MASTER'S THESIS

A high-sucrose diet enhances high-fat diet-induced osteoarthritis pathogenesis

Yunhui Min

Interdisciplinary Graduate Program of Advanced Convergence Technology &Science

GRADUATE SCHOOL

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A high-sucrose diet enhances high-fat diet-induced osteoarthritis pathogenesis

Yunhui Min

(Supervised by professor Young-Ok Son)

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This thesis has been examined and approved by

....

Thesis director, Dong-Sun Lee, Prof. of Faculty of Biotechnology, Jeju National University

....

Inhae Kang, Prof. of Food Science and Nutrition, Jeju National University

....

Young-Ok Son, Prof. of Animal Biotechnology, Jeju National University

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Date

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Technology &Science
GRADUATE SCHOOL
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국문 초록

퇴행성 관절염 (Osteoarthirits, OA)은 만성적으로 낮은 수준의 염증과 파괴된 연골을 특징으로 갖는다. 높은 지방의 섭취는 비만을 유발하고 무릎의 퇴행성 관절염 발생 위험도를 높인다. 그러나 높은 당분의 섭취이 퇴행성 관절염의 병리생성에 미치는 영향은 명확하게 설명되지 않았다. 따라서 실험적으로 유도한 퇴행성 관절염 모델에 고지방 식단 및 고지방+고당 식단을 제공하여 그에 대한 영향을 조사하였다.

8 주령 수컷 C57BL/6 생쥐에게 일반 사료 (Chow)와 고지방 사료 (HF), 고지방+고당 사료 (HF+HS)를 12 주동안 급여하였다. 이후 내측반월판절재술 (DMM)을 실시하여 각 개체에 실험적 퇴행성 관절염을 유도하였고, 8 주동안의 실험적 식이요법을 추가로 실시하였다. 식이 실험 종료 후 각 개체의 간에서의 염증성 사이토카인 수준 및 지질 대사의 분석을 통해 무릎의 퇴행성 관절염의 병리생성에 대해 조사하였다.

그 결과, 반월판 절제술을 받은 고지방+고당 식이 그룹에서 고지방 식이 그룹보다 간의 지질 축척이 증가하여 지방간 형성을 증가시켰다. 간에서 또한 염증성 사이토카인과 섬유 형성 표시 유전자의 발현 수준이 반월판 절제술을 받은 고지방 식이 그룹보다 반월판 절제술을 받은 고지방+고당 식이 그룹에서 증가하였다.

이와 같은 결과로, 고지방 식단의 추가적인 고당 섭취는 간에서 염증, 지질 추적 및 섬유화를 증가시켜 반월판 절제술을 통해 유도된 실험적 퇴행성 관절염 모델 생쥐의 퇴행성 관절염 병리발생을 촉진시킴을 알 수 있다. 이런 간 조직의 생화학적 변화는 퇴행성 관절염의 지표로서 작용할 수 있다.

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ABSTRACT

Objective: Osteoarthritis (OA) is marked by chronic low-level systemic inflammation and cartilage destruction. High dietary fat intake which causes obesity is reported to increase the risk of knee OA-development. However, the impact of high dietary sugar intake on the pathogenesis of OA have not been elucidated yet. Therefore, we investigated the effects of a high-fat and high-sucrose (HF+HS) diet in an experimental OA mouse model.

Design and methods: Eight-week-old male C57BL/6J mice were fed a standard chow diet (n = 6), high-fat (HF) diet (n = 5), or a HF+HS diet (n = 7) for 12 weeks, at which point they underwent surgical destabilization of the medial meniscus (DMM), and received the same experimental diets for an additional 8 weeks. The pathogenesis of knee OA, obesogenic parameters and inflammatory cytokines in the liver and adipose tissue were investigated in the experimental mice.

Results: HF+HS induced severe cartilage erosion with osteophyte development and subchondral bone plate thickening, which indicated HF+HS diet exacerbated OA. Despite of marginal differences in metabolic parameters, hepatic free cholesterol accumulation was increased in DMM-induced HF+HS diet-fed mice than in HF diet-fed mice. More strikingly, the inflammatory cytokines and fibrosis marker were increased in the livers of DMM-induced HF+HS diet-fed mice than control group. However, adipose tissue remodeling did not alter by HF+HS.

Conclusions: These findings indicated that excessive inclusion of the sucrose in HF diet triggered hepatic inflammation/fibrosis in non-alcoholic fatty liver disease (NAFLD), which contributes to the pathogenesis of OA in DMM-induced experimental OA mice

Keywords: osteoarthritis, high-fat, high-sucrose, inflammation, fibrosis

I.   INTRODUCTION

Cartilage destruction and chronic low-level of systemic inflammation are hallmarks of osteoarthritis (OA)(Collins, Hart et al. 2018). Obesity which is well known to associate with chronic systemic inflammation, is a risk factor for the development of knee OA(Coggon, Reading et al. 2001, Marshall, Bockstahler et al. 2009, Lauren K. King, Lyn March et al. 2013), mainly as a result of increased mechanical stress on the knee joints. The most noticeable impact of obesity in OA is a mechanical system including structural joint damage by inducing increased forces on the joint. This is accompanied by decreased muscle strength, metabolic imbalances, and increased levels of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α)(Emanuela, Grazia et al. 2012, Berenbaum, Eymard et al. 2013, Lauren K. King, Lyn March et al. 2013, Sellam and Berenbaum 2013).

According to the World Health Organization, 39% of adults over the age of 18 years were overweight in 2016, with increased intake of high-calorie foods, such as high-fat (HF) and high-sucrose (HS) fast foods, cited as a major contributing factor. Recent studies have suggested that a HF with high-glucose fast food diet can induce metabolic syndrome(Moreno-Fernandez, Garces-Rimon et al. 2018), a varied pattern of diseases associated with several risk factors, such as obesity, mixed dyslipidemia, and hyperglycemia(Dissard, Klein et al. 2013). Moreover, metabolic syndrome increases the incidences of type 2 diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD)(Moreno-Fernandez, Garces-Rimon et al. 2018). Therefore, many researchers have studied the effects of HF and HS diets, major contributing factors of metabolic syndrome, in various experimental disease models to prevent diseases. It is well known that a HF diet may lead to visceral obesity, muscle insulin resistance, and impaired glucose tolerance(Kim, Nolte et al. 2000). The metabolic syndrome comprises hyperglycemia, hyperinsulinemia, hypertension, and central adiposity, which is often associated with a HF diet(Collins, Martin et al. 2004). Emerging evidence demonstrate that a HF diet induces structural and symptomatic OA in mice, manifested as unique biomechanical,

neurobehavioral, and inflammatory changes(Griffin, Fermor et al. 2010). The pathogenesis of HF diet-induced OA involves systemic inflammation modulated by anti-inflammatory cytokines (IL-1Ra, IL-4) and adipokines (leptin and adiponectin)(Griffin, Fermor et al. 2010, Griffin, Huebner et al. 2012, Collins, Hart et al. 2018). In a previous study, accelerated cartilage degeneration was observed in HF diet-fed mice compared with that in lean diet-fed mice; this was accompanied by increased expression levels of leptin and poly (ADP-ribose) polymerase p85, an apoptotic marker, in the articular chondrocytes(Datta, Zhang et al. 2017). The effects of a HF diet on the development of various diseases have already been studied extensively. Chronic intake of HS reported to exacerbates HF diet-induced obesity and metabolic complication, such as NAFLD(Ragab, Abd Elghaffar et al. 2015). However, the effects of a HS diet have received less attention despite the high sugar content of HF-containing fast foods. Therefore, we investigated the effects of a HF+HS diet in mice with OA induced via surgical destabilization of the medial meniscus (DMM). We hypothesized that a HS diet promotes HF diet-induced OA via metabolic syndrome-related pathways. To test this hypothesis, we analyzed physical and biochemical changes in the knee joints, blood, livers, and adipose tissue of DMM-induced OA mice fed a HF or HF+HS diet.

