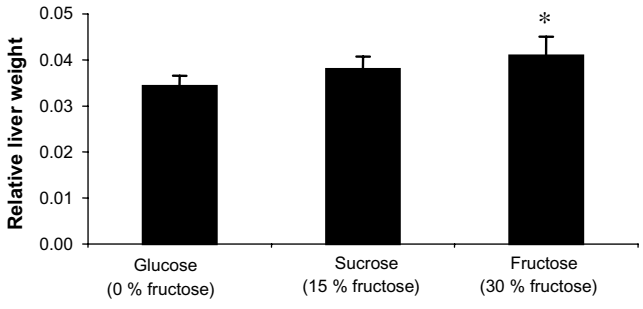

Fig. 1c. Relative liver weight. The relative liver weight is defined as liver weight divided by bodyweight. Sucrose is hydrolysed to fructose and glucose shortly after ingestion. If sucrose is considered as 50% fructose the liver weight increased dose-dependently with respect to fructose. Each point represents mean ± SD. *$P < 0.05$ compared to control.

dosed rats but no change was seen in rats given glucose (see Fig. 1c). If sucrose is considered 50% fructose the increase was dose-dependent with respect to fructose. There was no change in the level of C-peptide in plasma (see Table 2).

Multivariate models were applied to ^{1}H NMR spectra of plasma to identify possible data clustering and related metabolic changes. An unsupervised PCA model of the NMR spectra of all plasma samples was used to model the general variation of the spectra. The score plot from the two most important principal components (PC1 and PC2) in a PCA model is shown in Fig. 2, demonstrating that the fructose dosed animals are more separated from control animals than the glucose dosed animals are. Sucrose is in between, as it could be expected from its chemical composition. The difference in metabolite composition in the spectra explaining the large separation of fructose dosed animals from the controls is apparent when comparing the NMR spectrum of plasma from a control animal with the spectrum of plasma from a fructose dosed animal (Fig. 3). The spectrum of the fructose dosed animal (B) is dominated by high levels of triglycerides and very low density lipoprotein (VLDL) as assigned in the spectrum. Thus, the distribution of the fructose, sucrose and glucose dosed animals and their gradual separation from the control animals in Fig. 2 were caused by varying levels of plasma lipids. Having identified the main metabolic differences between the control and dosed animals, the changes in less dominating metabolites may also be of interest, and for that purpose CMPG spectra were used where the contribution from 'larger' metabolites such as lipids, has been suppressed. In Fig. 4 the score plot of an O-PLS-DA model of the CPMG spectra is shown. The O-PLS predictive component (Comp. 1) describes all the variation related to the discrimination of the control animals from all the carbohydrate dosed animals and the control animals are now clustering separated from all the dosed animals. Variation in the spectra not related to the main discrimination is modelled in Comp. 2, and it is clearly shown in Fig. 4 that this variation is

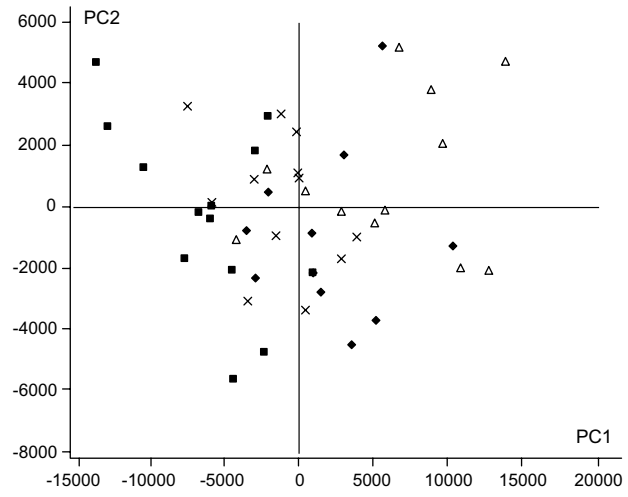

Fig. 2. Score plot of the first principal component (PC1) versus the second principal component (PC2) from a PCA model of pareto scaled data from NMR spectra of plasma samples. Controls (■); 30% Fructose (Δ); 30% Sucrose (◆); 30% Glucose (×). PC1 and PC2 explained 67% and 12% of the total variance in the spectral data, respectively.

