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ORIGINAL ARTICLE

Genistein-Attenuated Hepatic Steatosis and Inflammation in Nonalcoholic Steatohepatitis with Bilateral Ovariectomized Rats

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ABSTRACT

Objective: The objective was to investigate the anti-lipidemic and anti-inflammatory effects of genistein on rats with estrogen deficiency and nonalcoholic steatohepatitis (NASH).
Materials and Methods: Sprague-Dawley female rats (n = 48) were randomly divided into ovariectomized (OVX) and non-OVX groups, then again divided into three subgroups as follows: controls, rats fed with high-fat high-fructose (HFHF) diet (NASH group), and rats fed with HFHF diet plus daily 16 mg/kg genistein (genistein group). Liver tissues were used for histology, liver tissues were used for histology and measured of hepatic free fatty acid (FFA) by colorimeter, and nuclear factor kappa B (NFκB) expression by immunohistochemistry. Serum tumor necrosis factor-α (TNF-α) was evaluated by enzyme-linked immunosorbent assay.
Results: NASH group had increased serum TNF-α (171.62 ± 22.34 vs. 58.47 ± 14.83 pg/mL), %NFκB-positive cells (53.94 ± 11.89 vs. 13.73 ± 3.40), and hepatic FFA (9.07 ± 2.27 vs. 3.62 ± 0.77 nmol/mg tissue) when compared with control (P < 0.01). The most severe hepatic fat accumulation and inflammation was found in OVX with NASH group. Genistein treatment decreased serum TNF-α compared with NASH groups in both non-OVX and OVX groups (105.84 ± 29.77 vs. 171.62 ± 22.34 pg/mL and 73.07 ± 19.31 vs. 124.12 ± 16.04 pg/mL, respectively) (P < 0.01). Genistein reduced %NFκB-positive cells in NASH rats (31.84 ± 10.60 vs. 53.94 ± 11.89) and decreased hepatic FFA levels in OVX with NASH rats (6.50 ± 0.60 vs. 13.11 ± 1.65 nmol/mg tissue) when compared with NASH group, respectively (P < 0.01).
Conclusion: Estrogen deficiency is the contributing factor that worsens NASH. Genistein attenuated hepatic fat accumulation and inflammation. Moreover, genistein demonstrated to be more effective in estrogen deficiency with NASH than ovary-intact rats.
Keywords: Estrogen deficiency, genistein, nonalcoholic steatohepatitis, ovariectomized

SUMMARY

- This study are investigated the role of estrogen deficiency on NASH pathogenesis and effect of genistein attenuated HFHF diet-induced NASH rats. The results demonstrated the increasing of NASH severity in estrogen deficiency. Genistein could improvement NASH through alleviate hepatic fat accumulation, reduce inflammation, and decrease histological alterations.

parameters	HFHF	HFHF +Genistein	OVX	OVX +HFHF	OVX +HFHF +Genistein
Histopathology (NASH score)	↑↑	↓	↑	↑↑↑	↓
FFA	↑↑	↓	↑	↑↑↑	↓
TNF-α	↑↑	↓	↑	↑↑↑	↓
NFκB	↑↑	↓	↑	↑↑↑	↓

Abbreviations used: NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; non-OVX: Nonovariectomized; OVX: ovariectomized; HFHF: High-fat high-fructose; FFA: Free fatty acid; NFκB: Nuclear factor kappa B; TNF-α: Tumor necrosis factor-alpha; IL6: Interleukin-6; PPARγ: Peroxisome proliferator-activated receptor gamma; ERα: Estrogen receptor alpha; ERβ: Estrogen receptor beta; LDL: Low-density lipoprotein; TBARS: Thiobarbituric acid-reactive substances; ELISA: Enzyme-linked immunosorbent assay; DMSO: Dimethyl sulfoxide; DAB: Diaminobenzidine.