II.   MATERIALS AND METHODS

2.1 Chemicals and laboratory ware

Unless otherwise stated, chemicals and laboratory wares were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Becton-Dickinson (Franklin Lakes, NJ, USA), respectively.

2.2 Experimental OA model and diet

Male C57BL/6J mice were used for the experimental OA model. All procedures were approved by the Institutional Animal Care and Use Committee at the Jeju National University (2020-0014). The mice were fed a chow diet (n = 6), HF diet (n = 5), or HF+HS diet (n = 7) for 20 weeks [Fig. 1(A)]; experimental OA was induced after 12 weeks via surgical DMM(Glasson, Blanchet et al. 2007, Cho, Kang et al. 2019, Min, Kim et al. 2020). We prepared the fodder with Mouse 18% Auto LabDiet 5L79 (Orient Bio, Gyonggi, Korea) for the chow and the experimental diet groups. The isocaloric HF diet (HF group, 41% calories from fat) or HF diet containing sucrose (HF+HS group, 41% calories from fat, 37% calories from sucrose) were given to HF or HF+HS group. The fodder composition was adapted from the AIN-93M diet(Päivärinta, Pajari et al. 2006) (Supplementary Table S1). Mice were sacrificed for histological and biological analyses 8 weeks after DMM surgery, i.e., 20 weeks after starting the controlled diets.

2.3 Glucose tolerance test (GTT)

A glucose tolerance test (GTT) was performed on overnight fasted mice. Mice were fasted for 12 h before intraperitoneal injection of 10% d-glucose solution (0.5 g per kg body weight). The blood glucose levels were measured at 10, 20, 30, 60, and 120 min after d-glucose injection using a glucose meter (Midium Blood Glucose Analyzer; Kia Ace Co., Ltd, Gyeonggi, Korea).

2.4 Measurement of plasma and hepatic lipid levels

Upon completion of the experiment, the animals were fasted for 12 h and sacrificed by carbon dioxide asphyxiation. Blood was collected via cardiac puncture, and serum samples were aliquoted after coagulation followed by centrifugation. Serum TC (mg/dL) was analyzed using an enzyme assay kit (Asan Pharmaceutical Co., Seoul, Korea) and absorbance measurements at 500 nm.

To measure lipid contents in hepatic tissue lipids were extracted from 0.2 g of liver tissue using a chloroform:methanol (2:1) solution. The TG, TC, free cholesterol, non-esterified fatty acid contents were then analyzed using an enzyme assay kit (Asan Pharmaceutical Co., Seoul, Korea, Abcam, Cambridge, MA, USA and Wako Pure Chemicals, Osaka, Japan) and normalized to g liver (mg/g liver).

2.5 Reverse transcription-polymerase chain reaction (RT-PCR)

Upon completion of the experiment, 0.1 g of liver tissue and 0.2 g of the epididymal fat tissue were quickly transferred to a freezer (-80 °C) until required for RNA extraction using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA). The concentration of extracted RNA was measured using a Nano-200 Micro-Spectrophotometer (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou City, China), and cDNA was synthesized by reverse transcription kits (Applied Biosystems, Waltham, MA, USA). Gene expression was determined using real-time PCR (CFX96 RealTime PCR Detection System; Bio-Rad, Hercules, CA, USA, at Bio-Health Materials Core-Facility at Jeju National University). The PCR primers are summarized in Supplementary Table S2. The average values of hypoxanthine phosphoribosyltransferase (HPRT) or ribosomal protein lateral stalk subunit P0 (RPLP0) were used to represent the internal control gene.

2.6 Hematoxylin and Eosin (H&E) staining and adipocyte size analysis

The liver and epididymal adipose tissues were dissected from mice and immediately fixed in 10% buffered formalin. Tissue were embedded in paraffin, sectioned at 4 μm thicknesses and stained with hematoxylin and eosin (H&E) staining. All imaging of slides was performed using an LEICA DM 2500 microscope (Tucsen Photonics Co., Ltd.). H&E stained sections of epididymal adipose tissue was used for size determination by following the published protocol by Seo et al(Seo, Jo et al. 2021). Briefly, adipocyte size was examined by analyzing digital images of H&E-stained paraffin sections by using Image J software (NIH, USA).

2.7 Safranin O staining

The knee joints, livers, and abdominal adipose tissue from the experimental OA mice were fixed in 4% paraformaldehyde and embedded in paraffin. Knee joints were decalcified with 0.5 M ethylenediaminetetraacetic acid (pH 7.4) for 2 weeks. Five-micron-thick sections of the paraffin blocks were deparaffinized with xylene and hydrated with graded ethanol. The section of the knee joint was stained with Harris hematoxylin, Fast green, and safranin O (all obtained from Sigma-Aldrich). The liver and abdominal adipose tissue sections were stained with Mayer's hematoxylin (Dako, Carpinteria, CA, USA) and eosin Y (Sigma-Aldrich). The pathogenesis of OA was scored by five observers under unlabeled conditions using the Osteoarthritis Research Society International (OARSI) scoring system, including the OARSI grade (0–6), osteophyte maturity (0–3), and synovitis score (0–3)(Glasson, Chambers et al. 2010). The results of the OA pathogenesis grades were calculated using the average of observer's scoring in each mouse, and the representative image of safranin O was selected from the most developed lesion among sequential sections(Min, Kim et al. 2020).

2.8 Statistical analysis

　　Statistical analyses were performed using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, NY, USA). Data of histological experiments were analyzed using the non-parametric Mann–Whitney U test, one-way analysis of variance with Bonferroni's multiple comparison test, or Student's t-test. The 'n' number represents the number of independent experiments or mice. Statistical significance was set at $P < 0.05$.

