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Effects of high fructose intake on liver injury progression in high fat diet induced fatty liver disease in ovariectomized female mice

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ABSTRACT

Epidemiology shows that the morbidity of nonalcoholic fatty liver disease (NAFLD) is increased in postmenopausal women and chronic high fructose intake induces NAFLD progression. To analyze the effects of high fructose intake on estrogen deficiency, we evaluated liver disease progression using ovariectomized mice fed with a high fat diet (HFD) for 12 weeks. Hepatic steatosis developed in all HFD groups. Fructose intake significantly increased the liver weight and serum alanine aminotransferase, which was not exacerbated by ovariectomy alone. Ovariectomy enhanced the hepatic inflammatory activity shown by tumor necrosis factor α upregulation in the groups with or without fructose intake. Both fructose intake and ovariectomy increased the hepatocytes with ballooning degeneration and hepatic macrophage infiltration and activated hepatic stellate cells. Coexistence of fructose intake and ovariectomy markedly enhanced liver cell destruction, macrophage accumulation, and progression of fibrosis. Liver damage was ameliorated by 17β-estradiol supplementation. These findings suggest that high fructose intake enhanced the progression of NAFLD in ovariectomized female mice.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) has been increasing worldwide and becoming the most frequent disease of the liver. Nonalcoholic steatohepatitis (NASH) is a severe form of NAFLD characterized with sustained inflammation and fibrosis, eventually developing into liver cirrhosis and hepatocellular carcinoma (Sugimoto and Takei, 2011). To prevent liver related death in NASH patients, detailed understanding of factors associated with the progression of liver disease is necessary.

Insulin resistance and obesity are recognized as major factors that exacerbate NAFLD. In addition, menopause and unbalanced dietary carbohydrate contents are associated with NAFLD development (Suzuki and Abdelmalek, 2009). The prevalence of NAFLD is generally higher in men than in women, but epidemiological studies show that this sex difference disappears after menopause with increasing prevalence of NAFLD in women (Luo and Ishigami, 2015). In postmenopausal women, the presence of central obesity is significantly associated the risk of NAFLD (Chung et al., 2015). Because hepatic fatty acid oxidation is impaired in estrogen deficiency, reduced fat oxidation in postmenopausal women likely accelerates visceral fat, leading to obesity and NAFLD (Lovejoy et al., 2008; Nemoto et al., 2000). In addition to the effects on metabolism, estrogen modulates immune-inflammatory activity. Thus estrogen withdrawal may affect intrahepatic proinflammatory activity, leading to increased liver damage (Monteiro et al., 2014). These observations suggest that both the metabolic and immune-inflammatory changes in estrogen deficiency may exacerbate NAFLD development in postmenopausal woman.

Chronic high fructose intake induces hepatic steatosis (Lim et al., 2010; Castro et al., 2011). Fructose is principally metabolized in the liver, where it can be converted into fatty acids without physiological regulation by phosphofructokinase, stored in the form of triglycerides (TGs) (Softic et al., 2016). Fructose is believed to increase the generation of advanced glycation endproducts and reactive oxygen species

Abbreviations: αSMA, *alpha* smooth muscle actin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; hCLS, hepatic crown-like structure; HFD, high fat diet; MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OVX, ovariectomy; PCR, polymerase chain reaction; ROS, reactive oxygen species; SD, standard diet; SO, sham operation; TG, triglyceride; TGFβ, transforming growth factor-β; TNFα, tumor necrosis factor-α

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(ROS), thereby promoting the hepatocyte damage (Takeuchi et al., 2015; Kohli et al., 2010). The association of fructose and NAFLD is well known, however, the effects of chronic consumption of fructose on postmenopausal woman are not well understood. Because a nationwide survey in the US reported the increased dietary fructose intake in all sex and age groups from 1977 to 2004 (Marriott et al., 2009), high fructose intake may have contributed to the increased number of postmenopausal women becoming NAFLD patients. Thus, understanding the effects of high fructose intake during the postmenopausal stage may be helpful when considering fructose restriction to prevent NAFLD progression.

In this study, we evaluated the effects of high fructose intake on the progression of liver disease in ovariectomized female mice. To simulate NAFLD patients, mice were fed with a high fat diet (HFD) to induce obesity. We observed that either the fructose intake or ovariectomy enhanced the fatty liver disease; however, coexistence of these two insults further exacerbated liver damages and augmented hepatic fibrosis. Our observations suggest that high fructose intake accelerates the progression of NAFLD in estrogen deficiency.

