Sex hormone affects the severity of non-alcoholic steatohepatitis through the MyD88-dependent IL-6 signaling pathway

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Abstract

Recent research has shown that the occurrence of gender disparity in liver cancer associated with sex differences in MyD88-dependent IL-6 production, but the role of this signaling pathway in sex differences of non-alcoholic steatohepatitis (NASH) remains unknown. To investigate the effects of sex hormone-specific intervention on pathology and progression of NASH, and on the inflammatory TLR-MyD88-IL-6 signaling pathway NASH was modeled in C57/BL6 mice by feeding a methionine and choline-deficient (MCD) diet for 4 weeks. Male mice were subjected to sex hormone-related interventions such as orchidectomy, and orchidectomy combined with administration of either testosterone propionate or estradiol benzoate. Next, the degree of non-alcoholic fatty liver disease activity score (NAS), serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and the expression level of MyD88 and IL-6, were compared between these groups. Males developed more serious inflammatory problems and had a higher NAS than the females. Sex-specific intervention in male mice by orchidectomy reduced NAS, ALT, and AST, and the expression level of MyD88 and IL-6. But administration of exogenous androgen had no influence on either NAS or the expression of ALT, AST, MyD88, and IL-6. On the other hand, exogenous estrogen could alleviate the pathological damage caused by NASH, as well as reduce NAS, ALT and AST, and the expression of MyD88 and IL-6. The result show different sex hormone-related interventions affected the severity of NASH, possibly by modulating the level of sex hormones and regulating the TLR-MyD88-IL-6 signaling pathway.

Keywords: Non-alcoholic steatohepatitis, sex-associated differences, TLR-MyD88-IL-6 signaling pathway, sex hormone-specific intervention

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Introduction

Chronic liver diseases are caused by several factors, and have significant pathological differences between men and women.^{1–9} For example, the progression of chronic hepatitis type B is more rapid in men than in women. Also, men infected with the hepatitis B virus (HBV) are more likely to develop chronic infection and liver cancer than women.^{1,2} In addition, sex-related differences have also been found in alcoholic fatty liver disease, non-alcoholic steatohepatitis (NASH), and other kinds of chronic hepatitis.^{7–9} Nonetheless, the mechanisms that account for this gender disparity are unknown. With increasing reports of occurrence of NASH, there is an urgent need to study the molecular mechanism underlying sex-specific differences in disease manifestation and pathology.^{10–12}

Epidemiological surveys and studies of animal models have shown significant sex and age-dependent differences

in the onset of NASH.^{13–17} It is known that before 50 years of age, the incidence of NASH is higher in men than women, while after 60 years of age the incidence is higher in women. Hence, it has been hypothesized that the type and expression level of sex hormones might influence the sex-specific differences in NASH.^{13,14,18} However, currently available studies on the effect of sex hormones on the progression of NASH in animal models have provided contradictory results.^{19–23} This may be due to differences in intervention methods or the animal models used in these studies. Hence, this issue remains unresolved.

A recent study demonstrated that one cause of gender disparity in liver cancer is the difference in MyD88-dependent production of IL-6 (interleukin-6) between men and women. The main reason for this sex-specific difference is the inhibition of the inflammatory TLR-MyD88-IL-6 signaling pathway by estrogen in women; consequently, the

incidence of hepatocellular carcinoma is lower in female mice.²⁴ Several reports have confirmed the effect of TLR and IL-6 on the incidence and progression of NASH.^{25–29} However, it is unclear whether sex hormones, in a sex-specific manner, affect the progression of NASH through regulation of the TLR-MyD88-IL-6 signaling pathway. To investigate the pathogenesis of sex-related differences in NASH, we used a classical methionine and choline-deficient (MCD) diet-induced mouse model, and studied the effect of sex hormone-specific intervention on disease progression and on the TLR-MyD88-IL-6 signaling pathway. In this study, we investigated possible differences in the mechanisms underlying sex-dependent disease progression in different chronic liver diseases. Our findings will provide avenues for development of novel therapies against such disorders.