III.   RESULTS

3.1 HF and HF+HS diets induced body weight, glucose intolerance and hypercholesterolemia

Experimental OA was induced after 12 weeks via surgical DMM into C57BL/6J mice [Fig. 1(A)]. Mice were fed a HF or HF+HS diet for 20 weeks to analyze the effects on the pathogenesis of OA. After 20 weeks, various metabolic parameters were analyzed in DMM-induced OA mice. With expectation, the body weight was higher in the experimental groups than in mice fed a standard chow diet, but no differences in HF vs. HF+HS groups [Fig. 1(B–D)]. To test whether HF and/or HF+HS worsen glucose intolerance, we conducted glucose tolerance test (GTT). After intraperitoneal glucose administration, the 120-min glucose disposal rate was slower in the HF and HF+HS diet-fed mice than the chow diet-fed mice [Fig. 1(E)]. Next, we calculated the area under the curve (AUC) of GTT, an indication of intracellular glucose absorption(Dinger, Mohr et al. 2018); GTT AUC was larger in the HF and HF+HS diet groups than the chow diet group, but no differences in HF vs. HF+HS group [Fig. 1(F)]. Consistently, measurement of fasting TC levels in DMM-induced OA mice revealed significantly higher levels in the HF and HF+HS diet groups than the chow diet group, but no differences in HF vs. HF+HS group [Fig. 1G)]. These results suggested that HF and HF+HS diets showed glucose intolerance and dyslipidemia in DMM-induced OA mice(Ebihara and Nakao 2015).
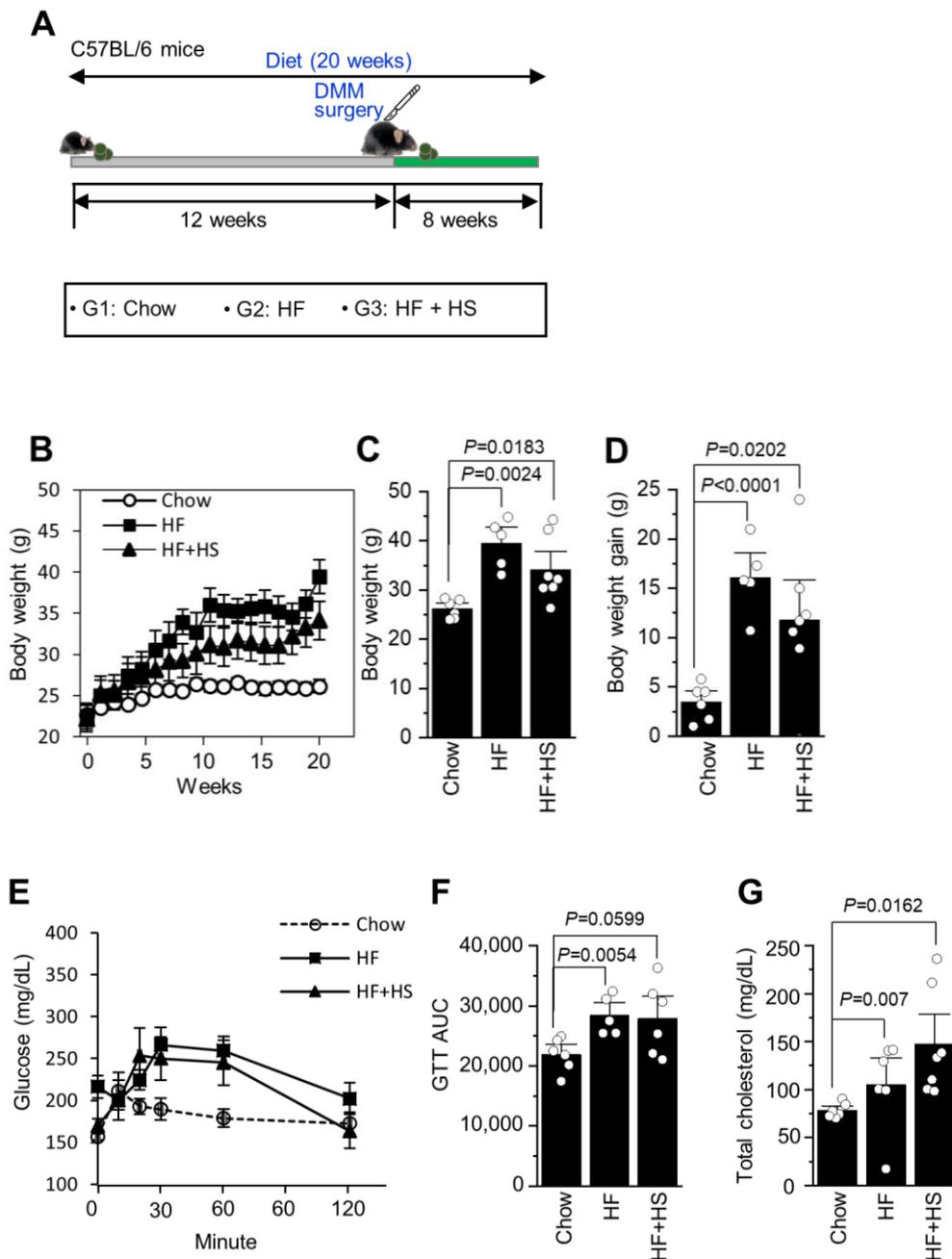
Figure 1. The effects of sucrose added to a high-fat (HF) diet in experimental osteoarthritis (OA) mice induced by surgical destabilization of the medial meniscus (DMM). **A,** Schematic diagram of 20-week controlled diets fed to DMM-induced mice, indicating the point of surgical intervention at 12 weeks. The mice were fed a standard chow diet, HF diet, or high fat + high sucrose (HF+HS) diet. **B,** Weekly body weight measurements. **C,** Final body weight. **D,** Body weight gain. **E,** Glucose tolerance test (GTT) in fasted mice. **F,** Fasting GTT

area under curve (AUC) values. **G,** Total cholesterol concentrations (mg/dL) in the blood of fasted mice. The values are presented as the mean ± standard error of the mean.

