Sex Differences in Choline-Deficient Diet-Induced Steatohepatitis in Mice

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Alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH) are common liver diseases in the United States. ASH and NASH occur more frequently in women than in men, and liver injury is also more severe in women. The role of estrogens in ASH has been well established, but their role in NASH has received relatively little study. The purpose of this study was to evaluate the effect of estrogens in methionine-choline deficient diet (MCDD)-induced steatohepatitis in mice. The degree of steatohepatitis was evaluated in males and in intact and ovariectomized females that were fed MCDD for 4 weeks, and in females that were fed MCDD containing tamoxifen. Hematoxylin and eosin-stained sections of livers showed marked steatohepatitis in all experimental groups. Compared to the control group, markers of hepatocyte injury such as aspartate transaminase (AST), alanine transaminase (ALT), and liver triglyceride levels increased significantly in males and in intact and ovariectomized female mice that were fed MCDD. Also, it was interesting that levels of AST and ALT increased much more in the MCDD + tamoxifen group than in the MCDD group. In female mice fed MCDD, hepatocyte proliferative and apoptotic indices increased slightly compared to mice that were fed a normal diet. Based on these results, it can be concluded that MCDD-induced steatohepatitis is comparable in male and female mice, and that ovariectomy or antiestrogen treatment had no protective effect in MCDD-induced steatohepatitis. Exp Biol Med 229:158–162, 2004

Key words: steatohepatitis; methionine- and choline-deficient diet; ovariectomy, apoptosis; bromodeoxyuridine

Macrovesicular fatty change (steatosis) is the most frequently encountered liver lesion in Western society. It is caused by a variety of conditions and is characterized by an increase in fat content. The most common causes of steatosis are alcohol, obesity, and diabetes (1). Steatosis is relatively benign and is easily reversible. However, with a secondary cellular stress (e.g., oxidative stress, endotoxin-mediated cytokine release), steatosis can progress to steatohepatitis, which is characterized by inflammation, necrosis, and fibrosis, a chronic liver disease (2, 3). Morphological features of steatohepatitis, whether they are caused by alcoholic steatohepatitis (ASH) or obesity and diabetes (nonalcoholic steatohepatitis; NASH), are similar and probably related to common pathogenetic mechanisms (4–6). In both ASH and NASH, hepatic metabolism of fats is altered through an imbalance in triglyceride synthesis and secretion and decreased fatty acid oxidation, resulting in fat accumulation (7, 8).

The severity of ASH and NASH appears to be more pronounced in women than in men, and the underlying mechanisms involved in the gender difference are not clear (9, 10). It has been suggested that estrogens that play an important role in lipid metabolism and inflammatory process may contribute to increased sensitivity in women (11, 12). Estrogens are shown to inhibit oxidation of fatty acids and promote synthesis of triglycerides in the liver (13). Furthermore, inhibition of mitochondrial β-oxidation of fatty acids by estrogens has been proposed as an important cause of acute fatty liver of pregnancy (14). In experimental animals, it has been shown that alcohol-induced liver injury was reduced in those with ovariectomy (15). The role of estrogens in ASH is further confirmed in experiments using toremifene, a potent antiestrogen (16). Concomitant administration of ethanol and toremifene resulted in a significant reduction of necro-inflammatory changes. The role of estrogens in pathogenesis of NASH or protective effect of antiestrogens in NASH has not been fully evaluated. In this study, we compared the morphological and biochemical changes in the liver induced by a methionine and choline-deficient diet (MCDD) in male and female mice, and examined the effects of ovariectomy and tamoxifen on steatohepatitis induced by MCDD in female mice. We elected to use tamoxifen because it is the most commonly used antiestrogen drug in the treatment of breast cancer.

