The Effect of Rat Strain, Diet Composition and Feeding Period on the Development of a Nutritional Model of Non-Alcoholic Fatty Liver Disease in Rats

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Summary
Non-alcoholic fatty liver disease (NAFLD) is an important cause of liver-related morbidity and mortality. The aim of this work was to establish and characterize a nutritional model of NAFLD in rats. Wistar or Sprague-Dawley male rats were fed ad libitum a standard diet (ST-1, 10 % kcal fat), a medium-fat gelled diet (MFGD, 35 % kcal fat) and a high-fat gelled diet (HFGD, 71 % kcal fat) for 3 or 6 weeks. We examined the serum biochemistry, the hepatic malondialdehyde, reduced glutathione (GSH) and cytokine concentration, the respiration of liver mitochondria, the expression of uncoupling protein-2 (UCP-2) mRNA in the liver and histopathological samples.

Feeding with MFGD and HFGD in Wistar rats or HFGD in Sprague-Dawley rats induced small-droplet or mixed steatosis without focal inflammation or necrosis. Compared to the standard diet, there were no significant differences in serum biochemical parameters, except lower concentrations of triacylglycerols in HFGD and MFGD groups. Liver GSH was decreased in rats fed HFGD for 3 weeks in comparison with ST-1. Higher hepatic malondialdehyde was found in both strains of rats fed HFGD for 6 weeks and in Sprague-Dawley groups using MFGD or HFGD for 3 weeks vs. the standard diet. Expression of UCP-2 mRNA was increased in Wistar rats fed MFGD and HFGD for 6 weeks and in Sprague-Dawley rats using HFGD for 6 weeks compared to ST-1. The present study showed that male Wistar and Sprague-Dawley rats fed by HFGD developed comparable simple steatosis without signs of progression to non-alcoholic steatohepatitis under our experimental conditions.

Key words
Fatty Liver • Oxidative stress • Cytokines

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Introduction
Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease in the Western world (Erickson 2009). The prognosis of NAFLD depends on the histological subtype of the disease. Primary NAFLD is considered the hepatic manifestation of the metabolic syndrome, whereas secondary NAFLD is a consequence of rapid weight loss, jejunoileal bypass, total parenteral nutrition, intestinal bacterial overgrowth or exposure to certain drugs and toxins (McClain et al. 2004).

The pathogenesis of NAFLD is not completely understood but two-hit (Day and James 1998) or multiple-hit theories have been considered. In brief, accumulation of lipids in the liver is an essential condition for developing NAFLD. Fatty hepatocytes become more sensitive to other factors, such as oxidative stress (Grattagliano et al. 2008),
mitochondrial dysfunction (Romestaing et al. 2008), cytokines and adipocytokines (Tilg 2010), and gut-derived lipopolysaccharide (Kirsch et al. 2006). These insults to hepatocytes may promote hepatocellular injury and liver inflammation.

Appropriate animal models of fatty liver are an essential tool for studying the mechanisms of NAFLD development and for testing potential drugs for NAFLD treatment. Such models should exert as much similarity to human NAFLD as possible. However, currently used models reflect human NAFLD only partially.

There are nutritional, drug-induced (Ferno et al. 2009), toxic, and genetic models (Forcheron et al. 2009) of NAFLD in rats. Nutritional models involve a high-fat diet, methionin cholin-deficient diet (MCDD) (Yoshioka et al. 2010), high saccharose, fructose (Kawasaki et al. 2009) and cholesterol diet (Jeong et al. 2005). Nutritional high-fat animal models seem to simulate human pathogenesis of primary NAFLD most closely, though they lack some features of human NAFLD (esp. spontaneous progression to non-alcoholic steatohepatitis (NASH) and cirrhosis, natural multifactorial etiology).