3.2 HF and HF+HS diet-mediated obesity promotes OA pathogenesis in mice

To investigate the effects of HF and HF+HS diets on OA pathogenesis, we performed surgical DMM on HF and HF+HS diet mice at 12 weeks after the initiation of a 20-week experimental diet(Min, Kim et al. 2020). We compared the OA parameters of the different groups using the OARSI grade (0–6), subchondral bone plate (SBP) thickness, osteophyte size, osteophyte maturity (0–3), and synovitis (0–3). Safranin O-stained images and OARSI grades indicated that the DMM-induced OA mice fed a HF+HS diet displayed a significantly greater degree of cartilage damage than chow diet-fed mice (p = 0.0350) [Fig. 2(A) and 2(B)]. Furthermore, the SBP was thicker in DMM-induced OA mice that received the HF+HS diet vs. standard chow (p = 0.0515), indicating increased subchondral sclerosis in the HF+HS diet group [Fig. 2(A) and (C)]. Osteophyte size (p = 0.0524) and osteophyte maturity (p = 0.0221), indicators of osteophyte formation, were increased in HF+HS diet-fed mice compared with that in chow diet-fed mice [Fig. 2(D) and 2(E)]. Additionally, there was a significantly higher degree of inflammation in the synovial membranes of the HF+HS diet-fed vs. the chow diet-fed mice after DMM surgery, based on safranin O-stained tissue sections [Fig. 2(F)] and the synovitis grading (p = 0.0140) [Fig. 2(G)]. All of the OA disease parameters were increased in DMM-induced HF and HF+HS diet-fed mice, but the differences were not significant compared with the parameters between HF vs. HF+HS.
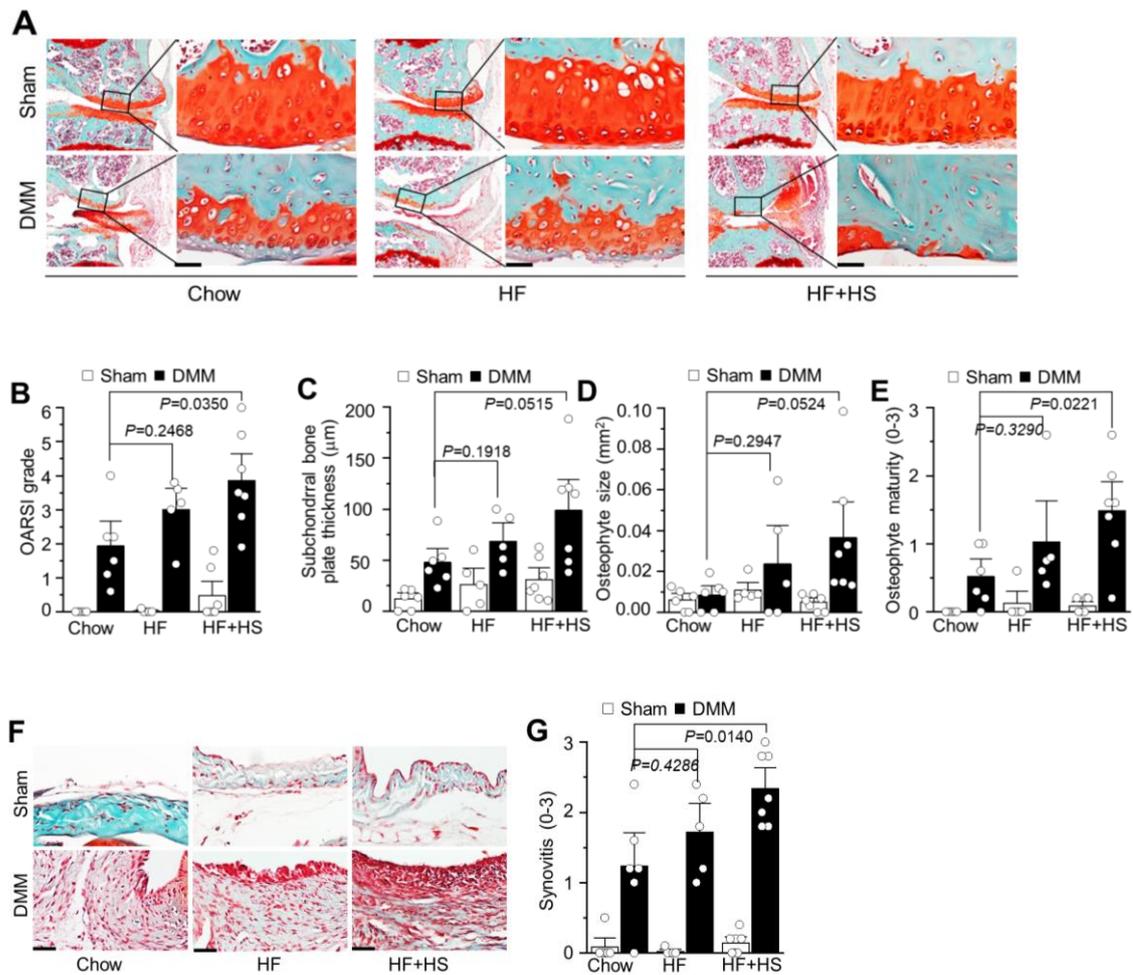
Figure 2. A high-fat, high-sucrose (HF+HS) diet promoted cartilage degeneration and synovitis in experimental osteoarthritis (OA) mice induced by surgical destabilization of the medial meniscus (DMM). Representative images of safranin O-stained frontal knee joints from DMM-induced mice fed a standard chow diet, HF diet, or HF+HS diet: **A,** Whole joint (50× magnification) and cartilage (200× magnification). Various OA disease parameters were assessed: **B,** OARSI grade. **C,** Subchondral bone plate thickness. **D,** Osteophyte size. **E,** Osteophyte maturity. **F,** Histological appearance of synovitis. **G,** Quantification of synovitis. The values are presented as the mean ± standard error of the mean and have been evaluated using the Mann–Whitney U test. D. Scale bar = 50 μm.

3.3 High sucrose consumption exacerbates HF diet-induced hepatic free cholesterol accumulation, inflammation and fibrosis

HS is well reported to disrupt hepatic lipid accumulation which consequently burdens the liver(Ipsen, Lykkesfeldt et al. 2018). To test whether HF+HS diet induces hepatic steatosis and stress, the H&E staining of liver tissue were investigated. We observed the enlarged hepatocytes and increased numbers of lipid droplets in the livers of HF and HF+HS diet-fed mice compared with those in chow diet-fed mice [Fig. 3(A)](Ragab, Abd Elghaffar et al. 2015). The hepatic lipid accumulation were confirmed by measuring the levels of hepatic TG, TC, free cholesterol and non-esterified fatty acid (NEFA)(Kim, Yun et al. 2017). Hepatic TG and NEFA were significantly increased in DMM-induced HF and HF+HS diet-fed mice compared with those in DMM-induced OA mice fed a standard chow diet, but no differences between HF vs. HF+HS [Fig. 3(B) and 3(E)].The levels of total cholesterol and diacylglycerol acyltransferase 2 (DGAT2) in liver, which is the markers for mammalian triglyceride synthesis(Suzuki, Tobe et al. 2005, Yen, Stone et al. 2008), were higher in the liver tissue of DMM-induced OA mice fed HF+HS diets vs. those fed on standard chow [Fig. 3(C) and 3(F)]. Free cholesterol in liver is well reported to associate with liver fibrosis aggravation by involving toll-like receptor 4 (TLR4) and/or transforming growth factor-β (TGF-β) mechanisms(Tomita, Teratani et al. 2014). We next wondered whether HF+HS diet-fed mice alter free cholesterol levels in liver. Interestingly, the levels of free cholesterol in liver were significantly higher in HF+HS-fed mice vs. those fed on HF-fed mice [Fig. 3(D)](Tomita, Teratani et al. 2014).
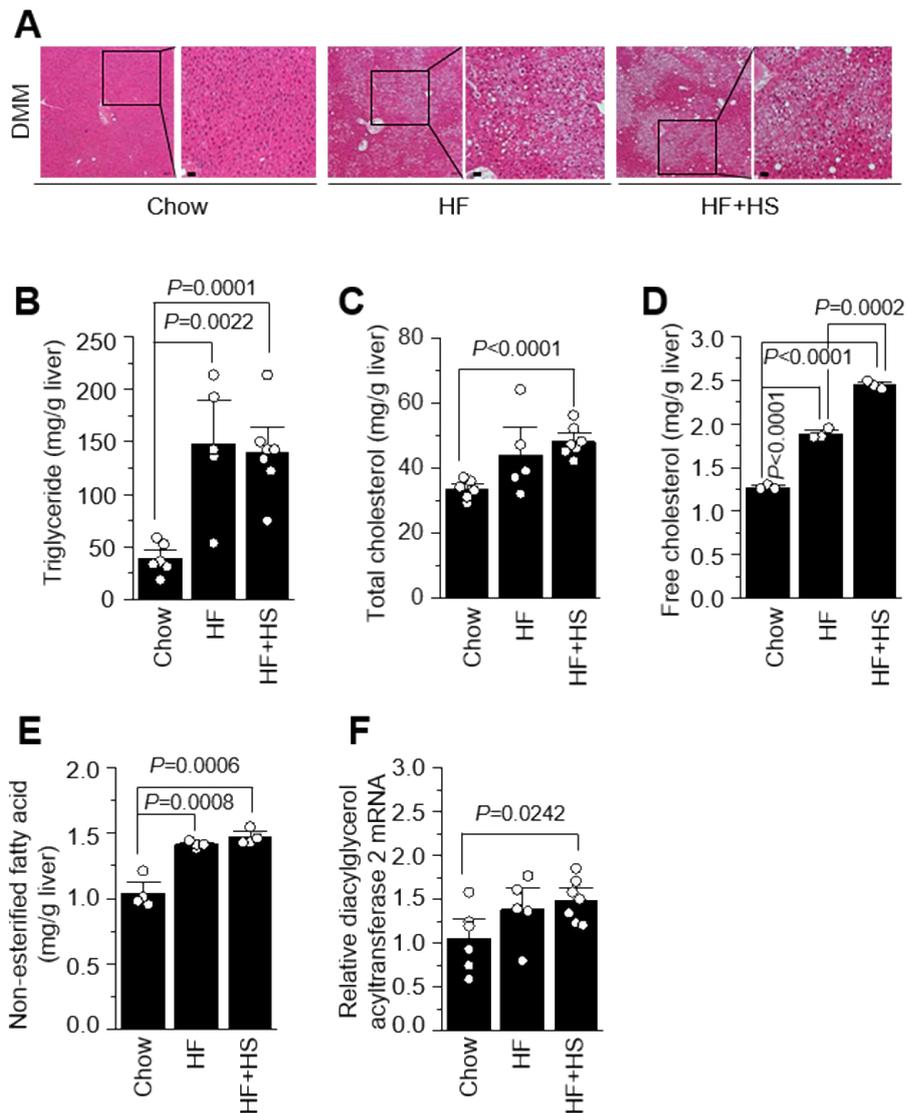
Figure 3. The effects of a high-fat, high-sucrose (HF+HS) diet on lipid metabolism in the liver tissue of experimental osteoarthritis (OA) mice induced by surgical destabilization of the medial meniscus (DMM). **A,** Hematoxylin and eosin-stained liver tissue sections (50× and 200× magnification) of DMM-induced mice fed a standard chow diet, HF diet, or HF+HS diet. **B,** Hepatic triglyceride concentration (mg/g liver). **C**, Hepatic total cholesterol concentration (mg/g liver). **D**, Hepatic free cholesterol concentration (mg/g liver). **E,** Hepatic non-esterified fatty acid (NEFA) levels (mg/g liver). **F,** lipid metabolism is presented by the expression of DGAT2 in the liver of OA-induced diet mice. The values are presented as the mean ± SEM and have been evaluated using the student's t-test. DMM, destabilization of the medial

