Functional recovery after liver resection
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chapter 3

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Arlène van Vliet
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Essential pathogenic and metabolic differences in steatosis induced by choline or methione-choline deficient diets in a rat model
ABSTRACT

Background: Choline deficient (CD) and methione-choline deficient (MCD) diets are rodent models for steatosis, with potentially dissimilar biochemical backgrounds. The aim of this study was to assess the metabolic and pathological derangements in rats fed CD and MCD diets.

Methods: Male Wistar rats received CD or MCD diet up to 7 weeks. Nutritional status, liver histopathology, Kupffer cell-mediated inflammation and injury, oxidative stress via thiobarbituric reactive species (TBARS), hepatic and plasma glutathione (GSH) and insulin homeostasis were assessed.

Results: In CD-fed rats, mainly microvesicular steatosis developed with occasional inflammatory cells. In MCD-fed rats, macrovesicular steatosis progressed to steatohepatitis (collagen deposition, activated stellate cells). Hepatic TBARS was increased and GSH decreased in the MCD-fed rats compared to no changes in the CD-fed rats. The CD-fed rats developed obesity, dyslipidemia and insulin resistance, in contrast to undetectable plasma lipids, unaffected insulin homeostasis and loss of body weight in the MCD-fed rats.

Conclusions: The CD diet induced uncomplicated steatosis as compared to progressive inflammation and fibrinogenesis in the MCD diet. CD and MCD diets represent two pathogenically different models of steatosis. Although equivalence for the outcome of both diets can be found in clinical steatosis, the results of models using these diets should be compared with caution.
Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder in the world, with a prevalence of approximately 20% in the general population and up to 95% among those with obesity.1–3 NAFLD represents a broad spectrum of liver disease ranging from mild steatosis to steatohepatitis featuring severe steatosis (>60% hepatocytes affected), hepatocellular injury, progressive chronic inflammation and fibrosis.3,4 Until recently, NAFLD was identified as a mild and reversible disorder without impact on liver surgery.5 However, several clinical studies have shown the significance of steatosis as patients having any degree of steatosis have an increased risk of postoperative mortality and morbidity after liver resection or liver transplantation.6,7 Animal models have greatly contributed to the understanding of mechanisms behind the increased susceptibility of steatotic livers to injury. However, the large variety of experimental models of steatosis is confusing and potentially obscures the comparison and interpretation of results. The most commonly used models are based on nutritional changes or on leptin deficiency (Zucker rats, ob/ob mice).8 However, in leptin-deficient rodents, the leptin deficiency and not steatosis per se leads to impaired hepatic regeneration after injury or resection.9,10 Therefore, the use of leptin-deficient rodents in surgical models relating to liver regeneration is questionable.

Nutritional models based on choline deficiency (CD) or methionine and choline deficiency (MCD) have been widely applied in surgical models of organ preservation,11 ischemia-reperfusion injury12–14 and liver regeneration.15,16 Existing data show that the impaired formation of very-low-density-lipoproteins (VLDL) contributes to the development of steatosis in MCD diet.17 However, choline can be biosynthesized via methionine and choline deficiency alone does not seem to impair the VLDL excretion.18 Therefore, the clinically relevant pathological features such as increased oxidative stress and the Kupffer cell-mediated inflammatory response contributing to the development of steatohepatitis, as described with the MCD diet, are not necessarily similarly induced by the CD diet.19 Furthermore, overall metabolic derangements induced by both diets are not well described in the light of new insights into NAFLD pathogenesis such as altered insulin homeostasis, a potential pathogenic feature of NAFLD.20–22

Our hypothesis is that the steatosis induced with the CD diet and that with the MCD diet are distinct in respect to clinically relevant pathological and metabolic derangements such as hepatic steatosis, oxidative stress, Kupffer cell-mediated hepatocellular injury and inflammatory response, insulin homeostasis, antioxidant capacity and the development of steatohepatitis. The aim of this study, therefore, was to assess these metabolic and pathological derangements in rats fed with these different diets.