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INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is the chronic progressive form of nonalcoholic fatty liver disease (NAFLD). The characteristics are demonstrated as necroinflammation, hepatocellular injury, and also cirrhosis at the end stage. Further progression of NASH may lead from hepatic steatosis and cirrhosis to hepatocellular carcinoma. A recent study conducted on a large middle-aged population showed that the prevalence of NAFLD and NASH is 20%–30%[1] and 12.2%,[2] respectively. Moreover, the development of NAFLD or NASH is strongly related with metabolic syndrome and obesity.[3] Since obesity is increasing worldwide, NASH has become the most common concern of liver diseases.

The mechanism of NASH pathogenesis is complex and involve many processes of hepatic lipid metabolism at the "first hit," resulting in inflammation and hepatocellular damage at the "second hit." However, the lipotoxicity from lipid accumulation is not the only process that accounts for the inflammation in the second hit. There are many molecular and metabolic alterations responsible for more progressive form of NAFLD such as insulin resistance, metabolic syndrome, gut-derived endotoxin, adipose tissue signals, and genetic factors. Therefore, the "multiple hits" hypothesis was proposed for more precise explanation of NASH pathogenesis.

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Specific diet components are important for NASH pathogenesis since they play a contributory role on hepatic lipid accumulation. However, previous studies reported that long-term consumption of increased percentage of fat with low percentage of carbohydrate did not increase the risk of NAFLD[4] and seemed to ameliorate elevated liver enzyme levels.[5] In contrast, carbohydrate overfeeding results in weight gain and hepatic steatosis in a short period of time. Moreover, dietary fructose enhanced cytotoxicity in methionine-choline-deficient diet-induced NASH mice which results in altered histological changes of liver and induced hepatocyte apoptosis.[6] These assume to the predominantly contributing effects of dietary fructose on NASH progression.

The persistence of nuclear factor kappa B (NFκB) activation was shown in animal models and patients with NASH.[7] Tumor necrosis factor-α (TNF-α) was shown to increase in patients with NASH[8] and was positively associated with histological severity of liver damage.[9] In contrast, interleukin (IL)-10, the anti-inflammatory cytokine, reported significantly lower effect in NAFLD patients.[8] The imbalance between pro-inflammatory and anti-inflammatory cytokines in NASH leads to hepatocyte necrosis and apoptosis, which finally turn into simple steatosis and NASH to more progressive forms.

Estrogen has an important role in lipid and glucose metabolism which might be linked with NASH. Mice with estrogen deficiency showed hypercholesterolemia and increased NASH progression.[10] In human studies, postmenopausal women had markedly increased visceral fat accumulation and a high incidence of metabolic syndrome.[11] Moreover, before 50 years of age, men had 1.8-fold increased risk of having greater NASH than women. However, this difference disappeared after 50 years of age or in the postmenopausal status. Therefore, estrogen could be another factor that involve and should be more concerned in the pathogenesis of NASH.

Although NASH is recently the most common liver disease, effective treatment for NASH has not yet been identified. Genistein is a major isoflavone in soybean that mimics the estrogen effect. It has been reported that genistein has not only estrogenic effect, but also hypolipidemic, antioxidant, and anti-inflammatory effects in both intact and estrogen-deficient rats.[12] Previous studies demonstrated that genistein decreased the levels of TNF-α, IL-6, and thiobarbituric acid-reactive substances (TBARS) in both serum and liver of high-fat diet-induced NASH rats.[13] Moreover, genistein reduced the activity of hepatic fatty acid synthetase.[14] Therefore, genistein might be useful to improve NASH pathogenesis; however, these effects of genistein need more studies to clarify in diet-induced NASH with estrogen-deficient rats.

The objective of this study is to investigate the effect of genistein on estrogen-deficient, high-fat diet-induced NASH rats.

MATERIALS AND METHODS

Animals and treatment

Sprague-Dawley rats, 4 weeks of age and weighing 60–100 g, were used in this study. All animal procedures were approved by the Animal Care and Use Committee, Faculty of Medicine, Chulalongkorn University. Rats were randomly allocated to six groups ($n = 8$ per group). Ovariectomized (OVX) rats group, rats were performed the bilateral ovariectomy with the double dorsolateral approach[15] under the anesthesia by thiopental sodium. Vaginal smear was performed at 8.00 am for 5 consecutive days to confirm the completion of bilateral OVX. Both non-OVX and OVX groups were then divided into three subgroups as follows: rats fed with standard diet, high-fat high-fructose (HFHF) diet-induced NASH rats, and HFHF diet-induced NASH rats with administration of 16 mg/kg body weight of genistein in 0.1% dimethyl sulfoxide (DMSO) via oral gavage once daily. All rats were fed with diet and water for 8 weeks *ad libitum*. After the rats were sacrificed, liver samples and blood were taken for histological and molecular analyses.