2. Materials and methods

2.1. Animal protocol

All female C57BL/6J mice (7 weeks old, 17 − 19g; Charles River Laboratories, Yokohama, Japan) were housed at 22–23 °C in a 12:12-h light: dark cycle. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and were approved by the Animal Care Committee of Kyushu University. The standard diet (SD) (Oriental Yeast, Tokyo, Japan) consisted of 58.2% carbohydrate, 4.8% fat, and 20.8% protein. The HFD (Oriental Yeast, Tokyo, Japan) consisted of 48.3% carbohydrate (no glucose, no sucrose), 30.1% fat (containing 4.3% cocoa butter, 3.0% lard, and 1.25% cholesterol), and 22.8% protein. The water containing 30% fructose was prepared using D- (−) - Fructose (Sigma, St. Louis, MO). At 1 week after arrival, mice (n = 40) underwent bilateral ovariectomy (OVX) (n = 16) or sham operation (SO) (n = 24). One week after the surgery, mice were divided into the following five groups (n = 8 per group): SO-SD group, SO mice with SD and tap water, SO-HFD group, SO mice with HFD and tap water, OVX-HFD group, OVX mice with HFD and tap water, SO-HFD-Fr group, SO mice with HFD and 30% fructose, OVX-HFD-Fr group, OVX mice with HFD and 30% fructose. All groups were fed for 12 weeks. The experimental design of the groups is shown in Fig. 1. In the estrogen replenishment study, mice (n = 15) were divided into three groups and underwent SO (n = 5), and OVX (n = 5), OVX with subcutaneous implantation of 17β-estradiol sustained release (0.5 mg/pellet, 90-day release, Innovative Research of America, Sarasota, FL) (n = 5). From 1 week after surgery, all mice were fed for 12 weeks with HFD and 30% fructose. At the end of the experimental period, the mice fasted overnight and then euthanized after being weighed. Blood was collected from the inferior vena cava, centrifuged (3000 rpm, 5 min, 4 °C), and serum was collected. The livers were harvested and fixed with 10% buffered formalin for histological examination or immediately frozen in liquid nitrogen for mRNA extraction and lipid analysis.

2.2. Measurement of serum biochemical markers and liver lipid content

Serum alanine aminotransferase (ALT) was measured using the transaminase CII test kit (Wako Pure Chemical Industries, Tokyo, Japan). Total lipids were extracted from the liver as described previously (Miura and Suzuki, 2014). Serum and liver TG were measured using TG E-test kit as manufacture's protocol (Wako Pure Chemical Industries, Tokyo, Japan).

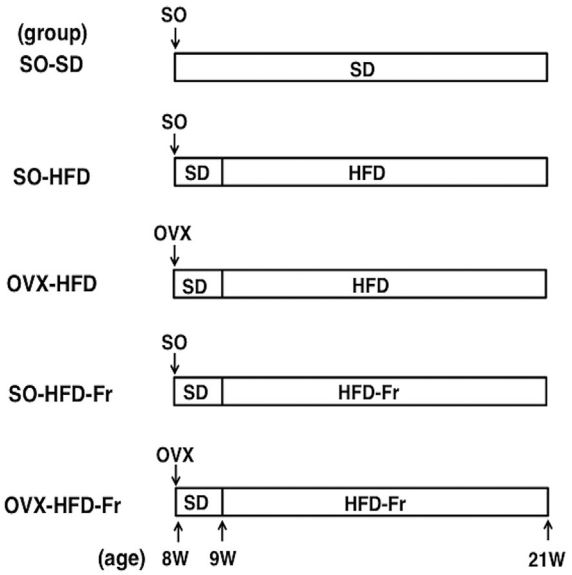

Fig. 1. Design of experimental groups. Female C57BL/6J mice (8 weeks old) underwent bilateral ovariectomy (OVX) or sham operation (SO). One week after the surgery, mice were divided into the following five groups (n = 8 per group); SO-SD group, SO mice with standard diet (SD) and tap water, SO-HFD group, SO mice with high fat diet (HFD) and tap water, OVX-HFD group, OVX mice with HFD and tap water, SO-HFD-Fr group, SO mice with HFD and 30% fructose, OVX-HFD-Fr group, OVX mice with HFD and 30% fructose. After a 12 week diet, histological and biochemical analyses were performed.

2.3. Histological and immunohistochemical analyses

The liver samples were fixed and embedded in paraffin. 5 μm thick sections were cut from each block and randomly assigned to 4 types of staining, i.e., hematoxylin and eosin (HE), Masson trichrome, and immunostaining for F4/80 and α-smooth muscle actin (αSMA). Anti-F4/80 antibody (1:100) (MCA497GA, Bio-Rad, Hercules, CA) and anti-αSMA antibody (1: 100) (Abcam, Cambridge, UK) were commercially purchased. The Histological scoring were assessed quantitatively by an expert hepatopathologist (H.S.) who was blinded to the sample's background. The observer evaluated in five fields of view at a magnification of 200× in each section, 5 sections for each animal. The numbers of ballooned hepatocytes and hepatic crown-like structure (hCLS) were quantified in HE stained sections (6 animals in each group) and F4/80 immunostained sections (6 animals in each group), respectively. The immune-positive area for F4/80 or αSMA was quantified in the same way as above in respective immunostaining (6 animals in each group) using Leica Application Suite Ver. 4.2 software (Leica Microsystems, Heerbrugg, Switzerland). The fibrosis score was determined by reference to the classification of Brunt et al. (Brunt et al., 1999; Kleiner et al., 2005). Magnifications of photographs shown in figures were 200×.

2.4. Quantitative real-time PCR

Total RNA was prepared from liver specimens using a TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 1 μg of RNA by GeneAmp RNA polymerase chain reaction (PCR) (Applied Biosystems, Hammonton, NJ). Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche, Basal, Switzerland). To control for variations in reactions, all PCR data were normalized against glyceraldehyde 3-phosphate dehydrogenase expression. The primer sequences used are listed in Table 1.

Table 1
Primers for real-time PCR.