Methods

Diet and animals

Male Wistar rats (250–300 g) (Harlan CPB, Zeist, the Netherlands) were acclimatized to laboratory conditions for a minimum of 7 days, maintained at constant 24°C with 12 h light-
dark cycle and fed a standard rodent chow (Hope Farms, Woerden, the Netherlands) with water ad libitum. After acclimatization, the rats were randomized into three experimental groups ($n = 4-6$ per time point/group) and fed with either choline deficient (CD) diet or methionine and choline deficient (MCD) diet (Harlan Teklad, Madison, WI, USA) up to 7 weeks ad libitum. A control group received isocaloric chow, containing adequate levels of choline and methionine, ad libitum (Harlan Teklad).

The daily food consumption and body weight were measured and after the designated period rats were killed. Blood was collected by vena cava puncture, centrifuged (10 min, 3000 r.p.m., 4°C) and plasma was stored at −80°C. Livers were removed, weighed and thin slices of all liver lobes were immersed in 10% formalin for light microscopy (HE and Sirius red staining) or frozen immediately in liquid nitrogen for immunohistochemistry or Oil Red O staining.

During all procedures the animals were treated according to the guidelines of the Dutch legislation and international standards for animal care and handling. The protocol was approved by the Animal Ethics Committee of the University of Amsterdam, the Netherlands.

**Biochemical analysis of plasma**

Plasma samples were analyzed in the Department of Clinical Chemistry using standard laboratory methods for alanine aminotransferase (ALT), total bilirubin (T-Bil), albumin, triglyceride, cholesterol and glucose.

**Assessment of hepatic lipid concentration**

Liver samples were homogenized in phosphate-buffered saline (pH 7.2) and centrifuged (4000 g, 10 min, 4°C). Hepatic lipids were extracted by the chloroform:methanol extraction method according to Folch et al. and measured enzymatically using commercial kits according to manufacturer's instructions (Trig/GB, Roche, Switzerland; Cholesterol Biomerieux, Boxtel, Netherlands). The protein concentration in the homogenates was measured with BCA Protein Assay kit (Pierce, Rockford, IL, USA) and expressed in mmol/mg protein.

**Assessment of oxidative stress and antioxidant response**

Hepatic and systemic oxidative stress was assessed by measuring lipid peroxidation as determined by malondialdehyde and measured as total thiobarbituric acid reactive substances (TBARS) according to Asakawa and Matsushita. Assays were performed in the presence of the antioxidant butylated hydrotoluene limiting the generation of new TBARS during the assay. Antioxidant response was assessed by plasma and hepatic total glutathione (GSH) concentrations. Liver samples were homogenized in meta-phosphoric buffer (pH 6.0), centrifuged (4000 g, 10 min, 4°C) and analyzed for GSH as previously described and expressed in nmol/mg protein.
Assessment of insulin homeostasis

Fasting plasma insulin levels were measured with enzyme-linked immunosorbent assay (Rat Insulin ELISA, Linco, St Charles, MO, USA). All samples were measured in duplicate in a 96-well microtiter plate and the concentrations were calculated from a standard curve. For assessment of insulin secretion and sensitivity, homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) were used, which correlate very well with the gold standard of the hyperglycemic clamp, the intravenous glucose tolerance test and the oral glucose tolerance test. The calculations were made as follows: HOMA-R = fasting insulin (IU/mL) x fasting glucose (mmol/L)/22.5 and QUICKI = 1/[log (fasting insulin) + log (fasting glucose)].

Proinflammatory cytokine response

Kupffer cell activation was assessed by plasma and hepatic tumor necrosis factor (TNF)-α content using an ELISA (Duoset Rat TNF-α, RnD Systems, Abingdon, UK) according to the manufacturer’s instructions. Liver samples were homogenized in buffer (NaPi 5 mmol/L, pH 6.0), centrifuged (10,000 g, 4°C, 10 min), measured in duplicate in a 96-well microtiter plate. The concentrations were calculated from a standard curve and expressed in pg/mg protein.