There are many experimental designs for developing fatty liver or NASH in rats using high fat diets. Various protocols differ in terms of the period of feeding, nutritional definition of diets, age, strain and sex of rats, method of feeding, and others. High-fat diets vary in the percentage of energy provided in lipids from 42 % (Buettner et al. 2006) up to 77 % (Zou et al. 2006). Variation can also be found in the preference of the composition of polyunsaturated, monounsaturated or saturated fatty acids (Lieber et al. 2004, Buettner et al. 2006, Romestaing et al. 2007). Rats are fed a high-fat diet from 3 (Lieber et al. 2004) to 14 weeks (Romestaing et al. 2007) or longer. Nutritional models using high fat diets are successful in developing simple liver steatosis but not in progressing to NASH in rats (Ahmed et al. 2009), the Lieber model being the exception (Lieber et al. 2004).

The present study characterizes and compares the effect of a high-fat diet (71 % kcal fat) on two strains of rats and in two time intervals of feeding.

Methods

Male Wistar (BioTest, Czech Republic) and Sprague-Dawley rats (AnLab, Czech Republic) with initial weight of 250±30 g were used throughout the study. The rats were housed at 23±1 °C, 55±10 % humidity, air exchange 12-14 times/h and 12h light-dark cycle. The animals had free access to tap water and different diets as described below. All the animals received care according to the guidelines set out by the Institutional Animal Use and Care Committee of Charles University in Prague and our study was approved by this committee.

The rats were divided into 12 groups (Table 1). Rats were fed a standard laboratory diet (ST-1, Velaz, Czech Republic; 10 % kcal fat, 30 % kcal proteins, 60 % kcal saccharides), medium-fat gelled diet (MFGD; 35 % kcal fat, 18 % kcal proteins, 47 % kcal saccharides; same composition as a standard liquid diet according to Lieber (Lieber et al. 2004)) or high-fat gelled diet (HFGD; 71 % kcal fat, 18 % kcal proteins, 11 % kcal saccharides; Lieber et al. 2004) for 3 or 6 weeks. MFGD and HFGD diets were prepared from ingredients purchased from MP Biomedicals (Solon, OH). All chemicals, unless otherwise mentioned, were analytical grade and were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Mean weight gain, food and water intakes were monitored 3 times a week. After starvation for 14 hours, the animals were sacrificed and liver and serum samples were taken. Liver was stored on ice between procedures (weighing, mitochondria isolation). Samples for consequent evaluation were immediately frozen in liquid nitrogen and stored at –80 °C until analysis.

**Table 1.** Definition of groups and number of animals in groups. ST-1 – standard diet, MFGD – medium-fat gelled diet, HFGD – high-fat gelled diet.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Feeding</th>
<th>Groups</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>3 weeks</td>
<td>ST-1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>MFGD</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>HFGD</td>
<td>5</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>3 weeks</td>
<td>ST-1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>MFGD</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>HFGD</td>
<td>6</td>
</tr>
<tr>
<td>Wistar</td>
<td>6 weeks</td>
<td>ST-1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>MFGD</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>HFGD</td>
<td>7</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>6 weeks</td>
<td>ST-1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>MFGD</td>
<td>7 (1 died during the experiment)</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>HFGD</td>
<td>7</td>
</tr>
</tbody>
</table>

**Serum biochemical measurements**

Serum glucose, total bilirubin, triglycerides,
alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activities were measured in the Institute for Clinical Biochemistry and Diagnostics, Faculty Hospital in Hradec Králové. Serum levels of interleukin-6 (IL-6) and transforming growth factorβ1 (TGFβ1) were analyzed using ELISA kits (Bender MedSystems, Austria).

**Measurement of oxygen uptake by isolated mitochondria**

Rat liver mitochondria were prepared from liver homogenates by conventional differential centrifugation (Schneider and Hogeboom 1950, Bustamante et al. 1977, Svátková et al. 1996). Oxygen consumption in isolated mitochondria was measured using High Resolution Oxygraph 2K (Oroboros, Austria). Measurements were performed in 2 ml of potassium-medium at 30 °C (Drahota et al. 2005). OROBOROS software (DatLab 3.1) was used to evaluate oxygen uptake.