Genes	Forward (5' – 3')	Reverse (5' – 3')
TNFα	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
MCP-1	AGGTCCCTGTCATGCTTCTG	GCTGCTGGTGATCCTCTTGA
TGFβ	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC
Col1a2	CCGTGCTTCTCAGAACATCA	GAGCAGCCATCGACTAGGAC
GAPDH	TGTGTCCGTGGATCTGA	TTGCTGTTGAAGTCGAGAG

TNFα, tumor necrosis factor α; MCP-1, monocyte chemoattractant protein-1; TGFβ, transforming growth factor β; Col1a2, collagen type 1-a2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2.5. Western blotting

Protein was extracted from liver using lysis buffer containing 25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate and cocktails of protease inhibitors. Immunoblotting was performed with anti- tumor necrosis factor-α (TNFα) antibody (Abcam, Cambridge, UK), anti-transforming growth factor-β (TGFβ) antibody (Abcam, Cambridge, UK) and anti-β-actin antibody (Santa Cruz Biotechnology, Dallas, TX). Immunoblots were detected and analyzed with ECL Prime Western Blotting Detection Reagent and Image Quant LAS 4000 mini (GE Healthcare, Little Chalfont, UK).

2.6. Statistical analyses

Statistical analysis was conducted using JMP 13 software (SAS Institute, Cary, NC). Results were presented as means ± SD. The differences of means were analyzed by one-way ANOVA and Tukey's post hoc test. $P < 0.05$ was regarded as statistically significant (Richard, 2008; Tukey, 1949).

3. Results

3.1. High fructose intake exacerbated fatty liver disease with serum ALT elevation in ovariectomized mice

The body weight of each group gradually increased during the experimental period, and reached in similar levels except for the OVX-HFD group, which showed slightly increased weight compared with the SO-HFD group (Fig. 2A, Table 2). Addition of 30% fructose significantly increased the caloric intake in both the groups with and without ovariectomy (Fig. 2B). However, ovariectomy reduced the caloric intake in both the groups with and without fructose intake. Ovariectomy slightly increased the body weights in the HFD groups, but did not do so in the HFD-Fr groups (Table 2). Meanwhile, high fructose intake significantly increased the liver weight in both groups with and without ovariectomy. Biochemical examination showed that serum ALT in all HFD groups was increased compared with that in the SO-SD group. Aspartate aminotransferase (AST) is also known as a major marker for liver injury, however, due to the localization of AST in hepatic and extrahepatic organs, we estimated ALT as a more specific marker of hepatocellular cell injury (Kwo et al., 2017). Fructose intake induced further increase of ALT (SO-HFD vs SO-HFD-Fr, $p < 0.05$, OVX-HFD vs OVX-HFD-Fr, $p < 0.05$), and the highest ALT was observed in the OVX-HFD-Fr group. Liver TG content in all HFD groups were increased three times more than that in the SO-SD group. Fructose intake showed a tendency to increase liver TG content, but ovariectomy did not increase liver TG content. This suggested that the highest ALT elevation in the OVX-HFD-Fr group was not simply attributed to liver TG abundance.

3.2. High fructose intake and ovariectomy augmented the ballooning degeneration of hepatocytes

Histological examination showed the centrilobular distribution of steatotic hepatocytes in the groups with the HFD (Fig. 3B, C, 3D, 3E). The hepatocytes with ballooned degeneration, characterized by enlarged cells with a wispy cleared cytoplasm and nucleus in the center, were frequently observed in the OVX-HFD group (Fig. 3C), which were similarly observed in the SO-HFD-Fr group (Fig. 3D). Coexistence of ovariectomy and fructose intake further increased the number of ballooned hepatocytes, accompanied by strong infiltration of inflammatory cells (OVX-HFD-Fr) (Fig. 3E and F). These observations showed that either the ovariectomy or fructose intake in HFD mice induced ballooning degeneration of hepatocytes, a pathological feature of NASH (Charlton et al., 2011). Furthermore, coexistence of these two insults not only increased the ballooned hepatocytes but also provoked inflammatory reaction into the liver.

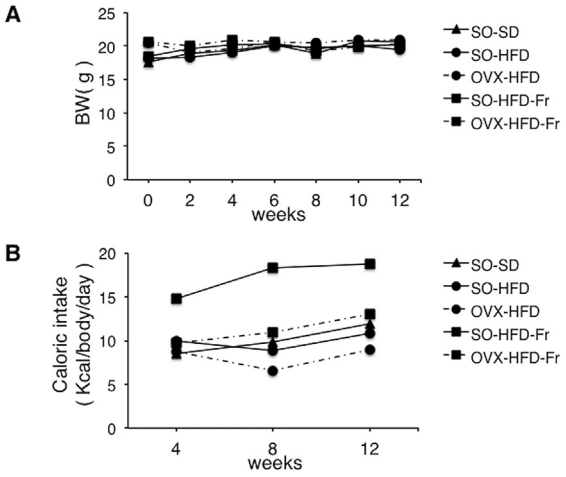

Fig. 2. Changes in body weight and energy intake. A: Time-course of body weight. Female mice underwent bilateral OVX or SO. One week after the surgery, mice were divided into five groups and fed the respective diet for 12 weeks. SO-SD group, SO mice with SD and tap water, SO-HFD group, SO mice with HFD and tap water, OVX-HFD group, OVX mice with HFD and tap water, SO-HFD-Fr group, SO mice with HFD and 30% fructose, OVX-HFD-Fr group, OVX mice with HFD and 30% fructose. The changes in body weight during the 12-week diet period were measured. B: Daily caloric intake. Caloric intake was calculated from the consumed weight of the diet. Data were presented as means in each group.