meniscus; HF, high-fat diet; HF+HS, high fat+high sucrose diet; NEFA, non-esterified fatty acid; DGAT2, diacylglycerol acyltransferase. Scale bar = 50 μm.

High fructose-mediated *de novo* lipogenesis is promoted by inflammation and consequently contribute to liver fibrosis(Todoric, Di Caro et al. 2020). To investigate the sugar addition on hepatic inflammation and fibrosis in DMM-induced OA mice, we analyzed the relative mRNA expression levels of various inflammatory and fibrosis-related factors. The mRNA expression levels of pro-inflammatory cytokines, such as TNF-α (p = 0.0212 and 0.0084 respectively) and IL-6 (p = 0.0295 and 0.00293 respectively), were increased in the livers of DMM-induced both HF and HF+HS diet-fed mice compared with those in DMM-induced chow diet-fed mice [Fig. 4(A) and 4(C)]. Consistently, the mRNA expression levels of transforming growth factor-beta (TGF-β), markers of fibrosis(Roehlen, Crouchet et al. 2020) has a similar tendency. Interestingly, the mRNA expression levels of monocyte chemotactic protein 1 (MCP1) was increased in DMM-induced HF+HS diet-fed mice (p = 0.0198) compared with those in DMM-induced chow diet-fed mice, but not in HF group [Fig. 4(B)]. Consistently, the mRNA expression levels of TLR4 was significantly increased in DMM-induced HF+HS diet mice (p = 0.0051) compared with those in DMM-induced chow diet-fed mice [Fig. 4(E)]. The mRNA expression levels of collagen type 1 alpha 1 (col1a1), one of the most characteristic substances seen in liver fibrosis, were also significantly increased in DMM-induced HF+HS group (p=0.0053), but not in HF-fed group, compared with those in DMM-induced chow diet-fed mice [Fig. 4(H)]. The mRNA expression levels of IL-1β and NOD-like receptor pyrin domain-containing protein 3 (NLRP3), an indicator of the inflammasome(Haneklaus and O'Neill 2015), were not altered in the experimental groups when compared with those in the chow diet-fed mice despite the trend of stepwise incline in mice fed with HF+HS>HF>Chow [Fig. 4(D) and (F)].

Collectively, these data demonstrated that addition of sugar to HF diet worsen the progression of simple NAFLD to hepatic fibrosis with inflammation.
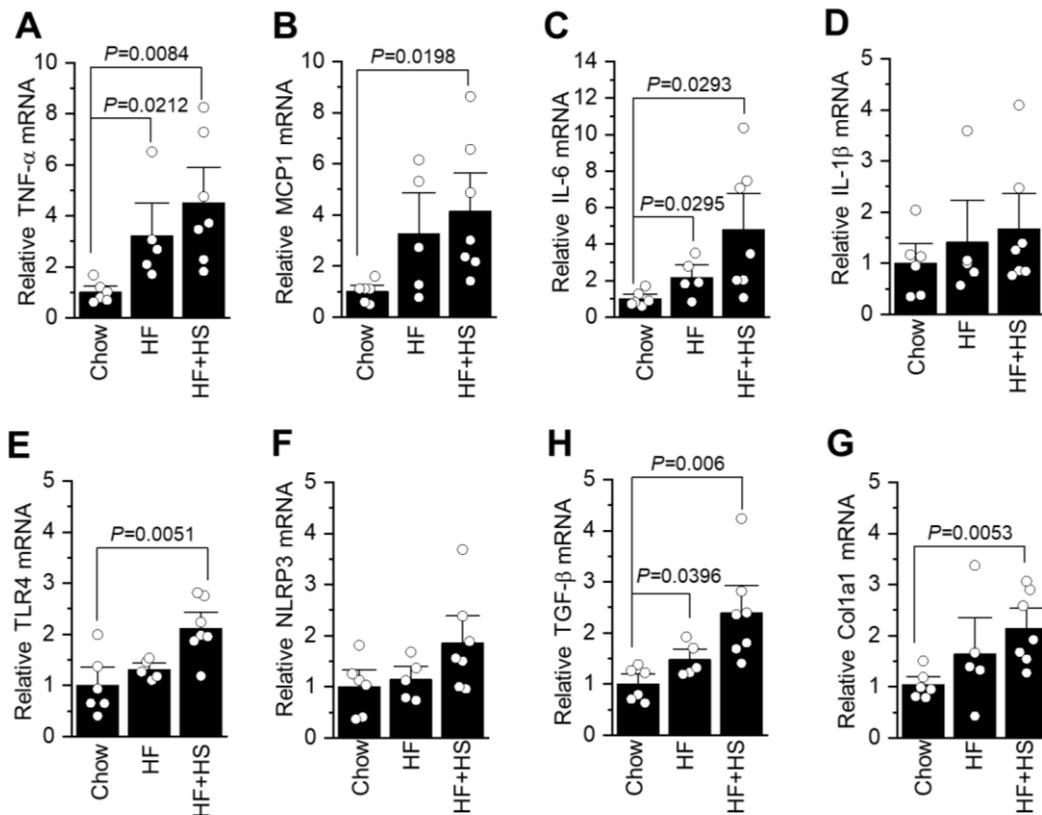
Figure 4. The effects of a high-fat, high-sucrose (HF+HS) diet on liver inflammation in experimental osteoarthritis (OA) mice induced by surgical destabilization of the medial meniscus (DMM). The relative mRNA expression levels of pro-inflammatory cytokines were measured in the liver tissue of DMM-induced mice fed a standard chow diet, HF diet, or HF+HS diet: **A,** Tumor necrosis factor-alpha (TNF-α); **B,** Monocyte chemotactic protein 1 (MCP1); **C,** Interleukin 6 (IL-6); **D,** Interleukin 1 beta (IL-1β). The relative mRNA expression levels of various inflammatory factors were evaluated: **E,** Toll-like receptor 4 (TLR4); **F,** NOD-like receptor pyrin domain-containing protein 3 (NLRP3); G**,** Transforming growth factor-beta (TGF-β). H**,** Relative mRNA expression levels of the extracellular matrix component, collagen type 1 alpha 1 (col1a1). The values are presented as the mean ± standard error of the mean and have been evaluated using the student's t-test.