**Determination of glutathione**

Liver homogenate was added into cold 10 % metaphosphoric acid, shaken and centrifuged (20 000 x g, 10 min, 4 °C). Glutathione in the supernatant was analyzed by modified fluorometric method (Hissin and Hilf 1976, Kanďár et al. 2007). Briefly, reduced (GSH) and oxidized (GSSG) glutathione were allowed to react with o-phthalaldehyde in phosphate buffer, and the fluorimetric detection was carried out (λ_{Ex}=350 nm, λ_{Em}=420 nm).

**Determination of tissue proteins**

Protein content was determined by the method of Lowry (Lowry et al. 1951) using bovine serum albumin as a standard.

**Determination of tissue cytokines and malondialdehyde**

Liver samples were homogenized in RIPA buffer and centrifuged (10 000 x g). Concentrations of liver IL-6 and TGFβ1 in the supernatant were measured by ELISA. Total tissue malondialdehyde (MDA) was analyzed using a slightly modified method of Pilz (Pilz et al. 2000, Popelova et al. 2008). Briefly, derivatization with 2,4-dinitrophenylhydrazine was performed after an alkaline hydrolyzation, and subsequent reversed-phase high-performance liquid chromatography (Agilent, USA).

**UCP-2 mRNA expression**

The total liver RNA was extracted by RNA Blue (Top-Bio, Czech Republic). Real-time two-step PCR was used for mRNA absolute quantification using plasmid standards. First-strand cDNA synthesis was performed with total RNA, which were reversely transcribed using oligo(dT) primer (Generi-Biotech, Czech Republic) and M-MLV reverse transcriptase (Top-Bio). ABI Prism 7900HT technology and TaqMan Universal Master Mix (Applied Biosystems, USA) were used to amplify cDNA. The specific UCP-2 primers and hydrolysis probe were designed by Generi-Biotech – forward primer 5’AAGACCACTTGACAAGAGGA3’, reverse primer 5’GCAAGGGAGGTCGTCTGTC3’ and probe 5’CCTAATTGGGCCCCAAATGCCG3’ labelled with FAM BHQ. For normalization, surfeit 1 gene (Generi-Biotech) was utilized.

**Liver histology**

Liver specimens were taken immediately after the rats were sacrificed and fixed by immersion in 4 % neutral formaldehyde. Paraffin sections were stained with hematoxylin-eosin to detect hepatic steatosis, inflammation and necrosis. Formaldehyde-fixed frozen sections stained with Sudan 3 were used for lipid detection. Fatty degeneration was graded according to the percentage of fat-containing hepatocytes - grade 1:0-25 %; grade 2:26-50 %; grade 3:51-75 %; grade 4:76-100 %. A degree of the inflammatory reaction and necrosis was indicated as the average counted in 10 randomly selected fields in each slide. Three-point scale was used for classification of these changes - 0=normal; 1=mild; 2=moderate; 3=severe (Avni et al. 2004).

**Determination of tissue triacylglycerols (TAG) and cholesterol**

Lipids from rat livers were prepared using chloroform-methanol extraction (Bligh and Dyer 1959). Total cholesterol and TAG were measured using commercial kits (Roche, Germany).

**Statistical analysis**

The results are expressed as the mean ± S.D. Comparisons were made among the groups using ANOVA followed by Tukey-Kramer’s post test (GraphPad Prism 4.03, GraphPad Software, CA) at the selected level of significance of P<0.05.

**Results**

**Food intake, initial and final weight**

The average food intake is shown in Table 2. Wistar rats fed with ST-1 diet consumed a significantly
higher amount of chow than the Sprague-Dawley strain. There was an increase in food intake in Sprague-Dawley rats fed with MFGD and HFGD for 3 weeks and HFGD for 6 weeks. There were no significant differences among Wistar rats fed by various types of diets.

There were no differences among groups of the same strain and time of feeding in the initial and final weights except for a lower final weight in Sprague-Dawley rats after 6 weeks of feeding with MFGD.