3.3. Coexistence of high fructose intake and ovariectomy induced accumulation of F4/80 positive macrophages

In F4/80 immunostaining, macrophages were sparsely observed in the SO-HFD group (Fig. 4B), and appeared more frequently in either the OVX-HFD group (Fig. 4C) or the SO-HFD-Fr group (Fig. 4D). In the OVX-HFD-Fr group, markedly increased macrophage infiltration was observed (Fig. 4E and F). In addition, we observed the appearance of hCLS, a histological feature of NASH, in the animals (Fig. 4G) (Itoh et al., 2013). hCLS was barely observed in the SO-HFD group, but occasionally observed in the groups with ovariectomy and/or fructose intake, suggesting the progression of liver disease from NAFLD to NASH

Table 2
Characteristics of experimental animals.

	SD	HFD		HFD-Fr	
	SO	SO	OVX	SO	OVX
n	8	8	8	8	8
Body weight (g)	20.6 ± 1.1	19.5 ± 0.9	20.9 ± 0.6*	20.2 ± 1.6	20.3 ± 1.3
Liver weight (g)	0.76 ± 0.1	1.7 ± 0.2	2.0 ± 0.2	3.3 ± 0.5*	3.6 ± 0.3†
Serum					
ALT (IU/l)	26.2 ± 12.7	59.8 ± 11.2	67.0 ± 35.1	95.9 ± 15.0*	147.3 ± 65.0†
Triglyceride (mg/dl)	37.9 ± 7.0	26.4 ± 7.7	17.3 ± 2.1	30.1 ± 8.7	26.1 ± 6.9
Liver					
Triglyceride (mg/g liver)	28.2 ± 7.2	104.5 ± 8.9	100.8 ± 13.9	113.5 ± 28.8	113.6 ± 27.9

SD; standard diet, HFD; high fat diet, HFD-Fr; HFD with 30% fructose, SO; sham operated, OVX; ovariectomized, ALT; alanine aminotransferase. *P < 0.05 compared with SO-HFD group, †P < 0.05 compared with OVX-HFD group. Data are mean ± SD.

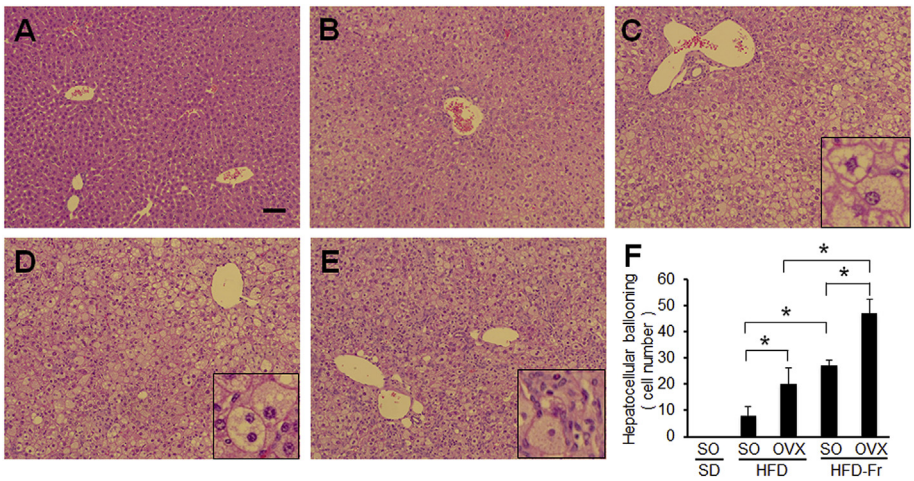

Fig. 3. Histological analyses of the livers in HFD fed female mice treated with ovariectomy and/or fructose intake. Representative photographs of hematoxylin and eosin staining of liver sections. A: SO-SD, B: SO-HFD, C: OVX-HFD, D: SO-HFD-Fr, E: OVX-HFD-Fr group. Scale bar = 100 μm. Insets show the hepatocytes with ballooning degeneration. F: Number of ballooned hepatocytes. The numbers of ballooned hepatocytes were quantified as described in Materials and Methods (6 animals in each group) and the mean numbers of ballooned hepatocytes in 5 fields of 200× magnification were shown. Data are presented as means ± SD. *$P < 0.01$.

(Fig. 4H). Analyses of mRNA expression of inflammatory cytokines showed that ovariectomy significantly upregulated tumor necrosis factor-α (TNFα) in the SO-HFD vs OVX-HFD and SO-HFD-Fr vs OVX-HFD-Fr group, and the expression of monocyte chemoattractant protein-1 (MCP-1) was markedly elevated in the OVX-HFD-Fr group (Fig. 5A). Immunoblotting of TNFα also showed that ovariectomy increased the protein levels, especially in fructose fed groups (Fig. 5B).