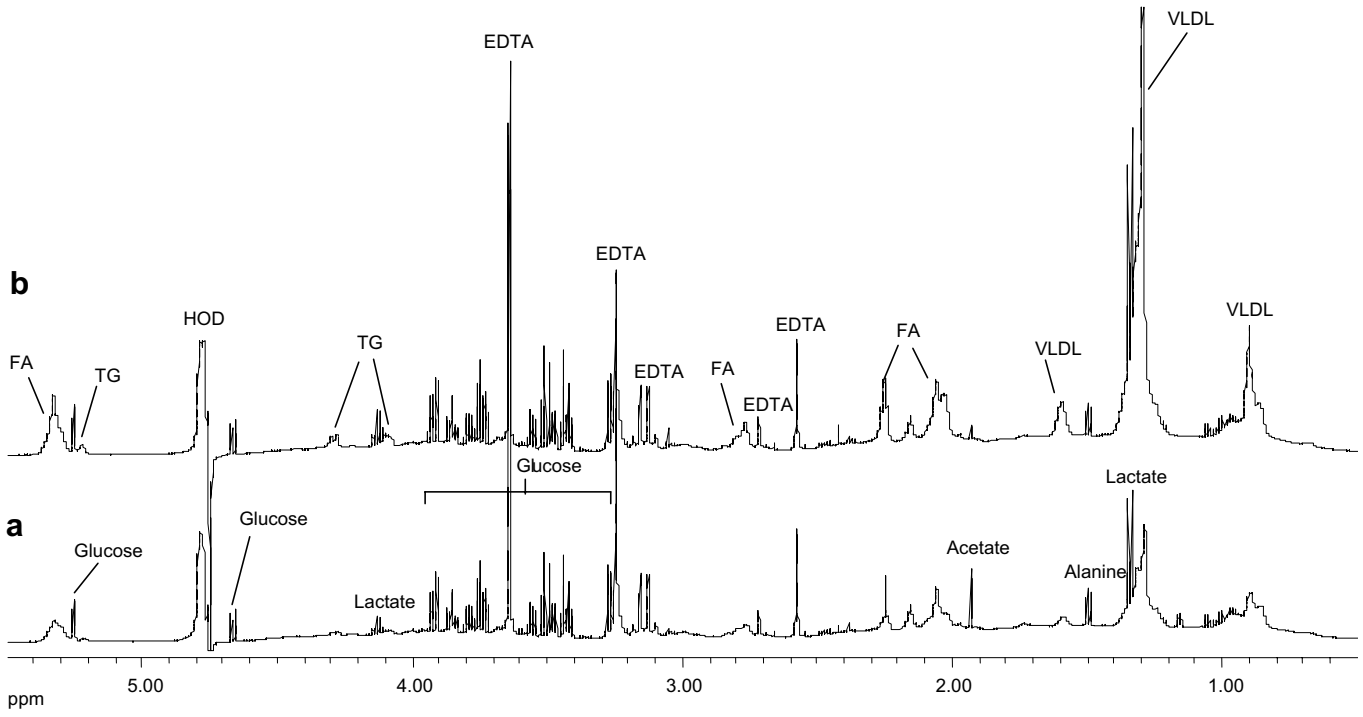

Fig. 3. ^{1}H NMR spectra (0.5–5.5 ppm) of a control plasma sample (a) and a sample from a rat given a 30% fructose diet. (b) Key: FA = fatty acids; HOD = suppressed water signal; TG = triglyceride; VLDL = very low density lipoprotein.

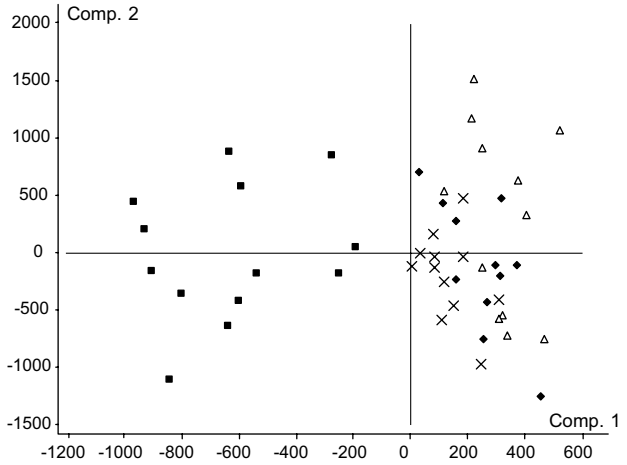

Fig. 4. Score plot of the predictive component (Comp. 1) versus the first orthogonal component (Comp. 2) from an O-PLS-DA model of pareto scaled data from CPMG spectra of plasma samples showing the discrimination of control rats from all rats fed with excess simple carbohydrates in Comp. 1. Controls (■); 30% Fructose (Δ); 30% Sucrose (◆); 30% Glucose (×). Comp. 1 explained 21% of the total variance in the spectral data.