Materials and methods

Animals and treatment

A mouse model of MCD diet-induced NASH was established, according to previously published methods. Ninety-one 14-week-old C57BL/6 mice (26 females and 65 males) were purchased from Laboratory Animal Center of Jilin University and maintained in the center's animal house under sanitary conditions. Mice were housed in plastic cages, in groups of three to five according to their sex, in a temperature and humidity controlled room with a 12-h light: 12-h dark cycle. Fifty-two mice (26 males and 26 females) were randomly divided into four groups: females fed with control diet, females fed with MCD diet, males fed with control diet, and males fed with MCD diet.¹⁵ Accordingly, 13 female and 13 male mice were fed an MCD diet (Cat No. A02082002B, Research Diets); the rest were fed the control diet (Cat No. A02082003B, Research Diets). Another group of 39 male mice were fed the MCD diet, and randomly divided into three groups for sex hormone-specific intervention: mice subjected to orchidectomy, orchidectomy plus subcutaneous injection of testosterone propionate (5 mg/kg/d dissolved in olive oil, Cat No. T1875, Sigma), and, orchidectomy plus subcutaneous injection of estradiol benzoate (0.5 mg/kg/d dissolved in olive oil, Cat No. E8515, Sigma).³⁰ All mice were permitted *ad libitum* consumption of water and were sacrificed at the end of 4 weeks. All experiments were conducted according to the protocol approved by the Animal Care and Use Committee of Jilin University.

Characterization of NASH-histological analysis and NAFLD activity score

Portions of liver from all mice were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (5 μ m thick) were stained with hematoxylin-eosin (H&E), osmic acid, and Masson's trichrome, to examine the histopathology, which was done double-blind by two persons. The NAFLD (non-alcoholic fatty liver disease) activity score (NAS) was recorded according to the following criteria proposed by the Pathology Subcommittee of the NASH Clinical Research Network:³¹ (1) Steatosis: score 0,

parenchymal involvement by steatosis <5%; score 1, 5–33% parenchymal involvement; score 2, 34–66% parenchymal involvement; score 3, >66% parenchymal involvement. (2) Lobular inflammation (inflammatory foci observed under 200 $\times$ magnification): score 0, no foci; score 1, <2 foci; score 2, 2–4 foci; score 3, >4 foci. (3) Hepatocellular ballooning: score 0, none; score 1, few balloon cells; score 2, many balloon cells/prominent ballooning. The sum of the above three scores is defined as NAS.

Measurement of serum levels of sex hormones and transaminases

Blood samples collected from mouse retroorbicular plexus were first allowed to coagulate in centrifuge tubes, then the supernatants were collected after centrifugation and analyzed by enzyme-linked immunosorbent assay (ELISA) to measure the concentration of estradiol and testosterone (Cusabio, USA, Cat No. CSB-E05109m and CSB-E05098m). Serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using an automatic biochemical analyzer (HITACHI 7180, Japan).

Two-step real-time RT-qPCR

Total RNA was extracted from frozen livers using TRIzol (Invitrogen, USA, Cat. No. 15596-026). One microgram of RNA was reverse transcribed into cDNA using the GoScriptTM Reverse Transcription System (Promega, USA, Cat. No. a5001). Real-time PCR was performed using the FS Universal SYBR Green Master (Roche, Germany, Cat. No. 4913914001) according to the following PCR protocol: 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 56.1°C for 45 s. GAPDH expression was used as an internal control. The relative expression (mRNA level) was calculated using the $2^{-\Delta\Delta C_t}$ method.¹⁶ The following primers were used for qPCR: IL-6-forward 5'-acaaagccagagtccttcagag-3', reverse 5'-attggatggctcttggtccttag-3'; MyD88-forward 5'-tggtggtggtgtttctgac-3', reverse 5'-gtccttctcatgccttggt-3'; GAPDH-forward 5'-ctgagagggaatcgtgcgt-3', reverse 5'-aaccgctcgttgccaatagt-3'.

Western blot

To isolate total protein from frozen liver samples, they were homogenized in 500 μ L protein homogenate and subjected to sonication. Following centrifugation at low temperature, the supernatants were collected and assayed with the Bradford reagent (Bio-Rad, USA, Cat. No. 5000202) to determine total protein concentration. Forty micrograms of protein per sample was denatured, separated by SDS-PAGE (SDS-polyacrylamide gel electrophoresis), and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA, Cat. No. HVPPEAC12). The membranes were blocked with TBS (tris-buffered saline) containing 5% milk for 1 h, then incubated with primary antibodies against MyD88, IL-6 or β -actin (Abcam, Britain, Cat. No. ab2068, ab83339, ab1801, respectively) overnight at 4°C. They were then incubated with the respective secondary antibodies conjugated to HRP (horse radish peroxidase)

(Abcam, Britain, Cat. No. ab6720) for 1 h at RT. The proteins separated on the PVDF membrane were visualized using the ECL chemiluminescent detection kit (Millipore, USA, Cat. No. 64-202) and a Tanon Imaging System (Tanon, China, Tanon-4100). The amount of protein was calculated after normalizing against β -actin, and expressed as the optical density ratio.

