Heliyon 10 (2024) e26055

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Genistein and sex hormone treatment alleviated hepatic fat accumulation and inflammation in orchidectomized rats with nonalcoholic steatohepatitis

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ARTICLE INFO

Keywords:
Genistein
Sex hormones
Nonalcoholic steatohepatitis
Bilateral orchiectomy
High fat and high fructose diet

ABSTRACT

Testosterone deficiency has been reported to accelerate nonalcoholic fatty liver disease (NAFLD). However, there are minimal data on the risk of NAFLD in transgender women and the treatment of NAFLD in this population. This study aimed to investigate the treatment effects and the mechanisms of action of genistein and sex hormones in orchiectomized (ORX) rats with nonalcoholic steatohepatitis (NASH) induced by a high fat high fructose diet (HFHF). Seven-week old male Sprague-Dawley rats were randomly divided into 7 groups (n = 6 each group); 1) control group, 2) ORX + standard diet group, 3) HFHF group, 4) ORX + HFHF group, 5) ORX + HFHF diet + testosterone group (50 mg/kg body weight (BW) once weekly), 6) ORX + HFHF diet + estradiol group (1.6 mg/kg BW daily), and 7) ORX + HFHF diet + genistein group (16 mg/kg BW daily). The duration of treatment was 6 weeks. Liver tissue was used for histological examination by hematoxylin and eosin staining and hepatic fat measurement by Oil Red O staining. Protein expression levels of histone deacetylase3 (HDAC3) and peroxisome proliferator-activated receptor delta (PPARδ) were analyzed by immunoblotting. Hepatic nuclear factor (NF)-κB expression was evaluated by immunohistochemistry. Rats in the ORX + HFHF group had the highest degree of hepatic steatosis, lobular inflammation, hepatocyte ballooning and the highest percentage of positive Oil Red O staining area among all groups. The expression of HDAC3 and PPARδ was downregulated, while NF-κB expression was upregulated in the ORX + HFHF group when compared with control and ORX + standard diet groups. Testosterone, estradiol and genistein treatment improved histological features of NASH together with the reversal of HDAC3, PPARδ and NF-κB protein expression comparing with the ORX + HFHF group. In summary, genistein and sex hormone treatment could alleviate NASH through the up-regulation of HDAC3 and PPARδ, and the suppression of NF-κB expression.

Abbreviations: DMSO, dimethyl sulfoxide; HDAC3, histone deacetylase 3; HFHF, high fat high fructose; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF-κB, Nuclear factor kappa B; ORX, orchiectomized; PPARδ, peroxisome proliferator-activated receptor delta.

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https://doi.org/10.1016/j.heliyon.2024.e26055
Received 13 February 2023; Received in revised form 25 January 2024; Accepted 7 February 2024
Available online 8 February 2024
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1. Introduction

Nonalcoholic steatohepatitis (NASH) is a progressive form of nonalcoholic fatty liver disease (NAFLD) that is characterized by hepatic inflammation, steatosis, and hepatocyte injury. NAFLD is becoming a major health problem worldwide and is considered a hepatic manifestation of metabolic syndrome. Therefore, NAFLD is associated with obesity, insulin resistance, and the increase in carbohydrate and fat consumption [1,2]. Because of its association with over nutrition, a high fat and high fructose (HFHF) diet has been widely used to induce NASH *in vivo* [3].

With the rising prevalence of male to female (MTF) transgender, the procedure to remove both testicles so called bilateral orchiectomy (ORX) has been performed more frequently. The resulting testosterone deficiency may aggravate NAFLD progression through the impairment of metabolic processes and the enhancement of oxidative stress and inflammatory responses [4]. The effect of orchiectomy on NAFLD, however, remains a matter of debate. A clinical study demonstrated that orchiectomy played a protective role on metabolic health in transwomen [5]. In contrast, an animal study showed that orchiectomy aggravated hepatic steatosis [4].

In transgender women, testosterone therapy is undesirable, and estrogen treatment is associated with a higher risk of breast cancer [6]; therefore, an isoflavone derivative compound called "genistein" might be a reasonable alternative to a hormonal therapy in this setting. Genistein is considered an alternative to a hormone replacement therapy for the treatment of menopausal symptoms and a potential treatment option of several diseases, such as cardiovascular disease, osteoporosis, impaired cognitive function, cancer and obesity [7]. As for NAFLD, genistein has been shown to suppress lipogenic gene expression more strongly than another isoflavone daidzein in an animal experiment [8]. Moreover, our previous preclinical study showed that genistein at the dose of 16 mg/kg improved NASH pathological features in male rats fed with a high-fat diet due to the upregulation of PPARγ, the inhibition of inflammatory cytokine production and the reduction of oxidative stress [9]. We also found that genistein could reduce hepatic fat accumulation, inflammation and fibrosis markers in an estrogen deficient rat model with NASH [10]. In male mice, genistein might be an androgen agonist or antagonist depending on the presence of intact testicular tissues and could be considered as a tissue specific androgen receptor modulator [11]. However, data are still lacking regarding the genistein action in the liver of castrated animals.