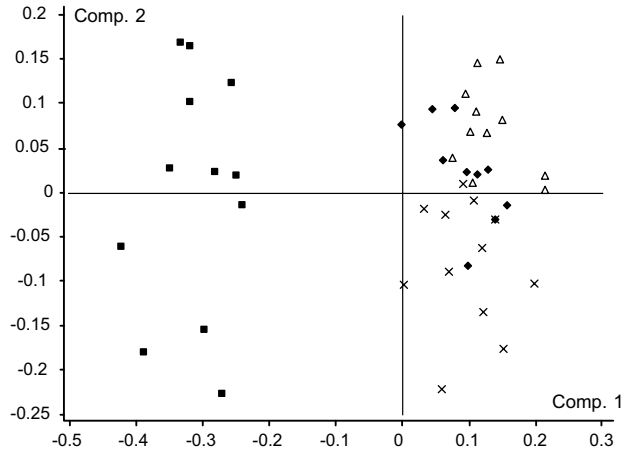

Fig. 5. Score plot of the predictive component (Comp. 1) versus the first orthogonal component (Comp. 2) from an O-PLS-DA model of pareto scaled urine NMR data showing the discrimination of control rats from all rats fed with excess simple carbohydrates in Comp. 1. Controls (■); 30% Fructose (Δ); 30% Sucrose (◆); 30% Glucose (×). Comp. 1 explained 26% of the total variance in the spectral data.

Table 3
Assignments of metabolites in plasma that highly contributed to the discrimination of controls and simple carbohydrate dosed animals in the O-PLS-DA model

Metabolites	^{1}H chemical shifts (δ) (ppm)	Increase (↑) or decrease (↓) dosed with simple carbohydrates
Acetate	1.92	↓
Methionine	2.14	↓
Serine	3.98, 3.96	↓
Valine	0.99, 1.04	↓
Lipoproteins (VLDL, LDL)	0.85–0.88, 1.2–1.3	↑
Lipids	1.7, 2.05, 2.78	↑

Table 4
Assignments of metabolites in urine that highly contributed to the discrimination of controls and simple carbohydrate dosed animals in the O-PLS-DA model

Metabolites[a]	^{1}H chemical shifts (δ) (ppm)	Increase (↑) or decrease (↓) dosed with simple carbohydrates
Acetate	1.92	↓
Formate	8.46	↓
2-oxoisovalerate	1.12	↓
p-Cresol glucuronide	5.08, 7.04, 7.08	↑
N-methylnicotinamide	4.48, 8.19, 8.96, 9.28	↑
Phenylacetylglycine	3.68, 3.76, 7.36	↑
Pyruvate	2.36	↑
Tryptophane	7.24, 7.28	↑

related to the separation of the three types of carbohydrates. In Table 3 the metabolites that were identified as the cause for the separation along Comp. 1 in Fig. 4 are listed. The main metabolite changes in plasma are decreased concentrations of acetate and the amino acids valine, serine and methionine. The lipids listed in Table 3 are remnants in the CPMG spectra, but they show the same increase in the carbohydrate dosed animals in the O-PLS-DA model as in the PCA model.

The urine spectra were also analysed with multivariate models. Low urea contents and pH shifts in samples from all treatment groups indicated some bacterial contamination. However, the PCA model of all the samples (model not shown) showed that the main variation of the PCA model was the separation of the four treatment groups, and there was no metabolite changes typically related to contamination, such as high concentrations of acetate, lactate and ethanol (Maiza et al., 1992). Thus we assume that the main variation in the metabolites was not affected. The O-PLS-DA model was used to model the data ensuring that only spectral variation related to the discrimination between control animals and carbohydrate dosed animals was modelled in the first component. The score plot of the O-PLS-DA model of the urine spectra is shown in Fig. 5. The control animals are clustering well separated from all the carbohydrate dosed animals along the predictive O-PLS component (Comp. 1), and the metabolites that are causing this separation are listed in Table 4. The main metabolite changes in urine are decreased concentrations of acetate, formate and 2-oxoisovalerate in carbohydrate dosed animals whereas *p*-Cresol glucuronide, *N*-methylnicotinamide, phenylacetylglycine, pyruvate and tryptophane concentrations are increased.