3.4 HF and HF+HS diets promote adipocyte hypertrophy in DMM-induced OA mice

To investigate whether inclusion of high sucrose to HF diet alters adipocyte hypertrophy in DMM-induced OA mice, we analyzed adipose tissue histology. Adipocyte size is significantly increased in the adipose tissue of the experimental diet groups compared with that in the adipose tissue of the chow diet-fed group. However, there were no differences between HF vs. HF+HS group [Fig. 5(A) and 5(B)]. Immune cell infiltrations in adipose tissue are crucial for the determination of adipose tissue dysfunction(Guzik, Skiba et al. 2017). Thus, we next checked the crown-like structure (CLS) density of the adipocytes, which represents macrophage accumulation(Murano, Barbatelli et al. 2008). CLS was increased in DMM-induced HF and HF+HS diet-fed mice compared with that in chow diet-fed mice, but no differences between HF vs. HF+HS group [Fig. 5(C)]. We further analyzed the expression levels of genes related to fatty acid synthesis and macrophage accumulation by using real-time PCR in adipose tissue. The expression levels of adipocyte protein 2 (aP2), related to insulin resistance and inflammation and also known as fatty acid-binding protein 4(Furuhashi 2019), was not altered in the experimental diet groups compared with those in the chow diet group (Supplementary Fig. S2(A)). The expression levels of DGAT2 and fatty acid synthase (FAS) related obesity(Boizard, Le Liepvre et al. 1998) was not altered in DMM-induced HF or HF+HS diet-fed mice (Supplementary Fig. S2(B) and 2(C)). The expression levels of macrophage accumulation-related factors, F4/80(Weisberg, McCann et al. 2003) and MCP1(Kanda, Tateya et al. 2006), were increased only in DMM-induced HF diet-fed mice (Supplementary Fig. S2(D) and 2(E)).
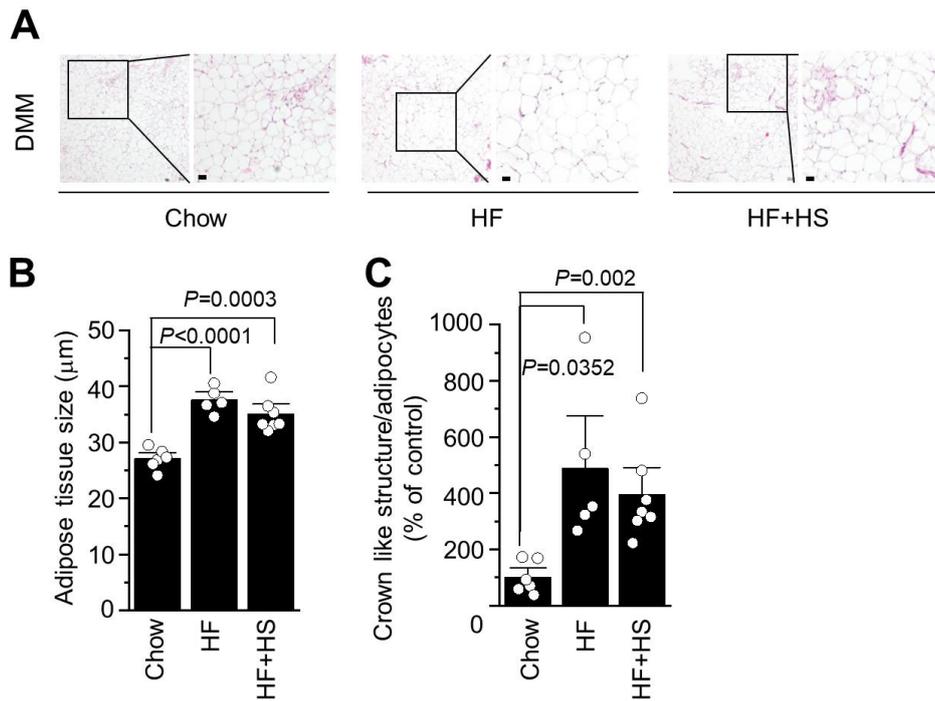
Figure 5. The effects of a high-fat, high-sucrose (HF+HS) diet on adipose tissue metabolism and inflammation in experimental osteoarthritis (OA) mice induced by surgical destabilization of the medial meniscus (DMM). **A,** Hematoxylin and eosin-stained adipose tissue sections of DMM-induced mice fed a standard chow diet, HF diet, or HF+HS diet. **B,** Quantification of adipose tissue size. **C,** Crown-like structure density. The values are presented as the mean ± standard error of the mean and have been evaluated using the student's t-test. Scale bar = 50 μm.

IV.   DISCUSSION

The "western diet", including high saturated fat, cholesterol and sugar, is well known nutritional patterns to increase the risk of obesity and its associated metabolic complications, such as cardiovascular diseases and type 2 diabetes. The pathogenesis of OA, both mechanically and physiologically, is closely related to obesity (Coggon, Reading et al. 2001, Berenbaum, Eymard et al. 2013, Lauren K. King, Lyn March et al. 2013). In this study, we investigated the effects of added sucrose on pathogenesis of OA and metabolic patterns in a HF diet fed to experimental OA mice induced via surgical DMM. Here, we demonstrated that the addition of sugar into HF diet (HF+HS) did not significantly increase body weight, glucose intolerance, dyslipidemia and/or adipose tissue remodeling compared to HF diet [Fig. 1 and 5]. Nevertheless, hepatic cholesterol, inflammation, and fibrosis were significantly higher in HF+HS diet-fed mice than in chow fed diet-fed mice, and no differences between Chow vs. HF group [Fig. 3 and 4]. Although the pathogenesis of OA did not reach statistical significance between HF vs. HF+HS, both HF and HF+HS group showed more pronounced signs of OA than chow diet-fed mice, including cartilage destruction, OARSI grade, SBP thickness, osteophyte size, and osteophyte maturity (trend of stepwise incline in HF+HS> HF> Chow) [Fig. 2]. To our knowledge, this is the first study to report the addition of sucrose which is made up of glucose and fructose worsens hepatic inflammation accompanied by hepatic cholesterol accumulation with pathogenesis of OA.