Immunohistochemistry

Briefly, after formaldehyde fixation and paraffin-embedding, 4 μm sections were deparaffinized. For the detection of activated stellate cells, sections incubated with α-smooth muscle actin (α-SMA) antibody (dilution 1:200, 60 min, DAKO Cytomation, Copenhagen, Denmark) or ED-1 for both hepatic resident macrophages (Kupffer cells) and circulatory macrophages (1:100, 60 min; Southern Biotechnologies, Birmingham, AL, USA). After incubation with secondary antibody (GAM-IgG2A, 1:100, 60 min; Southern Biotechnologies), Fast DAB (3,3-diaminobezidine; Sigma, Munich, Germany) was used together with hematoxylin counterstaining. The number of positive cells was calculated in 30 high power fields (HPF) at magnification of ×100 and was expressed as positive cells/HPF.

Histopathology

Liver sections were stained with hematoxylin-eosin for routine morphology and with Sirius red for collagen staining (0.1% Fast red in picric acid; Immunotech, Mijdrecht, the Netherlands). Liver histopathology was examined by light microscopy in 30 HPF per sample with a magnification of ×40.

Two blinded investigators evaluated the slides on a semiquantitative basis as follows. For steatosis: grade 0, none; grade 1, <25%; grade 2, 26–50%; grade 3, 51–75%; grade 4, >75%. For inflammation: grade 0, none; grade 1, scattered inflammatory cells; grade 2, <5 foci of inflammatory cells per HPF; grade 3, >5 inflammatory foci per HPF. For fibrosis: grade 0, no fibrosis; grade 1, moderately thickened centrilobular vein (CLV); grade 2, markedly thickened CLV (annular appearance of the vein wall with numerous fibrous extensions between hepatocytes); grade 3, cirrhosis.

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Statistical analysis
Data analysis was performed with GraphPad Prism 3.02 for Windows (GraphPad Software, San Diego, CA, USA). The results are presented as means ± SEM. Differences between groups were tested using the unpaired Student’s t-test or ANOVA (with appropriate post hoc analysis) for multiple comparisons. P-values less than 0.05 were considered significant.

Results
General features
In all groups, physical activity was similar and appearance of animals remained healthy. The mean body weight of the MCD-fed rats was significantly less than that of CD-fed rats or controls. The liver-to-body weight ratio was increased in CD-fed rats at 1 and 3 weeks compared to the MCD-fed rats and controls (P < 0.05) but after 7 weeks, showed no differences in all groups (P = 0.054). During the MCD diet, the rats sustained significant weight loss in contrast to a significant weight gain in the CD-fed rats compared to controls (MCD 221 ± 11 g, CD 442 ± 13 g, controls 367 ± 6 g; P < 0.05). No changes in plasma albumin levels were observed in both groups, indicating unaffected protein synthesis (data not shown).

Induction of steatosis and steatohepatitis
In controls, no pathological changes were seen and the inflammation score was significantly lower at all time points compared to the CD- and MCD-fed rats (Table 1).

<table>
<thead>
<tr>
<th>Time</th>
<th>Steatosis score</th>
<th>Inflammation score</th>
</tr>
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<tbody>
<tr>
<td>1 week</td>
<td>0.03 ± 0.03</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td>3 weeks</td>
<td>0.3 ± 0.1</td>
<td>1.5 ± 0.09</td>
</tr>
<tr>
<td>5 weeks</td>
<td>0.5 ± 0.1</td>
<td>1.8 ± 0.07</td>
</tr>
<tr>
<td>7 weeks</td>
<td>0.6 ± 0.04</td>
<td>2.1 ± 0.04</td>
</tr>
<tr>
<td>Control</td>
<td>0.01 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>

In the CD-fed rats, an increase of microvesicular steatosis with few inflammatory cells was observed at 1 and 3 weeks (P < 0.05; Fig. 1a). After 5 weeks, the CD-fed rats showed a microvesicular steatosis with few macrovesicular hepatocytes and inflammatory foci (P < 0.05). At 7 weeks in the CD-fed rats, only a slight increase in macrovesicular steatosis was detected with a slight increase in inflammatory cells (Fig. 1c).