Statistical analysis

Results are represented as mean $\pm$ SD. Statistical analysis was performed using SPSS19.0 and the least significant difference method. The Kruskal-Wallis test was used to analyze data from multiple independent groups. Mann-Whitney *U* test was used to perform pairwise comparison of data. *P* values <0.05 were considered significant.

Results

Sex-associated differences in MCD-induced NASH mouse model

Both male and female mice that were fed MCD diet exhibited steatosis and inflammation of the liver. Fatty change was mostly of a macrovesicular type, involving all zones of

the hepatic acinus. The inflammatory infiltrate was predominantly composed of foci of lymphocytes with occasional neutrophils randomly distributed in the lobule. Occasionally, we observed ballooning degeneration of hepatocytes in male mice that were fed the MCD diet (Figure 1(a)). While we found no significant difference in the steatosis score between the NASH afflicted liver of male and female mice ($P > 0.05$) (Figure 1(b)), the lobular inflammation score and NAS were significantly higher in males than in females ($P < 0.05$) (Figure 1(c) and (d)). No steatosis or inflammation was seen in the control diet-fed mice (Figure 1(a)). Our results demonstrate the successful establishment of the NASH mouse model, and the existence of sex-associated differences in disease pathology.

Effect of sex-specific intervention on serum levels of sex hormones, NAS, ALT, and AST

To study the effect of sex-specific intervention on the progress of NASH, male mouse models of MCD-induced NASH were subjected to orchidectomy to decrease the androgen level. The data showed that there were significantly lower serum levels of testosterone (Figure 2(a)), milder fatty change and inflammatory infiltrate

Figure 1 Sex-specific differences in MCD diet-induced NASH mouse model. (a) Histopathological changes in the liver of MCD diet-induced NASH mouse model. Liver samples from male and female NASH mice were treated and stained with HE (hematoxylin-eosin) and osmic acid. Representative pictures are shown at $200\times$ magnification. Black arrow depicts inflammatory infiltration and white arrow depicts ballooning. (b–d) Quantitative examination of liver histology. Graphs depict steatosis score (b), inflammation score (c) and NAS (d) in male and female MCD and control mice ($P < 0.05$). Bars, $50\ \mu\text{m}$. (A color version of this figure is available in the online journal.)

Figure 2 Effect of different sex-related interventions on serum sex hormone level and histopathology of liver in NASH mice. (a, b) Effects of orchidectomy (ori), orchidectomy+ testosterone (T) and orchidectomy+ estrogen (E) on serum testosterone level (a) and serum estradiol level (b), in comparison to control and MCD diet-mice (5 groups in total). Data are represented as mean $\pm$ SD. (c) Histopathological examination of the liver of male NASH mice following different forms of sex hormone-related intervention. Black arrow depicts inflammatory infiltration and white arrow depicts ballooning. (d) Effect of different sex hormone-related interventions on NAS ($P < 0.05$). (E–F) Effect of different sex hormone-related interventions on serum levels of ALT (e) and AST (f) ($P < 0.05$). Bars, 50 μ m. (A color version of this figure is available in the online journal.)

(Figure 2(c)), lower NAS (Figure 2(d)), lower ALT and AST (Figure 2(e) and (f)) in the surgically treated mice compared to untreated mice ($P < 0.05$). After treatment with exogenous testosterone propionate, the serum testosterone level was elevated to the level in mice without surgery (Figure 2(a)) and severe fatty change and inflammatory infiltrate was observed compared to that in the surgically treated mice, while no significant changes in NAS, ALT, and AST was observed ($P > 0.05$) (Figure 2(c) to (f)). However, when orchidectomy-treated male NASH mice were administered exogenous estradiol benzoate, there were a significant increase in serum estradiol level while only a little increase in serum testosterone level, and a significant decrease in fatty change and inflammatory infiltrate, NAS, ALT, and AST in comparison to the other three groups (male MCD mice, male MCD+orchidectomy mice, and male MCD + orchidectomy + testosterone propionate mice) ($P < 0.05$) (Figure 2(b) to (f)). Altogether, our results indicate that while orchidectomy can slow down the progress of MCD-induced NASH, exogenous administration of estrogen, but not androgen, can further improve the recovery.