Liver weight, relative liver weight, liver triglycerides and cholesterol (Table 2)

The absolute liver weight of the same strain and of period of feeding was higher only in the MFGD in comparison to the ST-1 group in Sprague-Dawley rats fed for 3 weeks. The relative liver weight was significantly higher in MFGD and HFGD groups of Sprague-Dawley rats fed for 3 weeks. Absolute and relative liver weights were lower in Sprague-Dawley rats compared to Wistar rats. Liver tissue triglycerides and cholesterol were higher in MFGD and HFGD in both strains and periods of feeding. The highest triglycerides contents were found in HFGD after 6 weeks in Wistar and Sprague-Dawley rats.

Serum characteristic (Table 2)

Feeding with MFGD or HFGD did not cause any significant changes in the serum activities of ALT and AST. There were no differences among serum glycemia or total cholesterol within groups; glycemia of Sprague-Dawley rats fed ST-1 was lower than the glycemia of Wistar rats fed the same kind of diet (p<0.05). Surprisingly, MFGD and HFGD caused a drop in serum triglycerides in all groups against ST-1. There were significantly lower levels of triglycerides in Sprague-Dawley rats in comparison with Wistar rat in both time periods.

Liver content of GSH and total MDA (Table 2)

A decrease in liver tissue GSH was found in Wistar rats fed HFGD for 3 weeks and Sprague-Dawley rats fed HFGD and MFGD for 3 weeks. There were no changes in GSH among rats within groups after 6 weeks. There was a significant increase in the total amount of MDA in the livers of Wistar rats fed HFGD for 6 weeks and Sprague-Dawley rats fed MFGD and HFGD for 3 weeks and HFGD for 6 weeks.

Liver and serum cytokines (Table 2)

Feeding with diets containing a higher percentage of fats increased liver IL-6, and significant changes were observed in HFGD in Wistar and Sprague-Dawley rats after 3 weeks and MFGD in Wistar rats after 6 weeks. Neither MFGD nor HFGD caused changes in tissue TGFβ1. Serum IL-6 and TGFβ1 levels were below detection limits of the kits.

Activity of respiratory complexes I and II (Fig. 1)

A significant decrease in the activity of respiratory complex I was found in Sprague-Dawley rats fed by HFGD for 3 weeks compared to ST-1 (p<0.05). There was a tendency of complex I to be lowered also in Sprague-Dawley and Wistar rats fed by MFGD, and MFGD or HFGD, resp. for 3 weeks, and in Sprague-Dawley rats fed by MFGD and HFGD for 6 week, but these changes were not significant.

UCP-2 mRNA expression (Fig. 2)

Increased expression of UCP-2 mRNA was observed in Sprague-Dawley and Wistar rats fed HFGD, and MFGD and HFGD, respectively, for 6 weeks.

Histology of the liver tissue (Table 3, Fig. 3a-3f)

In the liver tissue taken from control group of rats fed a standard diet (Fig. 3a, 3b), no signs of steatosis, inflammatory infiltrate or fibrosis were observed. There were no differences between ST-1 groups. Livers from Wistar rats fed MFGD for 3 or 6 weeks exhibited microvesicular steatosis without marks of inflammation or hepatocyte necrosis (Fig. 3c). However, MFGD did not induce significant steatosis in Sprague-Dawley rats (Fig. 3d). Livers from rats fed HFGD for 3 weeks displayed microvesicular steatosis without signs of progression to steatohepatitis in both strains. Microvesicular or mixed steatosis was not accompanied by inflammatory infiltrate, hepatocyte necrosis or fibrosis in the 6-week feeding period with HFGD in Wistar (Fig. 3e) and Sprague-Dalwey (Fig. 3f) rats.