5. Discussion

In a previous experiment where rats were given 13.45% sucrose in the diet for three-weeks we saw an increase in the mutation frequency and the level of bulky adducts in the colon epithelium on 2.0 fold and 2.1 fold, respectively

(Hansen et al., 2004). In this experiment the animals were given 30% simple carbohydrates for five weeks, which is a high dose compared to the estimated Danish human intake of 110 g sucrose/day (Fagt and Trolle, 2001; Matthiessen et al., 2003). As the mutation frequency in Big blue® animals is linearly dependent of the total dose of a mutagenic substance (Cosentino and Heddle, 2000), we expected an increase in the differences in mutation frequency between control and dosed animals compared to the previous experiment. This would increase the probability of revealing differences in the mutation frequency caused by the three simple carbohydrates. Although the increase in mutation frequency and level of bulky adducts in the colon was actually smaller in this experiment (1.3 fold and 1.6 fold, respectively) (see Table 2) we consider these results as a confirmation of the previously reported genotoxicity of simple carbohydrates in the rat colon (Dragsted et al., 2002; Hansen et al., 2004) and an indication that this effect cannot be specifically ascribed to either the fructose or the glucose moiety in sucrose. The most likely explanation for the increased weight gain and intake of energy in animals given simple carbohydrates is the low energy density the control feed. In this study we used potato starch in the control feed, which is partly fermented in the colon and thereby having a lower energy density than cornstarch, which was used in our previous experiments. As we have not seen increased weight gain in our previous experiments (Dragsted et al., 2002; Hansen et al., 2004), where the mutation frequency was increased more than in this experiment, it is unlikely that the increased intake of energy may be related the mutagenic effect. Since simple sugars are absorbed early in the gastrointestinal tract, well before they reach the colon, the genotoxic effect may likely be caused by a systemic effect, common to sucrose, fructose and glucose, or possibly through indirect changes in the colonic microenvironment.

The increase in the level of bulky DNA adducts in the colon of rats given fructose or glucose was of similar magnitude to that of sucrose and similar to levels observed in the liver. This is in accordance with our previous results with sucrose (Hansen et al., 2004). The chemical nature of these adducts is presently unknown. In commercial Brazilian sugar produced from sugarcane the concentration of PAH varies from not detected to 1.35 μg/kg (Tfouni et al., 2004). This content of PAH is probably due to the production technique where the sugarcanes are partially burned to facilitate the harvest. Sugar beets are not contaminated in this way. In commercial sugar produced from sugar beets in Denmark the concentration of PAH is below the detection limit of 0.1 μg/kg (personal communication, Danisco Sugar). As we have used analytical grade sugars, we find it unlikely that the increase in the level of bulky adducts should be caused by contamination of the sugars with PAH.

We observed increased pH and decreased concentrations of acetic acid and propionic acid, which may reflect changes in the microbiological environment in the colon. Also these changes were of similar magnitude for all sugars. As most of the substrate for the colonocytes is provided lumenally as SCFA and acetate and propionate serve as substrates for colonocytic lipogenesis (Zambell et al., 2003) the decreased level of these substances could perhaps result in increased permeability of the epithelial cell membrane, leading to an increase in the amount of genotoxic substances penetrating into the epithelial cells. This would be in accordance with our previous observations that sucrose does not change the mutation spectrum or the DNA adduct pattern (Dragsted et al., 2002; Hansen et al., 2004; Moller et al., 2003). The concentration of acetic acid was also decreased in plasma and urine indicating that the availability of acetate to the colonic cells was decreased systemically. Insulin increases the turnover and decreases the plasma concentration of acetate in healthy individuals (Piloquet et al., 2003). The decrease in plasma acetate may therefore reflect an increased insulin level in animals given simple carbohydrate rather than a decreased uptake of acetate from colon. However, the lack of effect of the sugars on plasma C-peptide, a marker integrating mean insulin levels over a time period, indicates that although insulin levels may have changed considerably as a result of the sugar intake, the mean concentration was unaffected and insulin resistance was not evident. Also the metabonomic analyses lends support to this conclusion since none of the urine markers observed previously in a rat model prone to diabetes (Williams et al., 2005) were observed in our rats given simple sugars. Finally, we did not observe an increase in cell proliferation in the colon epithelium, which is expected in insulin resistant rats. This confirm a study with a similar design where there was no effect on colon mutation frequency or level of bulky adducts in rats given 30% lard for 3 weeks (Vogel et al., 2003).