Experimental diet

Standard diet for NC and OC groups consisted of 7% fat, 47% carbohydrate, and 27% protein obtained from Perfect companion group Co., Ltd, Thailand. HFHF diet was prepared from the modification of Pickens MK formula.[6] The HFHF diet in this study consisted of 55% fat (vegetable oil), 35% carbohydrate (with 20% fructose), and 10% protein (albumin).

Histopathology

A few pieces of livers were fixed overnight in 10% formalin for paraffin embed. The 5 μm-thick sections were stained with hematoxylin and eosin for histological examination. Histopathologic lesion of liver samples was examined in all fields for grading and staging of steatosis, necroinflammation, and hepatocellular ballooning following the criteria of Brunt *et al*.[16] by an experienced pathologist in a blinded manner.

Tumor necrosis factor-alpha assay

Blood samples were collected by cardiac puncture and allowed to clot for 30 min at 25°C. Subsequently, clotted blood was centrifuged at 2000 g for 15 min at 4°C. Then, the serum was collected and stored at −80°C for TNF-α assay. TNF-α was assayed with the enzyme-linked immunosorbent assay technique using a colorimetric commercial kit (R and D system, Inc., MN, USA). The level of TNF-α was expressed as pg/mL.

Hepatic free fatty acid measurement

Lipid was extracted from the liver tissue with a lipid extraction kit (BioVision, Inc., CA, USA). The extracted lipid from the liver tissue was resuspended in 50 μl of lipid suspension buffer and sonicated for 15–20 min at 37°C. The solution was used to quantify the amount of free fatty acids (FFAs) by colorimetric assays (BioVision, Inc., CA, USA) and the amount of FFAs was expressed in nmol/mg of tissue.

Immunohistochemistry

The liver sections were deparaffinized with xylene. The endogenous peroxidase activity and nonspecific binding were blocked with 3% H_2O_2. The primary antibody of NFκB p65 was applied for 1 h at room temperature and incubated with secondary antibody for 30 min. The immunoreactivity was visualized with diaminobenzidine incubation and counterstained with hematoxylin. The number of positive stained cells was counted by Image Scope® program (Leica Biosystems Imaging, Inc., USA). The results were expressed as the percentage of positive immunoreactive cells.

Statistical analysis

The results were expressed as mean ± standard deviation.

One-way analysis of variance and Tukey *post hoc* test were used to compare the mean difference among experimental groups. Descriptive statistics were used for histological examination. $P < 0.05$ was considered statistically significant.

RESULTS

Genistein improved histological features of nonalcoholic steatohepatitis

Rats fed with HFHF diet demonstrated pathogenesis of NASH which increased the scores of steatosis, lobular inflammation, and hepatocellular ballooning in both non-OVX and OVX groups

[Figure 1a]. Moreover, histopathology showed the most severe fat accumulation and inflammation in OVX with HFHF diet-induced NASH group. Although steatosis score was not different between non-OVX and OVX rats fed with normal diet, H and E stain showed some lipid droplets in the liver of OVX rats. OVX rats, even fed with normal diet, had increased lobular inflammation (0.75 ± 0.71 vs. 0.00) and hepatocellular ballooning (0.62 ± 0.74 vs. 0.00) scores when compared with that of normal diet non-OVX rats [Figure 1b-d]. These suggested that OVX rats were naturally prone to be NASH. Administration of 16 mg/kg body weight of genistein in 0.1% DMSO significantly reduced steatosis and lobular inflammation scores in OVX rat groups as compared with OVX diet-induced NASH group (1.00 ± 0.54 vs. 3.00 ± 0.00 and 0.50 ± 0.54 vs. 1.50 ± 0.54; $P < 0.01$, respectively); however, statistical difference of hepatocellular ballooning score between NASH and NASH with genistein in OVX rats was not observed. There were no significant differences of all histological feature score between NASH and NASH with genistein groups of non-OVX rats.