After 1 week of diet intake, the MCD-fed rats initially developed mainly microvesicular steatosis, with a macrovesicular component, predominantly in the acinar zone 3 (Fig. 1b). At 3 weeks, macrovesicular steatosis was the main feature of steatosis showing scattered foci of inflammatory cells. After 5 weeks of diet intake, the MCD-fed rats showed a diffuse and extensive macrovesicular steatosis with increased numbers of mononuclear inflammatory cell foci and with occasional spotty necrosis. At 7 weeks, extensive
macroversicular steatosis throughout the section was observed. Steatosis was most evident in centrilobular areas together with accumulation of cellular debris and numerous clusters of inflammatory cells ($P < 0.03$; Fig. 1d)

Furthermore, in the MCD-fed rats, Sirius red staining showed increased perivenular fibrosis (Fig. 1e) with occasional centrilobular fibrosis (Fig. 1f). Activated stellate cells were detected by $\alpha$-SMA immunohistochemistry in the portal areas and occasionally also in the parenchyma (data not shown).

Figure 1 Representative histology of liver tissue (original magnification $\times 20$). HE-stained liver tissue of choline deficient (CD) diet-fed rat at (a) 1 week and (c) 7 weeks shows mainly microvesicular steatosis without prominent inflammation. HE-stained liver tissue of methionine-choline deficient (MCD) diet-fed rat at (b) 1 week and (d) 7 weeks shows many inflammatory foci. Sirius red stained liver tissue of (e) CD-fed and (f) MCD-fed rats at 7 weeks shows increased collagen deposition in the MCD-fed rats (arrows).
**Biochemical parameters**

Plasma ALT was slightly increased in the CD-fed rats at 3 and 5 weeks but no difference was seen at 7 weeks compared to controls. However, in the MCD-fed rats ALT increased 10-fold after 5 weeks ($P<0.05$; Fig. 2). Plasma bilirubin increased in the MCD-fed rats after only 1 week being fourfold higher after 7 weeks, in contrast to the CD-fed rats in which a twofold increase was seen only after 7 weeks ($P<0.05$, data not shown).

![Figure 2: Effect of choline deficient (CD) and methione-choline deficient (MCD) diets on hepatocytes. Hepatocellular damage assessed by plasma ALT was more prominent in the MCD-fed rats. Values are expressed as mean ± SEM. *$P<0.05$ compared to controls (CO); **$P<0.05$ compared to the other diet (ANOVA).](image)

Figure 3  Effect of choline deficient (CD) and methione-choline deficient (MCD) diets on (a) plasma triglycerides, (b) cholesterol, (c) hepatic tissue triglycerides and (d) cholesterol. In the CD-fed rats, a significant increase in hepatic triglycerides was observed in contrast to undetectable plasma triglyceride and cholesterol levels at 7 weeks in the MCD-fed rats. Values are expressed as mean ± SEM. *$P<0.05$ compared to controls (CO); **$P<0.05$ compared to the other diet (ANOVA).
In the CD-fed rats plasma triglyceride and cholesterol levels increased, whereas in MCD-fed rats the levels decreased below the detection limit (0.1 mg/dL) (Fig. 3a,b). Hepatic triglyceride content was increased in the MCD-fed rats at all time points compared to the controls and to the CD-fed rats, corresponding with the histopathological changes ($P < 0.01$; Fig. 3c). Hepatic cholesterol content was increased in the CD and MCD-fed rats at all time points compared to controls and was higher in the MCD-fed rats at 1 and 3 weeks compared to the CD-fed rats ($P < 0.01$; Fig. 3d).

**Oxidative stress and antioxidant response**

Hepatic TBARS was at all time points increased in the MCD-fed and the CD-fed rats as compared to controls and was significantly higher in the MCD-fed rats ($P < 0.01$, Fig. 4a). The plasma and hepatic glutathione levels were reduced in the MCD-fed rats ($P < 0.05$; Fig. 4b,c) whereas no significant changes were observed in rats having the CD diet, although there was a tendency towards increased levels at 3 and 5 weeks.