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Table 1. Comparison of Body Weights, Liver Weights, Serum Aminotransferases, and Liver Triacylglycerols in Male and Female Mice Fed Methionine-Choline Deficient Diet or Control Diet[a]

Group[a]	Body wt (g)	Liver wt (g/100 g body wt)	AST (U/L)	ALT (U/L)	Liver TG (mg/g of liver)
F+NC	19.2 ± 0.6	5.1 ± 0.1	73 ± 5	48 ± 3	20 ± 2
F+MCDD	12.2 ± 0.1[c]	4.8 ± 0.1	290 ± 40[c]	344 ± 60[c]	402 ± 15[c]
OV+MCDD	10.8 ± 0.2[c]	4.3 ± 0.1	291 ± 39[c]	448 ± 37[c]	387 ± 28[c]
F+MCDD+T	11.5 ± 0.3[c]	3.7 ± 0.2[c]	696 ± 80[c]	644 ± 10[c]	305 ± 35[c]
F+NC+T	18.9 ± 0.7	4.7 ± 0.1	72 ± 5	47 ± 3	22 ± 4
M+MCDD	16.9 ± 0.2[c]	4.3 ± 0.5	466 ± 61[bc]	540 ± 80[c]	487 ± 28[c]
M+NC	28.0 ± 0.6	4.0 ± 0.2	148 ± 53	56 ± 6	17 ± 1

[a] Mean + SEM of 4–5 mice.
[b] Groups: F+NC, female mice on normal chow; F+MCDD, female mice on methionine-choline deficient diet; OV+MCDD, ovariectomized mice on methionine-choline deficient diet; F+MCDD+T, female mice on methionine-choline deficient diet + tamoxifen; F+NC+T, female mice on normal chow containing tamoxifen; M+MCDD, male mice on methionine-choline deficient diet; M + NC, male mice on normal chow. (When tamoxifen was used, it was added to the diet at 0.01% concentration.)
[c] $P < 0.01$ compared with experimental groups and corresponding controls in males and females.
AST, aspartate transaminase; ALT, alanine transaminase; TG, triglyceride; F, female mice; NC, normal chow; MCDD, methionine-choline deficient diet; OV, ovariectomy; T, tamoxifen; M, male mice.

A choline deficiency model was used because the changes induced in the liver are reproducible, rapid, and similar to those observed in NASH and ASH, which are characterized by steatosis and necro-inflammation (17). Further more, NASH that develops in patients receiving total parenteral nutrition has been attributed to choline deficiency (7, 10). In this study we demonstrate that MCDD-induced morphological and biochemical changes are identical in males and females, and that ovariectomy and administration of antiestrogen had no protective effect in females. A preliminary account of this work was published previously (18).

Materials and Methods

Animals. Six- to seven-week-old male and female C57BL/6J mice were purchased from Harlan Teklad (Madison, WI) and maintained in the Northwestern University Medical School animal facility in accordance with the U.S. Public Health Service *Guide on the Care and Use of Laboratory Animals* and the protocols approved by the Northwestern University Animal Care and Use Committee. Mice were housed in groups of three to five according to sex, in plastic cages, in a temperature and humidity controlled room with a 12:12-hour light:dark cycle.

Experimental Procedure. During the first week of acclimatization all mice were fed Purina chow and divided into different experimental groups as shown in Table 1. A group of five female mice were subjected to ovariectomy under light ether anesthesia using aseptic conditions through a mid-abdominal wall incision. Ovariectomized mice were fed experimental diet beginning 1 week after the operation. Mice in groups of four to five were fed either a control diet, MCDD (Dyets, Bethelhem, PA), MCDD supplemented with tamoxifen (0.01%; Aldrich Chemical Co., Milwaukee, WI), or normal chow supplemented with tamoxifen for 4 weeks. All animals had free access to food and water until the experiment was terminated. One hour before sacrifice, all female mice were given bromodeoxiuridine (BrdU) (Sigma Chemical Co., St Louis, MO), dissolved in water, intraperitoneally at a dose of 100 mg/kg body weight. Body and liver weights were immediately recorded, and blood was obtained from the inferior vena cava for estimation of serum aspartate transaminase (AST) and alanine transaminase (ALT) using standard automated procedures. Liver triglyceride levels were measured using the triglyceride (TG) E-test as described before (19). Portions of liver were processed for morphological studies and the remainder was snap-frozen in liquid nitrogen and stored at −80°C and used to measure TG.

Morphological Studies. Portions of liver from all of the mice were fixed in 10% neutral buffered formalin for 18 to 24 hours and processed for light microscopy. Five-micrometer-thick paraffin sections were routinely stained with hematoxylin and eosin to evaluate steatosis and steatohepatitis. Fatty change and necro-inflammatory changes were graded according to published criteria (20, 21). In addition, paraffin sections from female mice were stained for BrdU to evaluate cell proliferation as described before (22), and for apoptotic cells using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Labeling and apoptotic indexes were obtained by counting 1000 hepatocytes in each liver and were expressed as a number of labeled nuclei per 1000 cells.