Genistein reduced hepatic lipid accumulation

Hepatic FFA level was used to examine the effect of genistein on lipid accumulation in liver. HFHF diet significantly induced hepatic lipid accumulation only in non-OVX group (9.07 ± 2.27 vs. 3.62 ± 0.77 nmol/mg tissue, $P < 0.01$). However, the comparable level of hepatic FFA between normal and HFHF diet was observed in OVX group (13.11 ± 1.65 vs. 13.89 ± 1.65 nmol/mg tissue, $P = 0.94$). These suggested that estrogen deficiency can be induced hepatic FFA production independent with diets and also confirmed that OVX rats were tended to NASH. In non-OVX rats, the reduction of hepatic FFA level was not found after received 16 mg/kg body weight of genistein (9.07 ± 2.27 vs. 9.77 ± 3.01 nmol/mg tissue, $P = 0.97$) when compared with NASH group. In contrary, in OVX groups, genistein significantly decreased hepatic FFA level when compared with NASH rats (6.50 ± 0.60 vs. 13.11 ± 1.65 nmol/mg tissue, $P < 0.01$). Interestingly, this reduction was significantly different even compared with OVX rats fed with normal diet (6.50 ± 0.60 vs. 13.89 ± 0.09 nmol/mg tissue, $P < 0.01$) [Figure 2].

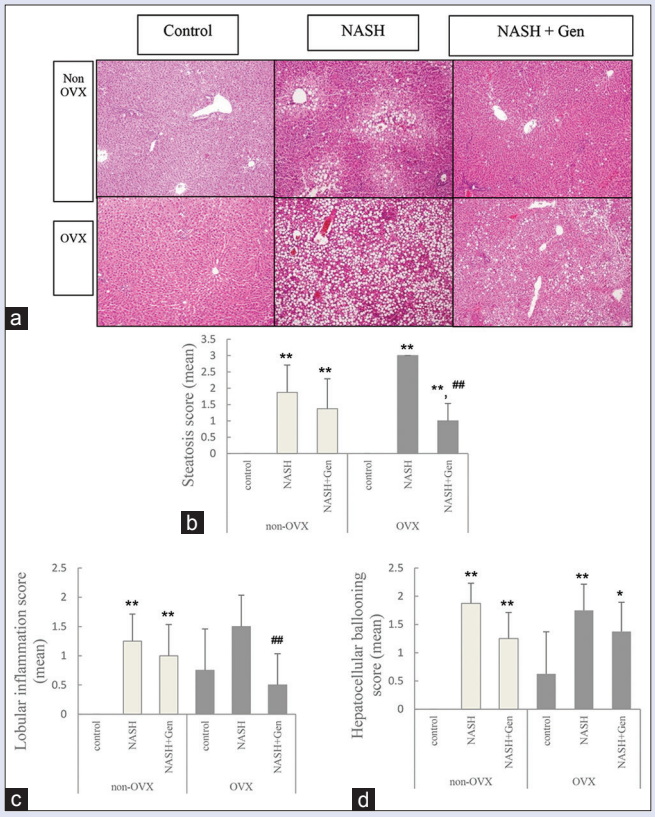

Figure 1: H and E staining and histological feature score following Brunt's criteria. (a) H and E (×100). (b) Steatosis scores between groups. HFHF diet-induced NASH significantly increased in both non-OVX and OVX rats ($P < 0.01$ compared with control group). Genistein significantly reduced steatosis scores in OVX rats ($P < 0.01$ compared with NASH group). (c) Lobular inflammation score. Genistein decreased lobular inflammatory score only in OVX rats ($P < 0.01$). (d) Hepatocellular ballooning score. *$P < 0.05$ compared with control group, **$P < 0.01$ compared with control group, ##$P < 0.01$ compared with diet-induced NASH group. HFHF: High-fat high-fructose; OVX: Ovariectomized; NASH: Nonalcoholic steatohepatitis