3.4. Coexistence of high fructose intake and ovariectomy enhanced the progression of liver fibrosis

Masson trichrome staining was performed to estimate hepatic fibrosis. Minimal pericellular fibrosis was shown in SO-HFD mice (Fig. 6B). In either OVX-HFD (Fig. 6C) or SO-HFD-Fr (Fig. 6D) mice, pericellular and periportal fibrosis marginally appeared. In OVX-HFD-Fr mice, pericellular fibrosis diffusely expanded around the liver (Fig. 6E and F). In αSMA immunohistochemistry, lobular and periportal distribution of activated hepatic stellate cells were observed in the OVX-HFD and SO-HFD-Fr groups (Fig. 7C and D). In accordance with the progression of fibrosis, strong lobular accumulation of activated stellate cells appeared in the OVX-HFD-Fr group (Fig. 7E and F). As shown in Fig. 8, coexistence of ovariectomy and fructose intake significantly upregulate the hepatic expressions of TGFβ and Col1a2. We also evaluated hepatic mRNA expressions of fetuin B and CD44, which are known to be involved in obesity induced NAFLD formation and regulation of inflammatory reactions, respectively. However, expressions of these genes were not significantly different among the groups (data not shown).

3.5. E2 replenishment ameliorated liver damage in OVX-HFD-Fr mice

To evaluate the role of estrogen deficiency, we prepared another set of mice with ovariectomy and fructose intake and supplemented estrogen by E2 pellet implantation. E2 supplementation significantly reduced the body weight, serum ALT, and liver TG content (Table 3). The hepatic mRNA and protein expression levels of TNFα were suppressed by E2 (Fig. 9). E2 supplementation also reduced the hepatic expressions of TGFβ, but the differences did not reach statistical significance.

4. Discussion

In this study, we evaluated the coordinated effects of high fructose intake and estrogen deficiency on the progression of HFD induced fatty liver disease, focusing on the involvement of inflammation and fibrotic reaction. Ovariectomy or fructose similarly induced hepatocyte ballooning, hCLS formation and hepatic stellate cell activation. However, hepatic TNFα upregulation was brought by ovariectomy and serum ALT elevation was induced by fructose intake. Coexistence of ovariectomy and fructose intake induced marked infiltration of F4/80 positive macrophages accompanied by liver fibrosis progression. Our observation suggests that chronic high fructose intake can be considered potent in exacerbating liver disease in estrogen deficient status.

High fructose intake increased the liver weight with a slight gain of liver TG content, indicating the accumulation of TG in the whole liver. Fructose is easily incorporated by the liver, resulting in fatty acids and triglyceride synthesis and accumulation (Softic et al., 2016). Fructose is highly toxic because it can induce nonenzymatic glycation to generate advanced glycation endproducts as well as intracellular metabolic disorder (Takeuchi et al., 2015). A high fructose diet induces hepatic steatosis in mice (Charlton et al., 2011), and induces hepatocyte