Discussion

High-fat diets have been used to model obesity, liver steatosis, dyslipidemia etc. in rats for many years. Since the discovery of NAFLD, high-fat diets in rodents have been used in an attempt to reproduce human NAFLD.
**Table 2. Basal characteristics of the dietary groups. The values represent the mean ± S.D. ST-1 standard diet, MFGD – medium-fat gelled diet, HFGD – high-fat gelled diet, GSH – reduced glutathione, MDA – malondialdehyde, IL-6 – interleukin-6, TGFβ1 – transforming growth factor β1, ALT – alanine aminotransferase, AST – aspartate aminotransferase.**

<table>
<thead>
<tr>
<th></th>
<th>Wistar - 3 weeks</th>
<th>Sprague-Dawley - 3 weeks</th>
<th>Wistar - 6 weeks</th>
<th>Sprague-Dawley - 6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ST-1</td>
<td>MFGD</td>
<td>HFGD</td>
<td>ST-1</td>
</tr>
<tr>
<td><strong>Rat characteristics</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Initial weight (g)</td>
<td>242±3</td>
<td>251±3</td>
<td>240±5</td>
<td>261±15</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>360±25</td>
<td>370±18</td>
<td>356±16</td>
<td>346±36</td>
</tr>
<tr>
<td>Final-initial weight difference (g)</td>
<td>118±22</td>
<td>119±17</td>
<td>116±15</td>
<td>84±23</td>
</tr>
<tr>
<td>Food intake (kJ/d/rat)</td>
<td>423±63</td>
<td>405±85</td>
<td>406±122</td>
<td>348±19###</td>
</tr>
<tr>
<td><strong>Liver characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute liver weight (g)</td>
<td>11.2±1.6</td>
<td>11.3±0.7</td>
<td>10.8±0.8</td>
<td>8.3±0.9###</td>
</tr>
<tr>
<td>Relative liver weight (% of body weight)</td>
<td>3.3±0.5</td>
<td>3.2±0.1</td>
<td>3.2±0.1</td>
<td>2.6±0.1###</td>
</tr>
<tr>
<td>Liver triglyceride (mg/g)</td>
<td>5.0±1.1</td>
<td>22.5±6.8##***</td>
<td>25.0±9.1***</td>
<td>6.4±2.5</td>
</tr>
<tr>
<td>Liver cholesterol (mg/g)</td>
<td>1.8±0.5</td>
<td>4.9±2.0**</td>
<td>4.7±2.0***</td>
<td>2.2±0.6</td>
</tr>
<tr>
<td>Liver GSH (mmol/kg liver)</td>
<td>13.3±1.5</td>
<td>11.8±2.5</td>
<td>8.7±1.3*</td>
<td>17.4±1.6</td>
</tr>
<tr>
<td>Total liver MDA (mmol/mg protein)</td>
<td>0.24±0.08</td>
<td>0.81±0.24</td>
<td>0.36±0.08</td>
<td>0.39±0.05</td>
</tr>
<tr>
<td>Liver IL-6 (ng/mg protein)</td>
<td>2.3±0.6</td>
<td>2.8±0.7</td>
<td>3.9±0.7**</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>Liver TGFβ1 (ng/mg protein)</td>
<td>0.53±0.2</td>
<td>0.67±0.11</td>
<td>0.66±0.14</td>
<td>0.40±0.01</td>
</tr>
<tr>
<td><strong>Serum characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.5±1.6</td>
<td>9.5±0.5</td>
<td>8.2±0.9</td>
<td>6.4±1.2#</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.95±0.33</td>
<td>0.46±0.10**</td>
<td>0.59±0.23</td>
<td>0.49±0.18###</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>1.55±0.18</td>
<td>1.52±0.40</td>
<td>1.57±0.27</td>
<td>1.39±0.18</td>
</tr>
<tr>
<td>ALT (μkat/l)</td>
<td>0.81±0.08</td>
<td>0.73±0.06</td>
<td>0.58±0.11</td>
<td>0.88±0.12</td>
</tr>
<tr>
<td>AST (μkat/l)</td>
<td>1.69±0.30</td>
<td>1.83±0.48</td>
<td>2.22±0.78</td>
<td>2.27±0.91</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001 compared to ST-1 in corresponding group, #P<0.05, ##P<0.01, ###P<0.001 compared to Wistar 3 weeks ST-1 group, +P<0.05, ++P<0.01, +++P<0.001 compared to Wistar 6 weeks ST-1 group.
Rodent animal models of NAFLD are essential tools for studying the mechanisms of NAFLD development. Currently, there is no animal model of NAFLD reflecting fully the histological and multifactorial pathophysiological characteristics of human NASH (Nanji 2004, London and George 2007). The present study has compared the time of feeding and strain differences in medium and high-fat nutritional models of NAFLD. Our nutritional models did not reproduce the liver changes typical of NASH. Feeding Wistar or Sprague-Dawley strains with HFGD for 3 or even 6 weeks caused simple microvesicular or mixed steatosis without inflammatory reaction or fibrosis. In contrast, Lieber et al. (2004), who used the same type of high-fat diet for only 3 weeks in Sprague-Dawley rats, reproduced typical hepatic lesions of human NASH. We did not induce significant inflammation or fibrosis even when Sprague-Dawley rats were fed HFGD for 6 weeks. The absence of severe inflammation and fibrosis in our experiment is confirmed by only slightly increased levels of liver IL-6 and similar hepatic content of TGFβ1 within the groups. Severe liver steatosis accompanied by inflammation has been regularly found in MCDD (Romestaing et al. 2007) but not characteristically in high-fat diet models, where either simple microvesicular or macrovesicular steatosis has been present (Buettner et al. 2006, Romestaing et al. 2007). Nevertheless, MCDD, which is quite often used as a model for the study of NASH, does not follow the natural course of the disease. Steatohepatitis in MCDD is the consequence of the accumulation of TAG in the liver, reduced synthesis of glutathione and impaired liver formation of VLDL. Moreover, MCDD leads to severe weight loss and cachexia, which are not typical symptoms for human NASH (Romestaing et al. 2007).