Statistical Analysis. Differences between different groups in body weights, liver weights, serum enzymes, liver TG, proliferative index, and apoptotic index were determined using the Student's t test, and were considered significant when $P < 0.01$.

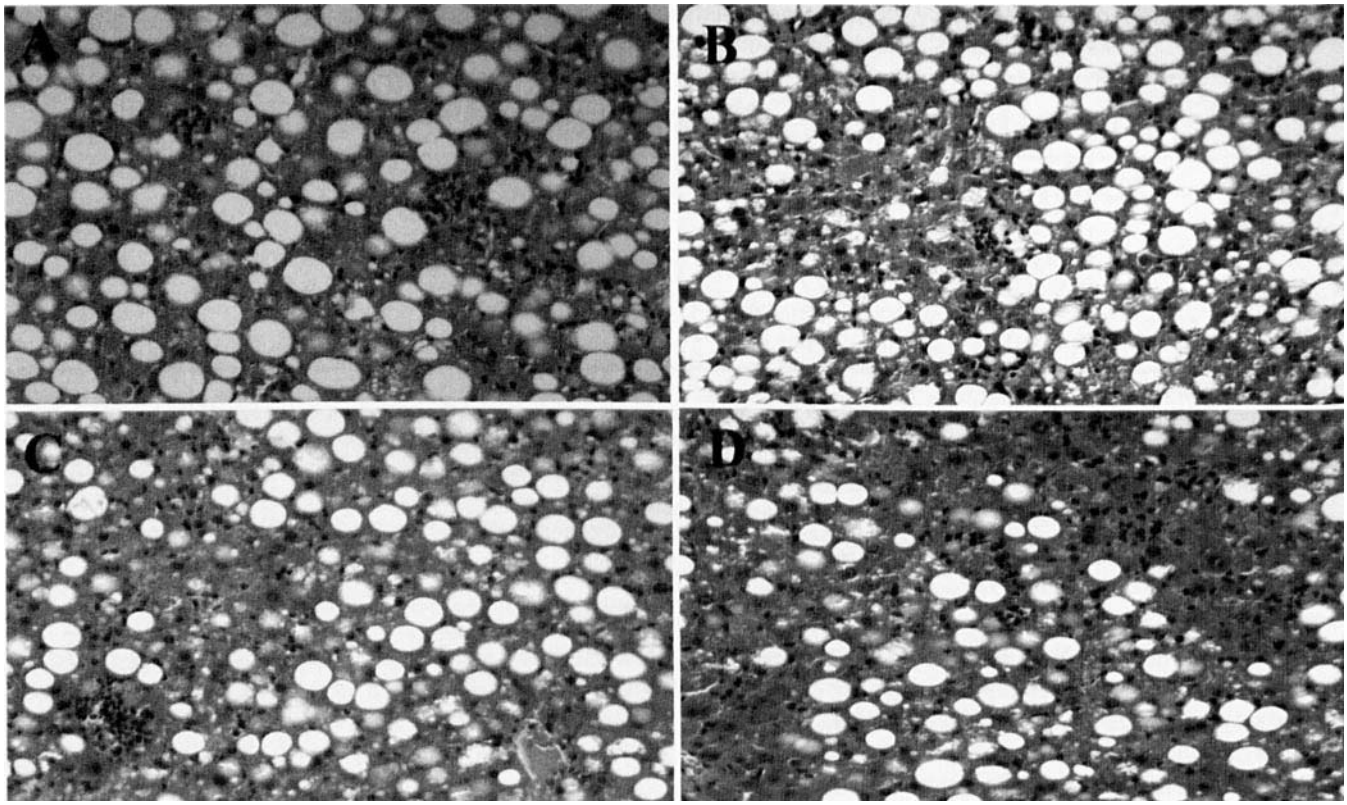

Figure 1. Hematoxylin and eosin–stained sections of liver showing marked steatohepatitis: (A) male mouse fed MCDD for 4 weeks, (B) female mouse fed MCDD for 4 weeks, (C) ovariectomized mouse fed MCDD for 4 weeks, and (D) female mouse fed MCDD containing 0.01% tamoxifen for 4 weeks (×200).