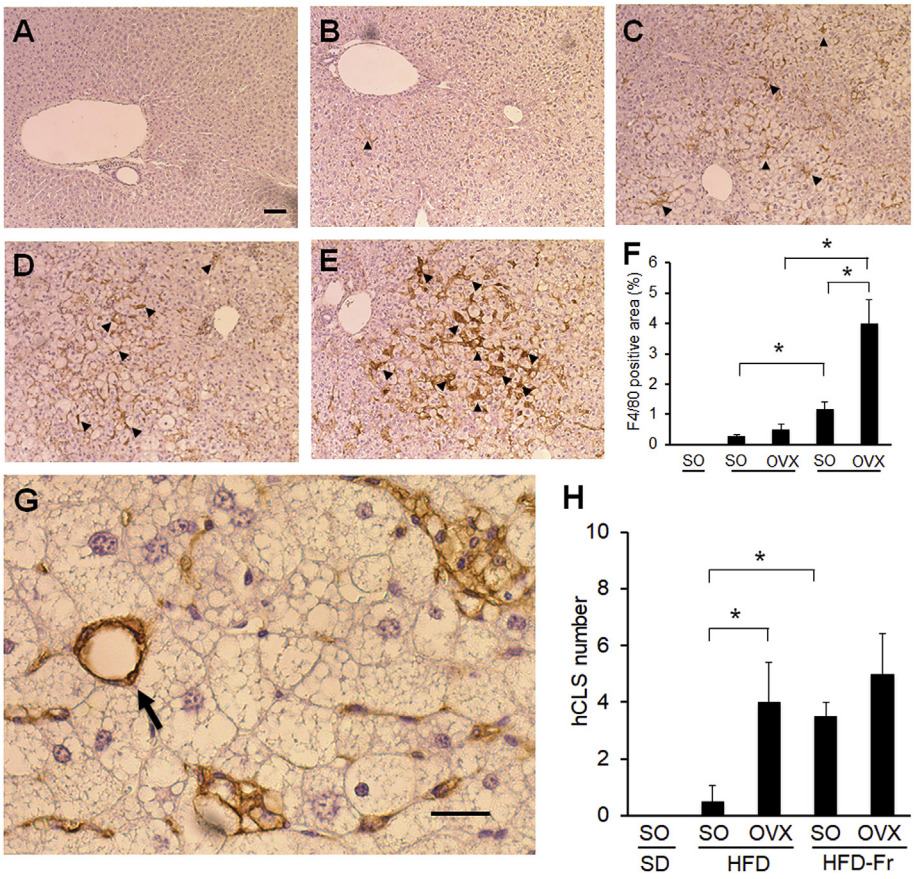

Fig. 4. Evaluation of macrophage accumulation. Representative photographs of F4/80 immunostaining of liver sections. A: SO-SD, B: SO-HFD, C: OVX-HFD, D: SO-HFD-Fr, E: OVX-HFD-Fr group. Scale bar = 100 μm. F: Quantifications of F4/80 positive cells. The immune-positive areas were evaluated and expressed as the percentage of the total area measured as described in Materials and Methods (6 animals in each group). Arrowheads, F4/80 immune-positive cells. G: Representative image of hepatic crown-like structure (hCLS) observed in the OVX-HFD-Fr group. Arrow, hCLS. Scale bar = 50 μm. H: Number of hCLS. The numbers of hCLS were quantified as described in Materials and Methods (6 animals in each group) and the total numbers of hCLS in 5 fields of 200× magnification were shown. Data are mean ± SD. *$P < 0.01$.

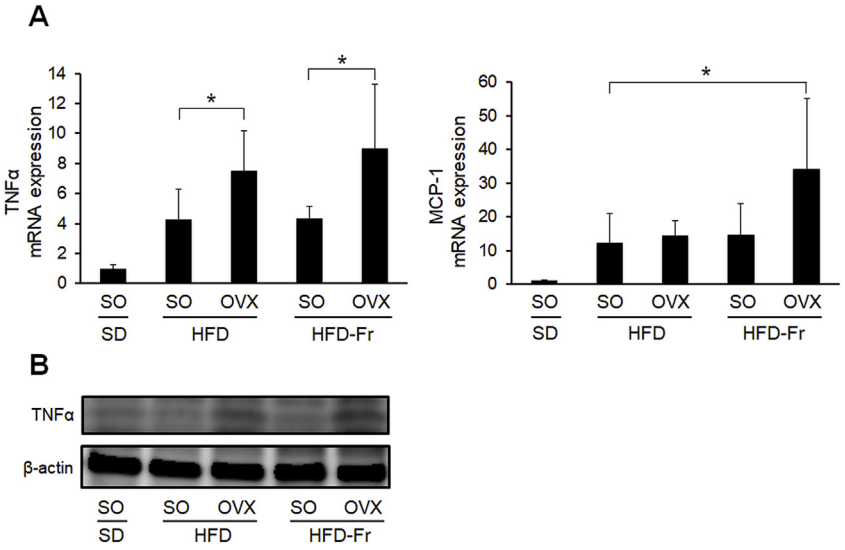

Fig. 5. Hepatic expression of inflammatory cytokines. A; mRNA expression levels of tumor necrosis factor-α (TNFα) and monocyte chemoattractant protein-1 were evaluated by real-time polymerase chain reaction (PCR). To control for variations in reactions, all PCR data were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The fold changes were calculated as the ratio of the expression levels of those in the SO-SD group. Data were presented as mean ± SD. *$P < 0.01$. Eight animals in each group. B; Representative immunoblotting for TNFα. Western blotting was performed to evaluate the protein expression levels in mice livers.

apoptosis with an increased Bax/Bcl2 ratio due to the development of oxidative stress (Choi et al., 2017). Increased hepatic ROS and proinflammatory macrophages in a high fructose diet drive the activation of hepatic stellate cells, which leads to hepatic fibrosis (Kohli et al., 2010). Here, we observed that fructose intake significantly increased serum ALT and αSMA positive hepatic stellate cells in the SO-HFD-Fr group, which were not accompanied by similar upregulation of hepatic TNFα and MCP-1. We speculate that the scarce presence of inflammatory reactions may be attributed to the type of cell death. While cell death can trigger and expand inflammatory reactions via damage-associated molecular patterns released from damaged cells (Kaczmarek et al., 2013), apoptosis has been considered a noninflammatory or even anti-inflammatory mode of cell death (Davidovich et al., 2014). Therefore, if apoptosis is the principal cause of cell death induced by fructose, inflammation would not be vigorously triggered, which would result in the low levels of the hepatic TNFα expression.

Ovariectomy affected the liver disease in a different manner. Reduced energy expenditure in estrogen deficiency is supposed to be associated with obesity and hepatic steatosis (Rogers et al., 2009; Witte et al., 2010; Melanson et al., 2015). While the energy intake in the OVX groups was reduced compared with that in the SO groups, body weight in the OVX-HFD group were slightly but significantly higher than that