Genistein alleviated hepatic inflammation

Serum level of pro-inflammatory cytokine; TNF-α, was significantly increased in non-OVX diet-induced NASH rats as compared with control (171.62 ± 22.34 vs. 58.47 ± 14.83 pg/mL, $P < 0.01$). Same as hepatic FFA level, OVX induced serum level of TNF-α independent with diets. Oral gavage of genistein significantly reduced the serum level of TNF-α in NASH rats both with and without ovaries (105.84 ± 29.77 vs. 171.62 ± 22.34 and 73.07 ± 19.31 vs. 124.12 ± 15.57 pg/mL; $P < 0.01$) when compared with diet-induced NASH group, respectively [Figure 3].

Changes of positive NFκB cells, expressed in percentage, were observed. Diet-induced NASH rats showed significant increase in the percentage of positive NFκB cells in only non-OVX rats (53.94 ± 11.89 vs. 13.73 ± 3.40, $P < 0.01$), whereas statistical significance was not found in OVX with NASH group when compared with normal control. Genistein significantly attenuated the percentage of NFκB -positive cells in non-OVX rats with HFHF diet-induced NASH (31.84 ± 10.60 vs. 53.94 ± 11.89, $P < 0.01$). In OVX rats, although statistical significance was not found between NASH and NASH with genistein groups, a reduction trend was demonstrated [Figure 4a and b].

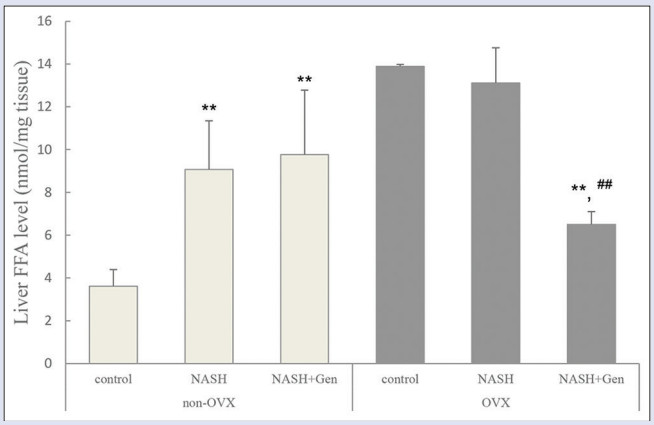

Figure 2: Hepatic free fatty acid level (nmol/mg tissue). Level of hepatic FFA was increased in non-OVX rats fed with HFHF diet ($P < 0.01$), whereas there was comparable level between control and NASH groups in OVX rats. Genistein was able to reduce hepatic FFA level in OVX rats ($P < 0.01$). HFHF: High-fat high-fructose; OVX: Ovariectomized; FFA: Free fatty acid; NASH: Nonalcoholic steatohepatitis

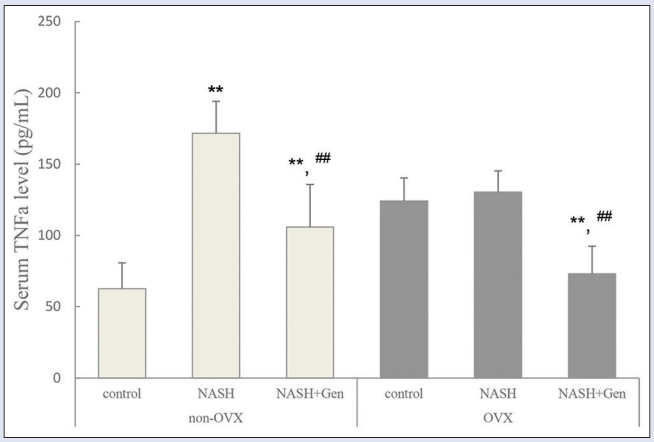

Figure 3: Serum level of TNF-α (pg/mL). In non-OVX groups, HFHF diet-induced NASH showed significantly raising level of serum TNF-α, while there was no difference between control and diet-induced NASH groups. The administration of genistein decreased TNF-α level in both non-OVX and OVX rat groups ($P < 0.01$). $^{**}P < 0.01$ compared with control group; $^{\#\#}P < 0.01$ compared with diet-induced NASH group. HFHF: High-fat high-fructose; OVX: Ovariectomized; FFA: Free fatty acid; NASH: Nonalcoholic steatohepatitis; TNF-α: Tumor necrosis factor-α