Results

General Features. Although the physical activity of mice in all groups was similar, the mean body weights of those that received MCDD were significantly less than those that were fed normal chow (Table 1). The liver weights relative to 100 g of body weight were comparable in all groups except for female mice in the MCDD + tamoxifen group, in which liver weights were significantly less than those of the control group maintained on normal chow ($P < 0.002$).

Liver Morphology. On gross examination, livers from mice fed MCDD with or without tamoxifen were enlarged, pale in color, soft, and greasy compared to livers from mice that were fed the control diet. Histological examination of livers from all MCDD groups, regardless of ovariectomy or tamoxifen treatment, showed marked steatohepatitis (Fig. 1). Fatty change was mostly a macrovesicular type, involving all zones of the lobule (grade 3 steatosis). Inflammatory infiltrate consisted of foci of acute and chronic inflammatory cells randomly distributed in the lobule. No ballooning change or fibrosis was present. Scattered apoptotic bodies were present in these livers. The results of BrdU labeling and apoptotic index in different groups in female mice are presented in Table 2. The BrdU labeling index of hepatocytes in MCDD group was significantly higher than in control mice (8.0 versus 2.3/1000 cells). The labeling indexes in ovariectomized + MCDD and MCDD + tamoxifen groups were, interestingly, slightly less than the MCDD group index. Apoptotic index was slightly higher in different groups that were fed MCDD, compared to control groups (not statistically significant).

Serum and Liver Biochemical Parameters. Levels of liver triglycerides and serum AST and ALT are presented in Table 1. Hepatic triglyceride levels were comparable in control male and female mice. In males and females of all groups maintained on MCDD, liver triglyceride levels increased significantly (28-fold in males and 14- to 20-fold in females), compared to those of mice that were fed normal chow, corroborating the histological findings. In mice that ate MCDD containing tamoxifen, hepatic triglyceride levels were slightly lower than in the mice that ate MCDD alone. Serum levels of AST and ALT, the markers of liver cell damage, increased significantly in all groups that received MCDD compared to those that ate normal chow. In males, AST levels increased 3-fold, and in females with and without ovariectomy, they increased by 4-fold. Interestingly, in mice that received MCDD + tamoxifen, AST levels increased 9.6-fold, although on histologic examination, the severity of steatohepatitis was comparable to that of MCDD group. Increases in ALT levels in groups that received MCDD ranged up to 7-fold to

Table 2. Proliferative and Apoptotic Index in Livers of Female Mice in Different Experimental Groups[a]

Group[b]	Proliferative index per 1000 cells	Apoptotic index per 1000 cells
F+NC	2.0 ± 0.3	1.0 ± 0.3
F+MCDD	8.0 ± 2.0[c]	2.0 ± 0.4
OV+MCDD	5.4 ± 0.7	2.0 ± 0.8
F+MCDD+T	3.2 ± 0.2	1.5 ± 0.6
F+NC+T	4.5 ± 1.0	0.7 ± 0.2

[a] MeanTSEM of 4–5 mice.
[b] Groups: F+NC, female mice on normal chow; F+MCDD, female mice on methionine-choline deficient diet; OV+MCDD, ovariectomized mice on methionine-choline deficient diet; F+MCDD+T, female mice on methionine-choline deficient diet + tamoxifen; F+NC+T, female mice on normal chow + tamoxifen.
[c] $P < 0.01$ compared with control group.
F, female mice; NC, normal chow; MCDD, methionine-choline deficient diet; OV, ovariectomy; T, tamoxifen.

9.6-fold in males and in females with and without ovariectomy, and to 13.7-fold in the MCDD + tamoxifen group.

Discussion

Increased macrovesicular-type fat in the liver is usually secondary to a combination of factors such as increased fatty acid availability, increased fatty acid synthesis, and conversion of fatty acids into TGs in the liver, and decreased export of TGs from the liver in the form of lipoproteins. It has been proposed that in choline deficiency, total protein synthesis and lipoprotein secretion are not affected, but secreted lipoproteins contain fewer TGs because of loss of function of newly synthesized phospholipids (23). It is generally believed that fatty change, irrespective of the cause, leads to lipid peroxidation (24). In both ASH and NASH there is induction of microsomal lipoxygenases that serve as a source of reactive oxygen radicals that cause lipid peroxidation (25, 26). End products of lipid peroxidation cause activation of proinflammatory cytokines and stellate cells, which lead to necro-inflammatory changes and fibrosis (27).