**Kupffer cell activation**

After 1 week, a twofold increase in hepatic TNF-$\alpha$ was seen in both the CD- and MCD-fed rats compared to controls ($P < 0.05$; Fig. 5). After 7 weeks, TNF-$\alpha$ levels had increased 10-fold.

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**Figure 4** Effect of choline deficient (CD) and methionine-choline deficient (MCD) diets on (a) activation of hepatic lipid peroxidation as measured by free TBARS in liver, and antioxidant response in (b) plasma and (c) liver as measured by total GSH. Hepatic TBARS was increased and both plasma and hepatic GSH decreased in the MCD-fed rats. Values are expressed as mean ± SEM. *$P < 0.05$ compared to controls (CO); **$P < 0.05$ compared to the other diet (ANOVA).
Figure 5 Effect of choline deficient (CD) and methionine-choline deficient (MCD) diets on hepatic Kupffer cell activation measured by tissue TNF-α levels. A significant and progressive increase was observed in the MCD-fed rats as compared to only a slight increase in the CD-fed rats. Values are expressed as mean ± SEM. *P < 0.05 compared to controls (CO); **P < 0.05 compared to the other diet (ANOVA).

Fold in the MCD-fed rats but stayed at the same level in the CD-fed rats throughout the whole diet period. In the MCD-fed rats, the levels were significantly higher after 5 and 7 weeks compared to the CD-fed rats (P < 0.05).

The increased hepatic TNF-α in the MCD-fed rats corresponded with an increased number of ED-1 positive macrophages in liver parenchyma compared to no differences between the CD-fed rats and controls (data not shown). Plasma TNF-α was undetectable in controls (detection level 5 pg/mL) but was increased 10-fold in both the CD and the MCD-fed rats after 1 week compared to controls and was highest after 5 and 7 weeks in the MCD-fed rats (P < 0.05) (data not shown).

Assessment of insulin homeostasis

HOMA-IR and QUICKI did not significantly differ from the controls in the MCD-fed rats at all time points, whereas in the CD-fed rats, significant increases in both indexes were observed after only 1 week (P < 0.05; Fig. 6).

Figure 6 Effect of choline deficient (CD) and methionine-choline deficient (MCD) diets on insulin homeostasis as assessed by (a) HOMA-IR and (b) QUICKI. In the CD-fed rats, QUICKI and HOMA showed significantly disrupted insulin homeostasis. No changes were detected in MCD-fed rats. Values are expressed as mean ± SEM. *P < 0.05 compared to controls (CO); **P < 0.05 compared to the other diet (ANOVA).

Discussion

The present study is the first to report essential differences in steatosis induced by CD and MCD diets in a rat model. The induction of hepatic steatosis, oxidative stress, insulin resistance, cytokine mediated hepatocellular damage and progression to steatohepatitis differed significantly between animals fed with one of the two diets. The MCD-fed rats...
developed the highest degree of steatosis corresponding with increased oxidative stress, decreased antioxidant response and progression to steatohepatitis. Consistent with previous studies, a significant weight loss was seen in the MCD-fed rats. In contrast, the CD-fed rats developed steatotic features similar to the human metabolic syndrome characterized by insulin resistance, dyslipidemia and obesity.

The proposed biochemical basis of fatty accumulation in choline and/or methione deficiency is impaired phosphatidyl choline (PC) synthesis, which is essential for hepatic VLDL secretion.\textsuperscript{30} PC synthesis occurs via two pathways: through direct incorporation of preformed choline into phosphatidyl compounds (CDP pathway) or through stepwise methylation of adenosyl methione.\textsuperscript{31} Interestingly, a recent study reported unimpaired PC synthesis via the CDP pathway in choline-deficient mice.\textsuperscript{32} The authors suggest that this is due to compensatory activation of the methylation pathway to maintain the PC synthesis and VLDL excretion, corresponding with the observed increased plasma lipids in the CD-fed rats. The exact mechanism of hepatic lipid accumulation in the CD-fed remains unclear.