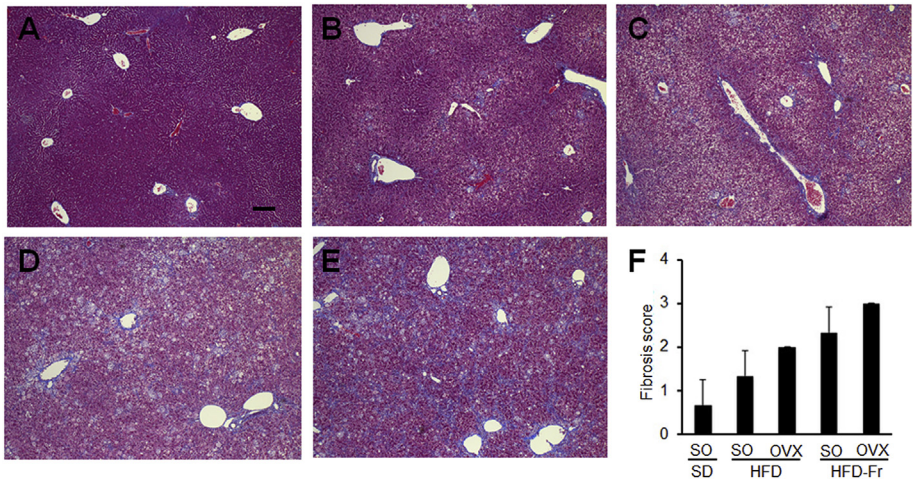

Fig. 6. Progression of liver fibrosis in HFD fed female mice treated with ovariectomy and/or fructose intake. Representative photographs of Masson trichrome staining of liver sections. A: SO-SD, B: SO-HFD, C: OVX-HFD, D: SO-HFD-Fr, E: OVX-HFD-Fr groups. Scale bar = 100 μm. F: Fibrosis scores in each group (n = 6). Fibrosis scores were evaluated by a hepatopathologist in observer-blinded study as described in Materials and Methods.

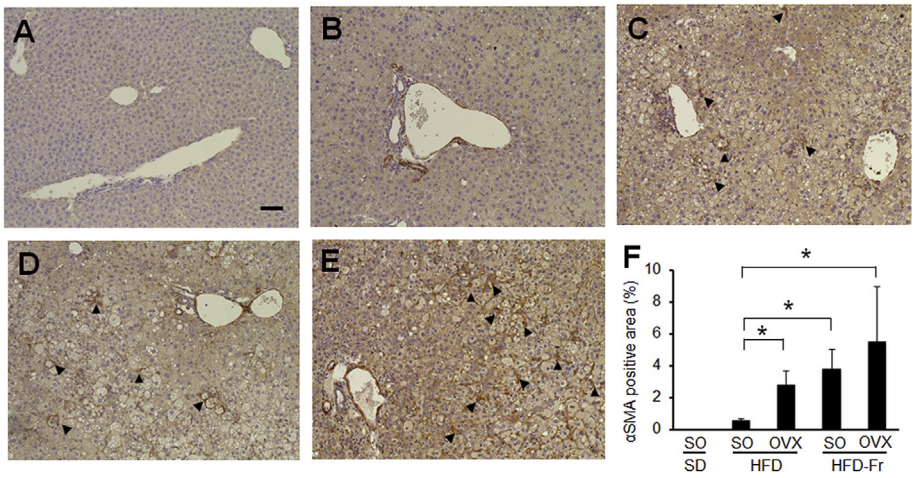

Fig. 7. Evaluation of activated hepatic stellate cells. Representative photographs of αSMA (α smooth muscle actin) immunostaining of liver sections. A: SO-SD, B: SO-HFD, C: OVX-HFD, D: SO-HFD-Fr, E: OVX-HFD-Fr groups. Scale bar = 100 μm. Arrowheads, αSMA immune-positive cells. F: Quantification of αSMA positive cells. The immune-positive areas were evaluated and expressed as the percentage of the total area measured as described in Materials and Methods (6 animals in each group). Data are presented as means ± SD. $^*P < 0.01$.

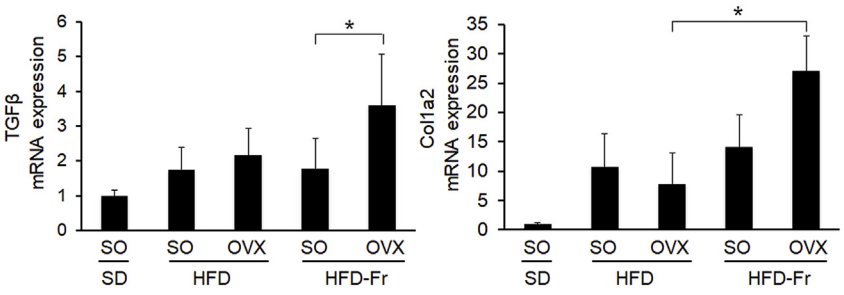

Fig. 8. Hepatic expression of fibrosis related gene. mRNA expression levels of transforming growth factor-β (TGFβ) and Col1a2 were evaluated by real-time PCR. To control for variations in reactions, all PCR data were normalized against GAPDH. The fold changes were calculated as the ratio of the expression levels of those in the SO-SD group. Data were presented as mean ± SD. $^*P < 0.01$. Eight animals in each group.