Effect of sex hormone-specific intervention on MyD88 and IL-6 mRNA level

After male NASH mice were subjected to orchidectomy and/or injected with exogenous testosterone propionate or estradiol benzoate, the mRNA level of MyD88 and IL-6 in the liver was analyzed. As shown in Figure 3, the expression of MyD88 and IL-6 was significantly reduced in MCD-diet male mice treated with orchidectomy, compared to those that were not ($P < 0.05$). Combining surgical treatment (orchidectomy) with administration of exogenous testosterone propionate did not change the mRNA level of MyD88 and IL-6 when compared to the surgically treated and untreated MCD-diet male mice ($P > 0.05$). However, when surgical treatment was supplemented with exogenous estradiol benzoate, we detected a significant decrease in MyD88 and IL-6 mRNA level in comparison to the other three groups (male MCD mice, male MCD + orchidectomy mice, and male MCD+orchidectomy+testosterone propionate mice) ($P < 0.05$). These results show that orchidectomy can decrease the expression of MyD88 and IL-6 in the liver, which can be further downregulated by

Figure 3 mRNA level of MyD88 and IL-6 in response to different sex hormone-related interventions. The relative mRNA levels of MyD88 and IL-6 analyzed by real-time qPCR and quantified using GAPDH as the internal control, is shown. Fold changes for each gene was calculated using the $2^{-\Delta\Delta C_t}$ method. Data are represented as mean $\pm$ SD

Figure 4 Expression of MyD88 and IL-6 in response to different sex hormone-related interventions. (a) Western blot analysis of MyD88 and IL-6 expression in total protein extracts of liver samples from all five groups of mice, is shown. β -actin indicates the amount of protein loaded in each well. Relative expression of MyD88 and IL-6 is represented as an optical density ratio normalized to β -actin

exogenous estrogen treatment, but not by administration of exogenous androgen.

Effect of sex hormone-specific intervention on MyD88 and IL-6 protein level

Next, we assayed the protein expression of MyD88 and IL-6 in the liver of male mice after they were subjected to the different kinds of interventions described previously. As shown in Figure 4, expression of MyD88 and IL-6 decreased

significantly in NASH model mice 4 weeks following orchidectomy, when compared to untreated male MCD-diet mice ($P < 0.05$). In mice where the surgical treatment was supplemented with exogenous estradiol benzoate, expression of MyD88 and IL-6 decreased significantly, when compared to the other three groups (male MCD mice, male MCD $\pm$ orchidectomy mice, and male MCD $\pm$ orchidectomy $\pm$ testosterone propionate mice) ($P < 0.05$). Administration of testosterone propionate

following orchidectomy had no additive, but a slightly negative effect ($P > 0.05$). Our results indicate that orchidectomy can decrease the expression of MyD88 and IL-6 in the liver of male NASH model mice, and that administration of exogenous estrogen, but not androgen, can enhance the beneficial effects of this procedure.

Discussion

Sex-specific differences identified recently in various chronic liver diseases have attracted considerable attention. In order to develop and optimize novel therapeutic strategies against such conditions, it is imperative to study underlying mechanisms and uncover disease pathogenesis. Although a series of studies have investigated this issue, most failed to draw precise conclusions. Naugler *et al.*²⁴ were one of the first to report that sex-specific differences in the onset and progression of liver cancer is associated with the activation of the TLR-MyD88 signaling pathway that induces Kupffer cells to produce IL-6, leading to inflammation and even hepatocellular carcinoma. Whether a similar MyD88-dependent mechanism contributes to gender-related differences in non-alcoholic steatohepatitis remains unknown.

Epidemiological surveys have shown that susceptibility to NASH at different ages differs with the sex of the individual. This report led researchers to speculate that sex hormones might play a key role in establishing sex-associated differences in the pathology of NASH. Subsequently, several studies were conducted to investigate the effect of estrogen on such a phenomenon. Kamada *et al.*¹⁹ established a high-fat and high-cholesterol diet-induced NASH disease model in female C57BL/6J mice. The authors implanted ovariectomized mice with hormone release pellets, and showed that estrogen exerted a protective effect on the pathological progression of NASH and partially alleviated liver damage in these mice. However, other reports have generated contradictory results and challenged above conclusions. For example, Kashireddy and Rao²⁰ established an MCD diet-induced steatohepatitis model in C57BL/6J mice, and examined ovariectomized mice treated with either estrogen or tamoxifen. Their data indicated that estrogen does not affect the pathogenesis of NASH. In addition, the effect of androgen on NASH has also been explored. Tian *et al.*²¹ examined the concentrations of total testosterone, free testosterone and estradiol in NASH patients, and performed a multiple regression analysis to conclude that androgen levels in the serum are not correlated with NASH pathology. In contrast, Mu and Zhang found that in a high-fat diet-induced NASH rat model, androgen does exert a protective effect on disease severity by decreasing fatty acid and cholesterol synthesis.^{22,23}

Animal models of steatohepatitis created by administering a high fat diet are, in general, simplified disease models with obvious hepatocyte steatosis, but lacking obvious inflammatory infiltrates. Hence, we believe that such a model is not ideal for studying NASH. In this study, we investigated the role of sex hormones on the etiology and pathology of NASH, in an MCD diet-induced male C57BL/6 mouse model whose histological features closely

resembled those seen in NASH patients.¹⁵ Specifically, we examined the effects of multiple sex-specific interventions in these animals.