The first metabolic impact of HS on DMM induced OA is that HF+HS diet promoted fatty liver disease in mice by increasing the expression levels of lipid metabolism-related genes in the liver, as evidenced by increased lipid accumulation compared with that in the HF diet-fed mice (Fig. 3). In particular, the both hepatic TG and NEFA levels were increased in DMM-induced HF and HF+HS diet-fed mice compared with those in chow diet-fed mice [Fig. 3(B) and 3(E)]. Hepatic total cholesterol content and mRNA expression levels of DGAT2 in the liver tissue were higher in DMM-induced HF+HS diet-fed mice [Fig. 3(C) and 3(F)]. These

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results suggest a link between hepatic steatosis, especially hepatic cholesterol accumulation and the pathogenesis of OA. In particular, cholesterol metabolism has been associated with the pathogenesis of OA(Triantaphyllidou, Kalyvioti et al. 2013, Choi, Lee et al. 2019, Villalvilla, Larranaga-Vera et al. 2020). High cholesterol levels have been observed in OA mice in a study that demonstrated the relationship between cholesterol metabolism and OA pathogenesis in a high-cholesterol diet OA mouse model(Choi, Lee et al. 2019). Other researchers demonstrated that high-density lipoprotein cholesterol-related factors (apolipoprotein A-I and lecithin: cholesterol acyltransferase) regulate catabolic factors (matrix metalloproteinase [MMP]-2, MMP-9, and MMP-13) and collagen II, an anabolic factor, and are closely related to non-alcoholic, fatty acid-associated accumulation of lipids in the liver(Triantaphyllidou, Kalyvioti et al. 2013). In accordance with this, our data indicated that high cholesterol levels in the blood and liver tissue of HF+HS diet-fed mice were closely associated with signs of OA and other indices of dyslipidemia.

Next, we postulated that liver damage/ inflammation is related to the pathogenesis of OA. Although the plasma level of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase, which are well-known markers for liver injury were not significantly different among groups (Supplementary Fig. S1), the HF+HS diet promoted liver inflammation, evidenced by increased mRNA expression levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) and other inflammatory factors (TLR-4, NLRP3, and MCP1 [Fig. 4(A–F)](Jia, Vianna et al. 2014, Nakamoto and Kanai 2014, Haneklaus and O'Neill 2015). The mRNA expression levels of col1a1 and TGF-β were significantly increased in DMM-induced HF+HS diet-fed mice compared with those in HF diet-fed mice [Fig. 4(H) and 4(G)]. Moreover, HF+HS diet-fed mice showed higher hepatic free cholesterol levels than HF diet-fed mice [Fig. 3(D)]. These data is well aligned with the notion that accumulation of hepatic free cholesterol is closely associated with hepatic stress and fibrosis in NAFLD(Tomita, Teratani et al. 2014). Accumulation of free cholesterol and oxidized low-density lipoprotein is associated with portal inflammation and fibrosis in nonalcoholic fatty liver disease(Ho, Ho et al. 2019).

Hepatic free cholesterol accumulation in especially hepatic stellate cells (HSC) accelerated hepatic fibrosis by upregulation of TLR4 and downregulation of endosomal-lyosomal degradation pathways, and thereby sensitizing activation of TGF-β-mediated pathways(Tomita, Teratani et al. 2014). Although we did not measure free cholesterol levels in HSC and/or HSC activation mechanisms, but it is plausible to assume free cholesterol levels in liver may affect fibrosis. However, it is still vague whether hepatic free cholesterol accumulation-mediated hepatic fibrosis affects pathogenesis is OA. Emerging evidence showed that about one-third of patients with arthritis have NAFLD and patients with psoriasis and psoriatic arthritis have a higher risk of NASH and fibrosis(Klujszo, Parcheta et al. 2020). It indicated that arthritis and liver stress mediated by NALFD have close relationship. One of the recent study mentioned that the multiplied organ failure are occurred by an overwhelming of systemic inflammation which mediated by endocrine and metabolic response(Singer, De Santis et al. 2004). Thus, it is plausible to assume that i) HS addition into HF diet would exacerbate HF diet-induced hepatic inflammation with NAFLD (especially free cholesterol accumulation in liver) and ii) hepatic and/or systemic inflammatory condition may affect the similar inflammation in knee to develop OA or *vice versa*. A future study is required to unravel the initiator of meta-inflammation to cause systemic inflammation.

Third metabolic consequences that we focused was adipose tissue remodeling. In our study, the visceral adipocyte size is increased in DMM-induced HF and HF+HS diet-fed mice compared with that in chow diet-fed mice, but the adipocyte size was slightly lower in HF+HS diet-fed mice than in HF diet-fed mice [Fig. 5(A) and 5(B)]. Likewise, the expression of lipid metabolism-related genes, e.g., *FABP4*, *DGAT2*, and *FASN*, was not altered in the abdominal adipose tissue of DMM-induced HF or HF+HS diet-fed mice compared with that in chow diet-fed mice (Supplementary Fig. S2(A–C)). The CLS density, which reflects the degree of macrophage accumulation in adipose tissue(Murano, Barbatelli et al. 2008), was increased in DMM-induced HF and HF+HS diet-fed mice compared with that in chow diet-fed mice; however, there was no significant difference between the HF and HF+HS diet-fed groups [Fig.

5(C)]. The mRNA expression levels of macrophage accumulation-related genes (*ADGRE1* and *MCP1*) was increased in DMM-induced HF and HF+HS diet-fed mice compared with that in chow diet fed-mice, but the levels were slightly lower in HF+HS diet-fed mice than in HF diet mice (Supplementary Fig. S2(D) and 2(E)). HS into HF is reported to exacerbate HF diet induced adipocyte hypertrophy and inflammation(Yang, Miyahara et al. 2012). Kang et al. showed adipocyte size, F4/80 and inflammatory protein profiles both in adipose tissue and blood were significantly increased in HF+HS group compared to HF group(Kang, Espín et al. 2016). The different findings on adipocyte hypertrophy and inflammation in our data vs. other report might be due to different duration of food intake and background condition. Since all of mice were induced OA by DMM, it is hard to exclude the possibilities whether surgical procedures would affect the susceptibility of HS response. Future study with Sham with different dietary groups and test of optimal dose and period for diet sensitivity are necessary.

V. CONCLUSION

In this study, we investigated the effects of a HF+HS diet in DMM-induced OA mice and found that compared with a HF diet, a HF+HS diet promoted OA. Moreover, our results showed that lipid accumulation, inflammation, and fibrosis were increased in the liver tissue of HF+HS diet-fed mice compared with those in HF diet-fed mice. Adipose tissue hypertrophy and macrophage infiltration were not altered in HF+HS diet-fed mice than in HF diet-fed mice. Our data suggest that inflammation, fat deposition, and fibrosis in the liver ware more closely related to OA pathogenesis than is lipid metabolism in adipose tissue. However, there are uncertain questions that we did not answer: i) do hepatic inflammation and OA development crosstalk and what mechanisms involved?, ii) do systemic levels or knee inflammation also increased?, iii) does the surgical procedure (DMM) and/or food intake affect the metabolic parameters? Those unanswered questions are necessary to answer in future study. We are currently undertaking animal experiments by using TLR-2/4 knock-out mice to gain additional insights into HF+HS on OA development. Nonetheless, our results demonstrate that the consumption of sugary foods as part of a HF diet contributes to the risk of developing OA. Furthermore, lipid metabolism, fibrosis, and inflammation of liver tissue may serve as indicators of OA disease progression.