in the SO-HFD group, suggesting reduced energy expenditure after ovariectomy. Decreased fatty acid oxidation and impaired very low-density lipoprotein assembly are thought to contribute the development of hepatic steatosis in estrogen deficiency (Paquette et al., 2009; Grist et al., 2002; Cote et al., 2014). In this study, neither liver weight gain nor hepatic TG increase was observed in the OVX group compared with the SO group. Because we used a HFD to induce NAFLD with obesity, ovariectomy could not further increase the hepatic lipid content. However, an increased number of ballooned hepatocytes and upregulation of TNFα were observed in the OVX groups compared with the SO groups. Ballooning degeneration of hepatocytes is attributed to the disturbed integration of the intermediate filament cytoskeleton (Lackner et al., 2008), which is believed to be induced by excess oxidative stress in steatotic hepatocytes (Lackner et al., 2008). Because estrogen is a strong endogenous antioxidant capable of reducing hepatic lipid peroxidation and ROS generation (Yoshino et al., 1987), estrogen withdrawal is likely to enhance oxidative stress in the liver, resulting in the progression of ballooning degeneration. Furthermore, loss of the immunomodulating activity of estrogen may be involved (Straub, 2007). Menopause is associated with the spontaneous increases in proinflammatory cytokines, such as interleukin-1, interleukin-6, and TNFα (Pfeilschifter et al., 2002). In addition, E2 inhibited the spontaneous secretion of these cytokines in cultures of peripheral blood (Rogers and Eastell, 2001). These findings indicate that the estrogen withdrawal provokes the proinflammatory reactions, such as the TNFα upregulation. Considering each aspect of the present study collectivity, we speculate that ovariectomy enhanced oxidative stress in hepatocytes and introduced a proinflammatory environment into the liver.

In this study, we observed that coexistence of fructose intake and estrogen deficiency markedly enhanced liver cell destruction, macrophage accumulation, and progression of fibrosis. In the OVX-HFD-Fr group, fructose damaged the liver with a serum ALT increase more than

Table 3
Characteristics of animals in E2 replenishment study.

	HFD-Fr		
	SO	OVX	OVX + E2
n	5	5	5
Body weight (g)	21.7 ± 1.4	24.0 ± 1.2	20.9 ± 2.0*
Liver weight (g)	2.3 ± 0.2	2.1 ± 0.1	2.8 ± 0.2
Serum			
ALT (IU/l)	138.8 ± 49.0	191.9 ± 53.0	118.4 ± 28.8*
Triglyceride (mg/dl)	42.2 ± 13.5	39.9 ± 15.2	31.4 ± 10.9
Liver			
Triglyceride (mg/g liver)	113.0 ± 21.4	123.3 ± 21.0	95.0 ± 6.7*

HFD; high fat diet, HFD-Fr; HFD with 30% fructose, SO; sham operated, OVX; ovariectomized, E2; 17β-estradiol, OVX + E2; OVX with E2 pellet implantation, ALT; alanine aminotransferase. *P < 0.05 compared with OVX-HFD-Fr group. Data are mean ± SD.

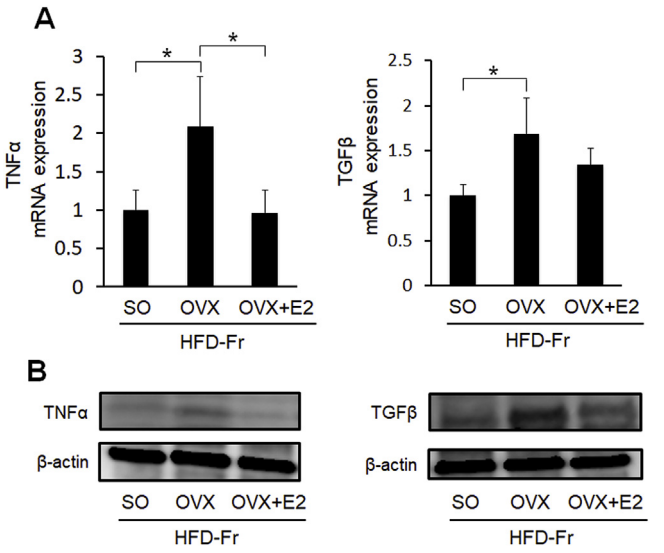

Fig. 9. Effects of E2 replenishment on hepatic cytokine expression in OVX-HFD-Fr mice. An E2 containing pellet was implanted into ovariectomized mice and the mice fed a HFD with fructose intake for 12 weeks. A; hepatic mRNA expressions of TNFα and TGFβ. To control for variations in reactions, all PCR data were normalized against GAPDH. The fold changes were calculated as the ratio of the expression levels of those in the SO-HFD-Fr group. Data were presented as mean ± SD. *$P < 0.01$. Five animals in each group. B; Representative immunoblotting for TNFα and TGFβ. Western blotting was performed to evaluate the protein expression levels in mice livers.

two times than that of the OVX-HFD group. This liver damage was accompanied by marked inflammation of F4/80 positive macrophages, probably induced by upregulated MCP-1. We speculate that high fructose intake and ovariectomy synergistically enhanced liver damage; high fructose intake predominantly induced hepatocyte death by increased oxidative stress, and ovariectomy mainly expanded tissue destruction via sustained proinflammatory activation.

Ballooning degeneration of hepatocytes, hCLS, and fibrosis are the pathological characteristics for NASH. Either high fructose intake or ovariectomy on HFD mice modestly induced these pathological features, and coexistence of these insults increased liver disease progression. Our observations would be helpful to understand nutritional and hormonal correlations involved in the pathogenesis of NAFLD, however, we cannot simply apply our interpretations derived from this mice study to human liver disease; fructose was given an extremely high amount and estrogen withdrawal was induced by ovariectomy. Further investigation is necessary to evaluate the utility of fructose restriction in postmenopausal women.

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Conflicts of interest

The authors declare that there is no duality of interest associated with this manuscript.

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