Our results show that following orchidectomy, the serum levels of testosterone, ALT and AST, as well as NAS in male NASH model mice decreased significantly compared to the untreated male MCD-diet mice, thus proving its validity as a therapeutic strategy against NASH. When these surgically treated mice were administered testosterone propionate, although the serum testosterone level increased, there was no significant difference in NAS, ALT or AST, and hence no change in the severity of NASH, when compared with mice subjected to orchidectomy alone. Theve *et al.*³² reported similar findings, while studying the influence of sex hormones on animal models of hepatitis created by infecting with *helicobacter hepaticus*. Our data suggests that the effect of androgen on the progression of NASH in male mice is complex and multifactorial, involving both direct and indirect mechanisms. Further studies are needed to elucidate the underlying molecular pathways. Moreover, in this study exogenous sex hormones were administered by subcutaneous injections, as opposed to the natural hormone secretion seen in mice. Additional improved methods for exogenously supplementing mice with sex hormones might be necessary in order to perform more accurate studies of the underlying mechanism. Interestingly, when mice subjected to orchidectomy were administered estradiol benzoate, not only did the serum estradiol level increase, but the NAS, as well as concentrations of ALT and AST reduced significantly in comparison to the other three groups – male MCD mice, male MCD+orchidectomy mice, and male MCD+orchidectomy+testosterone propionate mice (Figure 2(b), (d) to (f)). These results suggest that different sex-specific interventions could affect NASH differently, which is in turn related to the level of sex hormones, especially estrogen, in blood.

In 2007, Naugler *et al.*²⁴ reported that sex-specific differences in liver cancer were associated with the TLR-MyD88-IL-6 signal transduction pathway. TLRs, or Toll-like receptors, are expressed by many types of liver cells; they enable cells to recognize invading microorganisms or pathogen-associated molecular patterns and activate the adaptor protein MyD88. Next, MyD88 activates mitogen-activated protein kinase (MAPK) and inhibits Ikappa B kinase (IKK) signaling pathways, resulting in the expression of inflammatory genes (including IL-6) by Kupffer cells.^{33–37} IL-6 not only plays an important role in defending cells from invasion by microorganisms and pathogens, but also functions in inducing chronic liver injury and fibrosis, even leading to cirrhosis and hepatocellular carcinoma.

Interestingly, several studies have previously reported that TLRs and/or IL-6 might affect the progression of NASH.^{25–29} However, whether intervention through manipulation of sex hormone levels to halt the progression of NASH is associated with alterations in the TLR-MyD88-IL-6 pathway, was unclear. In this study, we investigated such an association in a mouse model of NASH.

Both mRNA and protein levels of MyD88 and IL-6 in the liver, assayed by RT-qPCR and Western Blot respectively, decreased significantly in MCD-diet male mice subjected to

orchidectomy. Androgen supplement did not affect the expression of MyD88 and IL-6 compared to both the MCD-diet mice and those treated with orchidectomy. On the other hand, estrogen supplement significantly decreased the expression of MyD88 and IL-6, providing an additional protective effect against the progression of NASH in MCD-diet male mice. Our data demonstrates the existence of sex-associated differences in C57BL/6 NASH mouse model, and that different interventions aimed at manipulating the level of sex hormones can modulate the severity of NASH. We have shown that this effect is due to the regulation of the inflammation-causing TLR-MyD88-IL-6 pathway by sex hormones, especially estrogen. Estrogens decrease the activity of NF- κ B and C/EBP β and, consequently, IL-6 promoter activity. Although administration of estrogens reduces IL-6 production and attenuates liver damage, estrogen is still cannot be applied to the NASH because of the side effects of estrogen such as heart disease, strokes, and blood clots. So looking for more safety estrogens replacement therapy, such as implant estrogens and selective estrogen receptor agonist, may provide a novel area for therapeutic interventions for NASH.

In conclusion, our results show that estrogen supplement and orchidectomy may improve the pathological liver damage associated with NASH, and that the effect of sex hormones on NASH might be mediated through the TLR-MyD88-IL-6 pathway. Our study provides the basis for future research on sex-associated differences in the etiology and progression of NASH and other chronic liver diseases, and for the development of novel therapeutic strategies.

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GX and SQ contributed to the work equally and should be regarded as co-first authors.

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