Deprivation of methionine and choline in mice resulted in marked steatohepatitis, characterized by panlobular steatosis, acute and chronic inflammatory infiltrate, scattered apoptosis, and increased mitosis. These findings are similar to those observed in rats treated with a choline-deficient diet (19, 28). The possible mechanism of choline deficient diet–induced steatohepatitis in rats and MCDD-induced steatohepatitis in mice is through the generation of oxygen free radicals and oxidative stress, a mechanism similar to that proposed in ASH and NASH in humans (26, 29–32). Because ASH and NASH are purported to be aggravated by estrogens, the present study was undertaken to investigate the sex differences in MCDD-induced steatohepatitis in mice and to evaluate the possible role of ovariectomy and antiestrogen treatment in MCDD-induced liver changes in female mice.

In both male and female mice that were fed MCDD, weight loss (37% less than controls) and microscopic changes induced in the liver (fatty change and necro-inflammation) were comparable. Morphological changes observed in the livers of MCDD-treated male and female mice are further corroborated by comparable serum ALT and AST and liver TG levels. These findings are in contrast to alcohol-induced steatohepatitis in male and female rats. Yin *et al.* (15) have shown that alcohol-induced steatohepatitis developed more rapidly and severely in females than in male rats.

In addition, to directly test the role of estrogens, we evaluated the effect of ovariectomy on MCDD-induced steatohepatitis. The degree of steatohepatitis (both fatty change and inflammation) and TG levels in the liver and serum AST and ALT levels in ovariectomized mice were comparable to those of mice with intact ovaries. It is interesting that, unlike in MCDD treated mice, ovariectomized rats treated with alcohol developed a lesser degree of steatohepatitis and that this protection was reversed by estrogen administration (15). However, the effect of antiestrogens on the liver is controversial. Tamoxifen, the commonly used drug in the treatment and prevention of breast cancer, is implicated in steatohepatitis and cirrhosis (33, 34), whereas toremifene, an antiestrogen that is more potent than tamoxifen, was shown to have partial protection against alcohol-induced steatohepatitis in rats (16). The results of our study showed that administration of tamoxifen alone had no effect on body weight, liver weight, or liver morphology, and that all these values were comparable to those of the control group. The only difference was a slight decrease in the hepatocyte apoptotic index in mice treated with tamoxifen (0.7 versus 1.0/1000, in tamoxifen and control groups, respectively). However, Carthew *et al.* (35) reported an increase in apoptosis in some strains of rats treated with tamoxifen for 3 months at a dose of 420 ppm. In mice that received tamoxifen concomitantly with MCDD, the degree of steatohepatitis was comparable to that of the group that received MCDD only. However, serum ALT and AST levels were significantly higher in the tamoxifen + MCDD group compared to the MCDD group, suggesting more liver cell damage. The apoptotic index was, interestingly, lower in the tamoxifen + MCDD group (1.5/1000 hepatocytes) than in the MCDD group (2.0/1000 hepatocytes). These findings clearly indicate that, unlike in humans, tamoxifen has neither a hepatotoxic nor a protective effect against MCDD-induced steatohepatitis in mice.

Unlike in MCDD-treated mice, in alcohol-treated animals, significant differences in steatohepatitis between males and females were reported (15). The differences between males and females in MCDD and alcohol models are probably due to differences in the mechanism of induction of liver injury. Alcohol-induced steatohepatitis is dependent on factors such as oxidative injury, through the

generation of reactive oxygen radicals and generation of cytokines (26, 36). In females, in addition to an increase in the ethanol-inducible form of hepatic P-450 enzymes, more endotoxin-induced cytotoxins from the estrogen effect may account for more liver injury. MCDD-induced steatohepatitis is mostly due to induction of microsomal lipo-oxygenases that generate free radicals, mitochondrial dysfunction, and a decrease in hepatic glutathione levels (17, 29, 31). It is possible, unlike in alcohol-treated animals, that MCDD may not cause an increase in gut endotoxin levels. Estrogens may influence the gut permeability to endotoxin, resulting in increased levels in the serum, leading to activation of Kupffer cells and cytokine production. This may explain the lack of differences in steatohepatitis in MCDD-treated male and female mice and ovariectomized mice. Further studies are required to confirm this hypothesis.

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