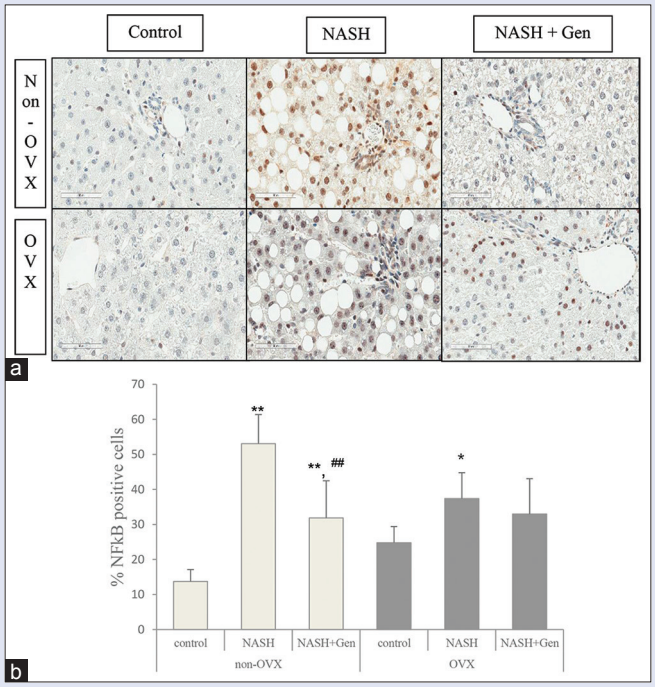

Figure 4: NFκB-positive cells. (a) Immunohistochemistry (×400). Bar, 60 μm. NFκB immunostaining was rare in control groups (both non-OVX and OVX groups), whereas NFκB-positive cells increased in number in NASH groups. Number of NFκB-positive cells was decreased in NASH with 16 mg/kg body weight genistein. (b) Percentage of NFκB-positive cells was determined using Image Scope®. In non-OVX rats, diet-induced NASH increased the percentage of NFκB-positive cells ($P < 0.001$) which significantly reduced after administration of genistein ($P < 0.001$). In OVX rats, diet also increased the percentage of NFκB-positive cells ($P < 0.05$); however, genistein could not decrease NFκB-positive cells. $^{*}P < 0.05$ compared with control group; $^{**}P < 0.01$ compared with control group; $^{\#\#}P < 0.01$ compared with diet-induced NASH group. NFκB: Nuclear factor kappa B; NASH: Nonalcoholic steatohepatitis; OVX: Ovariectomized

DISCUSSION

High-fat high-fructose diet induced nonalcoholic steatohepatitis

In this study, we demonstrated the role of fructose together with fat overfeeding in the pathogenesis of NASH. Histopathology showed changes in liver tissue of rats fed with HFHF diet in both non-OVX and OVX groups. All histological features following Brunt's criteria worsen after consumption of HFHF diet as identified by increased each histological features score. High fat dietary intake is well accepted for inducing obesity, insulin resistance, and metabolic syndrome, which are the contributing factors on hepatic lipid accumulation in NASH pathogenesis. However, long-term consumption of increased percentage of fat with low percentage of carbohydrate did not increase the risk of NAFLD.[4] Contrarily, high carbohydrate consumption results in weight gain and hepatic steatosis in a short period of time.

The predominant carbohydrate component in our daily diet is sucrose, which further digests into a monosaccharide, fructose. Since liver is the major site of fructose metabolism, persisting high fructose ingestion may be implicated in the rise of hepatic fatty acid through *de novo* lipogenesis stimulation and inhibition of lipid oxidation. This causes fatty accumulation in liver. The key enzyme that is responsible for the interaction of fructose metabolism and hepatic fat accumulation might be fructokinase.[17] Since fructose metabolism requires large amount of adenosine triphosphate (ATP), high fructose influx to liver can lead to elevated metabolic stress by ATP depletion. These are involved in the production of many pro-inflammatory cytokines (such as TNF-α, IL-6, and NFκB) these are involved in more production of many pro-inflammatory cytokines (such as TNF-α, IL-6, and NFκB) which causative in the progression of NAFLD to NASH.