Supplementary Table S1. The composition of diets for HF or HF+HS group.

|	HF diet				HF+HS diet			
	gm	kcal	kcal(%)	g(%)	gm	kcal	kcal(%)	g(%)
Casein	195	780			195	780		
L-Cystine	3	12	17	20	3	12	17	19.8
Sucrose	0	0			435	1740		
Corn Starch	435	1740			0	0		
Maltodextrin	50	200	41	49	50	200	41	48.6
Lard	175	1575			175	1575		
Soybean oil	39	351	41	21	39	351	41	21.4
Cellulose	40	0			40	0		
HPMC	10	0			10	0		
Mineral Mix	35	0			35	0		
Calcium Carbonate	4	0			4	0		
Vitamin Mix	10	40			10	40		
Choline bitartrate	2	0			2	0		
Total	998	4698			998	4698		
	gm(%)	kcal(%)			gm(%)	kcal(%)		
Protein	19.84	16.86			19.84	16.86		
Carbohydrate	58.72	42.15			58.72	42.15		
Fat	21.44	41			21.44	41		
total	100	100			100	100		
	4.7kcal/g				4.7kcal/g			
Cholesterol content	126mg/g				126mg/g			

*Cholesterol in lard = 0.72mg/gram.

*Basic formulation was followed by AIN-93M diet.

Supplementary Table S2. PCR primer

Genes	Strand	Primer sequences (5'-3')	Origin
Dgat2	Forward Reverse	CCGCAAAGGCTTTGTGAAG GGAATAAGTGGGAACCAGATCA	Mouse
Acox1	Forward Reverse	CTTGGATGGTAGTCCGGAGA TGGCTTCGAGTGAGGAAGTT	Mouse
Tlr4	Forward Reverse	AGTGGGTCAAGGAACAGAAGCA CTTACCAGCTCATTTCTCACC	Mouse
Tnf-α	Forward Reverse	GGCTGCCCCGACTACGT ACTTTCTCCTGGTATGAGATAGCAAAT	Mouse
Mcp1	Forward Reverse	AGGTCCCTGTCATGCTTCTG GCTGCTGGTGATCCTCTTGT	Mouse
IL-6	Forward Reverse	CTGCAAGAGACTTCCATCCAGTT AGGGAAGGCCGTGGTTGT	Mouse
Nlrp3	Forward Reverse	ATGCTGCTTCGACATCTCCT AACCAATGCGAGATCCTGAC	Mouse
Col1a1	Forward Reverse	CAAGAACAGCAACGAGTACCG GTCACTGGTCAACTCCAGCAC	Mouse
Tgf- β	Forward Reverse	TTGCCCTCTACAACCAACACAA GGCTTGCGACCCACGTAGTA	Mouse
Fabp4	Forward Reverse	AGCATCATAACCCTAGATGGCG CATAACACATTCCACCACCAGC	Mouse
Fans	Forward Reverse	GGAGGTGGTGATAGCCGGTAT TGGGTAATCCATAGAGCCCAG	Mouse
Adgre1	Forward Reverse	CTTTGGCTATGGGCTTCCAGTC GCAAGGAGGACAGAGTTTATCGTG	Mouse
Hprt	Forward Reverse	TTGCTCGAGATGTCATGAAGGA AGCAGGTCAGCAAAGAACTTATAGC	Mouse
Rplp0	Forward Reverse	GGATCTGCTGCATCTGCTTG GGCGACCTGGAAGTCCAACT	Mouse

DGAT2: Diacylglycerol Acyltransferase 2, Acox1: Acyl-CoA oxidase 1, TLR4: Toll-like receptor 4, TNF- α: Tumor necrosis factor alpha, MCP1: Monocyte chemotactic protein 1, IL-6: Interleukin 6, IL-1β: Interleukin 1 beta, NLRP3: NOD-like receptor pyrin domain-containing protein 3, Col1a1: Collagen type 1 alpha 1, TGF- β: Transforming growth factor beta, FABP4: Fatty acid-binding protein 4, FASN: Fatty Acid Synthase, Adgre1: Adhesion G Protein-Coupled Receptor E1, HPRT: Hypoxanthine phosphoribosyltransferase, RPLP0: Ribosomal protein lateral stalk subunit P0
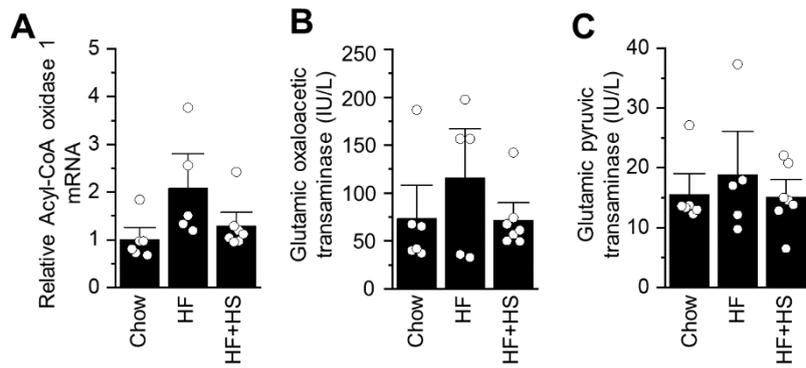
Supplementary Figure S1. Analysis of liver damage marker genes in the experimental mouse model. (A) The lipid metabolism of acyl-CoA oxidase 1 in liver tissue from the DMM-induced diet mice was analyzed. The damage of the liver is presented by the value of glutamic oxaloacetic transaminase (B) and glutamic pyruvic transaminase (C) in the liver from DMM-induced diet mice. The values are presented as the mean ± SEM and have been evaluated using the student's t-test. HF, high-fat diet; HF+HS, high fat +high sucrose diet.
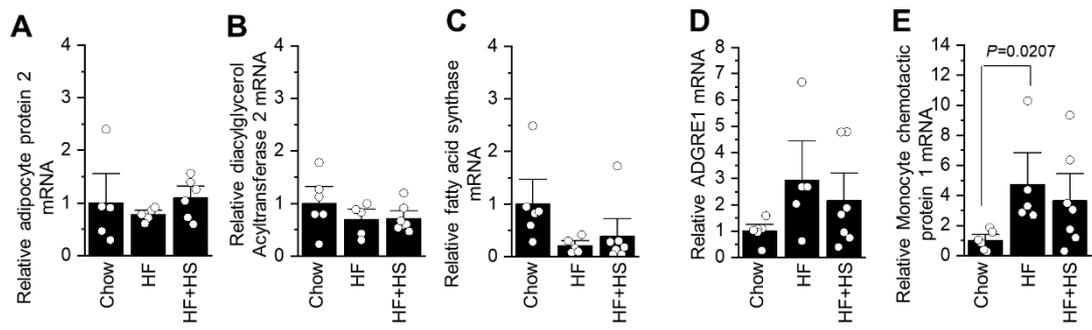
Supplementary Figure S2. The analysis of fatty acid synthesis-related genes and macrophage accumulation-related genes in adipose tissue from DMM-induced high fat or high fat+ high sucrose diet mice. The mRNA expression levels of adipocyte protein 2 (A), diacylglycerol acyltransferase 2 (B), fatty acid synthase (C), Adgre1 (adhesion G protein-coupled receptor E1) (D), and monocyte chemotactic protein 1 (E) were analyzed. The values are presented as the mean ± SEM and have been evaluated using the student's t-test. HF, high-fat diet; HF+HS, high fat +high sucrose diet.

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