Lieber et al. (2004) used MFGD (35 % kcal fat) diet as a standard diet. However, standard diets for laboratory rats, as recommended by the American Institute of Nutrition, normally contain only about 10 % of energy provided by fats (Reeves et al. 1993), while 30 to 35 % kcal fats is recommended for human beings. Feeding MFGD for 3 or 6 weeks induced significant microvesicular steatosis in Wistar rats but not in Sprague-Dawley rats. Wistar rats seem to be more sensitive to developing steatosis when consuming diets with a higher fat content in comparison with Sprague-Dawley rats. Our findings of the inability of MFGD to induce fatty liver changes in Sprague-Dawley rats are consistent with findings of other studies (Lieber et al. 2004, Ahmed et al. 2009).

Although Sprague-Dawley rats fed with MFGD and HFGD for 3 weeks and HFGD for 6 weeks had significantly higher food intake in comparison with the ST-1 group, we did not observe increase in final body weight. This could be related to the ability in particularly young rats to increase energy expenditure through increased production of heat (Berry et al. 1985). UCP-2 is a protein located in the inner mitochondrial membrane. UCP-2 causes the uncoupling of substrate oxidation in mitochondria from ATP formation. This is done by dissipating proton gradients and converting fuel to heat. Increased heat production occurs also in brown adipose tissue and skeletal muscles, where raised UCP-1 and UCP-3 expression and activation can be found after intake of foods rich in fats (Turner et al. 2007). The liver

Fig. 1. Effect of feeding with MFGD and HFGD on the activity of respiratory complexes I and II. ST-1 – standard diet, MFGD – medium-fat gelled diet, HFGD – high-fat gelled diet. * p<0.05 vs. ST-1 in Sprague-Dawley rats fed for 3 weeks.