Estrogen deficiency tends to nonalcoholic steatohepatitis

Estrogen is the primary female sex hormone which is involved not only in reproductive but also in many biological functions, including lipid metabolism. Therefore, estrogen deficiency leads to dysregulation of metabolism and is associated with the alteration of lipid accumulation throughout the body which may involve in several diseases. This study results showed the comparable level of serum TNF-α, percentage of NFκB -positive cells, and hepatic FFA between rats fed with HFHF and normal diet in bilateral OVX groups. Moreover, estrogen deficiency with HFHF intake increased hepatic FFA level more than HFHF intake without estrogen deficiency. These findings confirmed that estrogen deficiency is the contributing factor for NASH pathogenesis. In liver, E2 signals are related to low-density lipoprotein (LDL) receptor expression, which results in the reduction of serum LDL-cholesterol. Previous studied in patients with Turner syndrome; the genetic condition that affects female characteristic development, revealed excess visceral fat and lipid droplets in liver.[18] OVX rats treated with estrogen (E2) and estrogen receptor alpha (ERα) agonists have been shown to decrease body weight and total cholesterol level.[19] Moreover, men have an increased risk of having greater NASH than menstrual women; however, this difference vanishes in postmenopausal status.[11] In addition, estrogen deficiency affects the immune system. OVX mice were reported to enhance TNF-α production from T-cells[20] which can further stimulate NFκB signaling pathway for more inflammatory stimulation. Together with increased fat accumulation and pro-inflammatory cytokine production, estrogen deficiency plays a role in the progression of NASH.

Genistein improved nonalcoholic steatohepatitis pathogenesis

Genistein administration in our study has been demonstrated to improve histopathology scores in steatosis and lobular inflammation in OVX rats fed with HFHF diet. However, this improvement was not found in non-OVX rats. Moreover, genistein attenuated hepatic FFA in NASH with estrogen deficiency but not in NASH with intact ovaries. Genistein also reduced the percentage of NFκB-positive cells in both NASH with intact ovaries and NASH with estrogen deficiency; however, statistical significance was only observed inovaries-intact with NASH rats.

Since estrogen is essential for lipid metabolism, insufficiency or deficiency of estrogen may lead to increase in fat accumulation. Genistein is one of the isoflavones that can mimic the physiological functions of estrogen via binding with estrogen receptor. Genistein has been reported to attenuate hyperlipidemia in male hamsters fed with high-fat diet by upregulation of both ERβ and ERα in liver[21] and reduced food intake in OVX mice, resulting in decreased weight gain.[22] Female OVX Wistar rats treated with ERβ agonist, ERα agonist, or genistein and fed with high-fat diet demonstrated reduction of lipogenesis and triglyceride accumulation in liver and muscles.[23] Moreover, it increased the expression of PPARγ, which is the hepatic transcription factor that regulates fat metabolism and inflammation in NASH.

Furthermore, it has been found that genistein showed antioxidant effect and decreased renal inflammation in streptozotocin-induced diabetic mice.[24] In addition, genistein decreased the plasma level of TNF-α and IL-6 in rats fed with fructose,[25] and also inhibited IL-1 β, IL-6, and TNF-α mRNA levels. Furthermore, macrophages and neutrophils contributing to the inflammatory response, attenuate their activation by genistein.[26]

CONCLUSION

This study suggests that genistein administration to HFHF diet-induced NASH in both estrogen-deficient and ovarian intact rats improves histopathological features, alleviates hepatic fat accumulation, and reduces pro-inflammatory cytokines (TNF-α). Moreover, genistein demonstrated to be more effective in estrogen-deficient NASH rats compared to rats with intact ovaries.

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Conflicts of interest

There are no conflicts of interest.

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