Previous studies have indicated that sucrose does not induce oxidative stress in either colon, liver or plasma (Dragsted et al., 2002; Hansen et al., 2004; Moller et al., 2003) and this is in accordance with the present study, in which 2-amino-adipic-semialdehyde levels, a specific marker of protein carbonyl formation, were unchanged in the colon epithelium of sugar-dosed animals. The lack of oxidative stress may indicate that glycoxidation as a result of altered glucose metabolism is a less likely mechanistic explanation for the genotoxicity of simple carbohydrate, although more direct markers should be applied before a final conclusion may be drawn.

We found increased levels of plasma VLDL and triglycerides, especially in the fructose dosed animals. This finding has also been seen in insulin resistant rodents (Taghibiglou et al., 2000; Yoshino et al., 1997). The effect in our study was weaker with sucrose and absent with glucose in accordance with the effect on liver weight. We believe therefore that this is a specific effect of fructose on the liver, rather than an indirect effect through the insulin pathway, and that it is unrelated to mutagenesis. Increased liver weight is usually not reported in animals dosed with sucrose or fructose, and we did not observe this effect in our previous studies where effects of sucrose and cornstarch were compared. In the present study we fed the control group

with potato starch, which is degraded more slowly than cornstarch, and the effect may therefore be more readily observable. The increased liver weight associated with fructose in the present study is in accordance with the increased hepatic VLDL production and increased plasma triglycerides.

The increased level of *N*-methylnicotinamide in the urine was seen with all three sugars but most prominently for fructose, and may indicate a direct effect of these simple carbohydrates on the kidney function, since the substance has been suggested as a biomarker for renal function (Maiza et al., 1992). However, more recent research shows a relationship to liver peroxisome proliferation in rats and to liver cirrhosis in humans, indicating that this may primarily be ascribed to fructose mediated liver toxicity.

Several of the metabolites in urine were identified as causing the same changes in all the carbohydrate dosed animals. Similar changes in urine metabolite patterns have previously been observed in urine from mice infected with *Schistosoma mansonii*, a parasite affecting the function of liver and kidney and disturbing the metabolism of amino acids and carbohydrates (Wang et al., 2004). This pattern included an increased excretion of *p*-cresol glucuronide, phenylacetylglycine, tryptophan and pyruvate and a decreased excretion of acetate and 2-oxoisovalerate. The decreased level of urine 2-oxovalerate may be a direct consequence of the decreased concentration of valine in plasma. Also the increased urine level of pyruvate and tryptophane and decreased level of plasma serine and methionine indicate a seriously disturbed amino acid metabolism. Depletion of methionine and valine is known to affect the capacity for DNA and RNA synthesis in fast growing cells (He et al., 2003). It may be speculated that a decreased availability of valine and methionine might specifically affect the DNA repair capacity in the growing colonocytes, however previous studies found no changes in the expression of DNA-repair genes in sugar-dosed Big blue® rats (Dragsted et al., 2002). Schistosomiasis is generally thought to be related mainly to bladder cancer, by a high excess risk of colon cancer in Schistosomiasis patients has been reported in some studies from China (Cheever, 1981; Guo et al., 1993; Qiu et al., 2005; Xu and Su, 1984).

Further studies are needed to elucidate this potential relationship and the mechanisms behind the effects of high sugar intake on amino acid depletion.

In conclusion, the simple carbohydrates, sucrose, fructose and glucose, have similar genotoxicity in the rat colon and alter the colonic chemical environment to the same extent. They equally affect the metabolism of amino acids and also affect the liver function. The genotoxicity may be related the altered chemical environment, which may affect the microbiological environment in the caecum and thereby also in the colon, or to alterations in amino acid metabolism but probably not to insulin resistance or to oxidative stress. Further studies are needed in order to assess the effects on colon genotoxicity of altered amino acid metabolism and whether an altered chemical environment characterized by high pH and lowered levels of SCFA's in the lower parts of the gut is sufficient to cause mutation.

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