Fig. 2. Effect of feeding with MFGD and HFGD on the expression of UCP-2 mRNA. S-D Sprague-Dawley rats. UCP-2 – uncoupling protein-2, ST-1 – standard diet, MFGD – medium-fat gelled diet, HFGD – high-fat gelled diet. *, **, and *** p<0.05; 0.01, and 0.001, resp. vs. ST-1 in appropriate group.
is the main organ involved in fatty acid metabolism, and fatty acids are implicated in the regulation of gene transcription in the liver (Pégorier et al. 2004). Saturated and monounsaturated fatty acids induce expression of lipogenetic genes in the liver, while the effect on the expression of genes involved in fat oxidation is various (Buettner et al. 2006). A polyunsaturated fatty acid-rich (PUFA) diet increases transcription of genes related to lipid oxidation (Buettner et al. 2006). PUFA activates fatty acid oxidation via the PPARα (peroxisome proliferator-activated receptor α) pathway more strongly than saturated or monounsaturated fatty acids (Duplus et al. 2000). UCP-2 expression in the liver is also regulated via PPARα, which is implicated as a regulator of lipid metabolism and energy homeostasis.

The decrease in serum TAG is not typical for patients suffering from NAFLD or in other nutritional animal models of NAFLD (Buettner et al. 2006), where serum neutral lipids are usually elevated. In explanation of lower serum TAG levels in MFGD and HFGD groups, we should take into account that samples were taken after 14 hours of starvation. Decrease in serum TAG can be caused principally by blockage of VLDL (very low density lipoprotein) secretion in the liver, the main lipoprotein transporting TAG in the plasma of fasting animals, and by elevated clearance of TAG by peripheral tissues. Secretion of VLDL in the liver is dependent on production of Apolipoprotein B-100 (Apo B-100) and availability of microsomal triglyceride transfer protein (MPT). Feeding with diet containing higher amount of fat leads to the development of insulin resistance. Changes in insulin sensitivity of peripheral tissues (skeletal muscles) and plasma hyperinsulinemia precede manifestation of other aspects of metabolic syndrome (Barnard et al. 1998). Acute hyperinsulinemia attenuates Apo B-100 synthesis in the liver (Cummings et al. 1995). MTP catalyzes the transfer of TAG to Apo B-100 and thus has a pivotal role in the assembly of VLDL. Insulin is known to inhibit MTP expression in the liver (Allister et al. 2005). High-fat feeding of rats is associated with metabolic adaptation of skeletal muscles for more efficient lipid uptake which leads to the accumulation of intramuscular lipids with a determinantal effect on insulin action and the development of peripheral insulin resistance (Hegarty et al. 2002). Nevertheless, further experiments are required to clarify the drop in serum TAG after MFGD and HFGD in rats. The activities of serum ALT and AST of rats fed MFGD or HFGD are within the physiological ranges which are commonly found in patients suffering from liver steatosis.

Higher levels of oxidative stress in the liver are found in animal models of NAFLD (Lieber et al. 2004, Nanji 2004). Oxidative stress is a result of increased hepatic fat load, decreased content of antioxidants, reduced activities of enzymes involved in the metabolism of reactive oxygen species (ROS), raised proinflammatory cytokines, activation of Kupffer cells, and mitochondrial dysfunction etc (Malaguarnera et al. 2009).

Redox changes in the fatty liver can be monitored by reduced glutathione content, or by changes in the ratio of GSH and GSSG. Intracellular glutathione seems to play an important role not only in scavenging ROS, but also in regulating gene expression (Franco et al. 2007) and in fibrogenic processes (Zheng et al. 2007). We found a decrease in liver tissue GSH levels in HFGD after 3 weeks in both strains, but these temporary changes were not observed after 6 weeks. This could be a consequence of metabolic adaptations of the liver to longer feeding with excessive fat. Lipid peroxidation, evaluated in our experiment by the hepatic content of MDA, can directly damage hepatocytes as well as increase

### Table 3. Average histological grades of steatosis, inflammatory infiltrate, and necrosis of hepatocytes. ST-1 – standard diet, MFGD – medium-fat gelled diet, HFGD – high-fat gelled diet.