In the MCD-fed rats, increased oxidative stress and a decreased antioxidant response were observed in the presence of steatohepatitis (activation of hepatic stellate cells and collagen deposition) but these features were significantly less induced in the CD-fed rats. Oxidative stress and lipid peroxidation are generally considered as the most pathogenic mechanisms of nonalcoholic steatohepatitis (NASH).\textsuperscript{33} For prevention of oxidative stress, a balance between reactive oxygen species (ROS) production and antioxidant response is essential. The important sources for oxidative stress are intracellular fatty acids, via direct toxicity or via induction of cytochrome p-450 microsomal lipoxygenase 2E1 (CYP2E1) and peroxosomal beta-oxidation.\textsuperscript{34,35} CYP2E1 and beta-oxidation generate ROS promoting cell membrane damage via malondialdehyde, the end-product of lipid peroxidation. Malondialdehyde causes further structural changes in the mitochondrial matrix leading to mitochondrial dysfunction.\textsuperscript{36,37} These further activate the proinflammatory cytokine release of Kupffer cells and stellate cells leading to necroinflammatory changes in hepatic parenchyma. Indeed, in the MCD-fed rats the observed increased lipid peroxidation corresponded with marked hepatocellular damage and increased hepatic TNF-\(\alpha\). In the CD-fed rats, the antioxidant scavenging of ROS was apparently sufficient as less lipid peroxidation and hepatocellular damage was observed.

Interestingly, another important pathogenic feature proposed to play a role in progression of NASH, insulin resistance, was observed in the CD-fed rats. This insulin resistance is probably due to a combination of factors determining hepatocyte sensitivity to insulin such as increased TNF-\(\alpha\) and hypertriglyceridemia. In the CD-fed rats, no significant inflammation was found but another source for TNF-\(\alpha\) in the absence of inflammation, namely adipose tissue, was present. TNF-\(\alpha\) reduces the expression of Glut4, an insulin-dependent glucose transporter and decreases the phosphorylation of insulin-receptor substrate-1 (IRS-1).\textsuperscript{39} Also hypertriglyceridemia potentially contributed to the insulin resistance in the CD-fed rats as triglycerides directly inhibit peripheral glucose uptake via down-regulation of IRS-1.\textsuperscript{22}

It has been hypothesized that, in the absence of mitochondrial dysfunction, peripheral insulin resistance will not lead to the development of NASH but only to uncomplicated...
steatosis. Consistent with this hypothesis, less lipid peroxidation, also indirectly reflecting mitochondrial damage, was detected in the CD-fed rats with insulin resistance. Opposite to this hypothesis, no insulin resistance was present in the MCD-fed rats, as has also been previously reported, even though extensive lipid peroxidation and fibrinogenesis was observed.\textsuperscript{40} It seems that insulin resistance in this model does not play a crucial role in the development of NASH. However, additional studies are needed to clarify this aspect.

The profound differences in metabolic derangements must have significant impact when these diets are applied in surgical models of steatosis as used in ischemia-reperfusion and partial hepatectomy studies. Impaired glucose metabolism alters liver metabolism and affects the immune system and is also considered to be an independent risk factor for acute liver failure after extended liver resection.\textsuperscript{41,42} Furthermore, oxidative stress and lipid peroxidation (important pathogenic features of human NAFLD) are already present in MCD-fed rats several weeks before necroinflammatory changes are seen at histopathological examination. In contrast, CD-fed rats do not develop significant inflammation or fibrosis nor show mitochondrial changes. This might lead to underestimation of the actual implications of steatosis particularly in situations when unaltered mitochondrial function is crucial such as for hepatocyte proliferation.

In conclusion, this study shows that steatosis induced by the CD or MCD diets differ profoundly in features clinically relevant to human NAFLD and NASH. The CD diet induced uncomplicated steatosis with features of the metabolic syndrome in contrast to the MCD diet with induction of progressive Kupffer cell-mediated inflammation and fibrinogenesis. Although CD and MCD diets represent two pathogenically different models of steatosis, equivalence with clinical steatosis can be found for the outcome of both diets. However, the results of studies applying these diets in rats should be compared with caution.

Acknowledgment
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