<table>
<thead>
<tr>
<th></th>
<th>ST-1 MFGD HFGD</th>
<th>ST-1 MFGD HFGD</th>
<th>ST-1 MFGD HFGD</th>
<th>ST-1 MFGD HFGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>1.0 3.3±0.5***</td>
<td>3.3±0.8***</td>
<td>1.0±0</td>
<td>1.6±0.7xxx</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
</tbody>
</table>

*P<0.05, ***P<0.001 compared to ST-1 in appropriate group, xxx P<0.001 compared to Wistar 3 weeks MFGD group, & & P<0.01 compared to Wistar 6 weeks MFGD group.
hepatic inflammation (Lee et al. 2007) and can mediate fibrogenesis (George et al. 2003). Increased production of MDA does not correlate well with changes in liver glutathione. Changes in hepatic MDA correlate better with tissue TAG, cholesterol, and IL-6. Liver steatosis could affect lipid composition and fluidity of mitochondrial
membranes (Ghoshal and Farber 1993) and enhance generation of lipoperoxidation products. Extra loads of fatty acids also raise fatty acid oxidation by less efficient peroxisomal pathways (Akbiyik et al. 2004), which can further increase the oxidative stress in the liver.

Accumulating evidence indicates that hepatic mitochondrial dysfunction is crucial to the pathogenesis of NAFLD (Vendemiale et al. 2001). ROS may be produced by electron leakage through complex I-III (Sato 2007). Free radicals mediate mitochondrial damage and mitochondrial dysfunction leads to higher production of free radicals (Feillet-Coudray et al. 2009). ROS can cause oxidative lesions in mtDNA (mitochondrial DNA) and may deplete mtDNA (Gao et al. 2004) which may decrease the synthesis of mtDNA-encoded respiratory chain polypeptides of complexes I, III, IV, and V, thus further blocking electron flow in the respiratory chain, and further increasing mitochondrial ROS formation (Pessayre 2007).

We observed a significant decrease in the activity of respiratory complex I in Sprague-Dawley rats fed HFGD for 3 weeks. Similar results of reduced activity of complex I were found in ob/ob mice (Garcia-Ruiz et al. 2006). More pronounced changes in respiratory complex I rather than in complex II in NAFLD can be explained by the higher sensitivity of complex I to oxidative stress (Drahota et al. 2005) and by depletion of mtDNA. Impairment of the F0F1-ATP synthase complex found in steatotic livers can lead to reduced ATP levels and can be related to the oxidative stress occurring in the mitochondria isolated from fatty liver (Vendemiale et al. 2001). Increased production of ROS by mitochondria depletes hepatic GSH. We discovered lowering of liver GSH after 3 weeks of feeding with diet containing higher amount of fat, but not after 6 weeks. Changes in GSH correlate well with alterations of expression of UCP-2. The up-regulation of UCP-2 is thought to act as a protective mechanism against elevated mitochondrial formation of ROS (i.e. superoxide anion radical) by diminishing the redox pressure on the mitochondrial electron transport, but exposes the liver to chronic depletion of ATP (Serviddio et al. 2008) by decreasing the efficiency of energy synthesis. Moreover, mild increase in formation of lipoperoxidation products may induce mitochondrial uncoupling and thus prevent further lipid peroxidation (Echtay et al. 2003).

In conclusion, the present study showed that male Wistar and Sprague-Dawley rats developed comparable steatosis without signs of inflammation, necrosis, or fibrosis after 3 or 6 weeks of HFGD feeding. Other markers evaluated, such as liver tissue content of TAG, cholesterol, reduced glutathione, IL-6, and TGFβ1, were also similar in both strains. These rat nutritional models of NAFLD did not reproduce the typical liver lesions of human NASH in the Wistar or Sprague-Dawley strain of rats. The absence of progression of simple steatosis to NASH and the lacking of multifactorial etiology are the main disadvantages of most animal models of NAFLD. Spontaneous development of steatohepatitis does not occur in nutritional and metabolic models of steatosis (Farrel 2005). Nevertheless; animal models of NAFLD are useful for understanding the etiology, pathophysiology, and natural course of the disease as well as for assessing therapeutical approaches to treating human non-alcoholic fatty liver disease.

Conflict of Interest
There is no conflict of interest.

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Abreviations
References