Recent studies demonstrated that genistein and hormonal treatment may alter the progression of NASH through the action of peroxisome proliferator-activated receptors (PPARs). Moreover, the reduction of epigenetic modification of histone deacetylase 3 (HDAC3) has been shown to play a role in NAFLD development [12]. A previous study reported that the reduced binding of HDAC3 on PPARα target genes in aged liver promoted the up-regulation of lipid synthesis genes leading to the development of hepatic steatosis. However, the effects of genistein and sex hormone treatment on a PPARδ subtype and HDAC3, and their roles in regulating hepatic lipid metabolism and inflammation remain uncertain. Furthermore, nuclear factor-κB (NF-κB), the intracellular signal transduction that plays a crucial role in inflammatory responses, can be activated by varied pathogenic factors, thus promoting the NASH progression [13,14]. To the best of our knowledge, data are scarce regarding the effects of hormonal therapy and genistein on PPARδ, epigenetic modification of HDAC3, and NF-κB signaling pathways in an *in vivo* testosterone deficiency NASH model. Therefore, the aim of this study was to evaluate the treatment effects of genistein in an animal model of transgender women with NASH and its mechanisms of action in comparison with standard sex hormone treatment.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (aged 7 weeks, 200–250g body weight (BW)) were purchased from Nomura Siam International Co. Ltd., Thailand. The experimental procedures were conducted in accordance with the guidelines for experimental animals by the National Research Council of Thailand and approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Chulalongkorn University (License No.006-2564). This study was reported in accordance with the ARRIVE guidelines. All rats were housed in cages under a standard condition (room temperature at 25 °C with 12:12 light-dark cycle) and fed with a standard diet for one week for the purpose of acclimatization. After the acclimatization period, bilateral orchiectomy was performed in ORX groups under anesthesia using isoflurane inhalation.

2.2. Experimental design

Forty-two male Sprague-Dawley rats were divided into seven groups: 1) control group fed with a standard diet, 2) ORX + standard diet group, 3) HFHF group, 4) ORX + HFHF diet group, 5) ORX + HFHF diet with testosterone treatment (50 mg/kg BW subcutaneous injection once weekly), 6) ORX + HFHF diet with estradiol treatment (1.6 mg/kg BW oral gavage once daily) and 7) ORX + HFHF diet with genistein treatment (16 mg/kg BW oral gavage once daily). G power data analysis program was used to calculate the number of rats in each group by using TNF-α levels from a study by Ji G et al. [15] as shown in Supplementary Fig. 1. We used the hepatic TNF-α levels, as determined by ELISA assay, in each group as follows: 1) control group, 2.87 ± 1.01 pg/mg, 2) NASH group, 19.94 ± 4.17 pg/mg, 3) NASH + Genistein 4 mg/kg, 13.89 ± 2.81 pg/mg and 4) NASH + Genistein 8 mg/kg, 11.17 ± 1.90 pg/mg. Before the calculation process, we converted standard error of mean (SEM) to standard deviation (SD) and then used the mean and SD of each group to calculate a sample size. As a result, the sample size was 6 rats in each group (42 in total). However, one rat died from an unidentified cause during the experiment which resulted in the final number of 5 rats in the HFHF group.

Rats were allowed to freely access food and water ad libitum for 6 weeks. At the end of the experiment, rats were fasted overnight (6–8 h) and then euthanized with overdose (>50 mg/kg BW) thiopental sodium via intraperitoneal injection (IP). Then, whole liver

tissue was immediately harvested and washed with ice-cold normal saline solution (NSS). Liver tissue was used for histopathological examination and protein analyses. For the histological examination, liver tissues were fixed in 10% formalin overnight, transferred into paraffin embedding and cut at 5-μm thickness before staining with a hematoxylin and eosin (H&E) method. The paraffin-embedded tissue was also used to determine NF-κB expression by immunohistochemistry. The remaining parts of liver tissue were used to examine the protein expression levels of PPARδ and HDAC3. The degree of hepatic lipid deposition was evaluated by Oil Red O staining.

A HFHF diet was prepared by adopting the formula from a previous study [16]. In this study, HFHF consisted of 55% fat, 35% carbohydrate (20% fructose and 15% starch) and 10% protein. A standard diet is composed of 7% fat, 47% carbohydrate, and 27% protein (Perfect companion group Co., Ltd, Thailand). HFHF diet was started after the acclimatization period in HFHF diet, ORX + HFHF diet, ORX + HFHF diet + testosterone, ORX + HFHF diet + estrogen, and ORX + HFHF diet + genistein groups.

Testosterone enanthate (Bayer Pharma AG, Germany) was administered once weekly at the dose of 50 mg/kg via subcutaneous injection for 6 consecutive weeks. This dosage was adopted from our pilot study. Estradiol valerate (Bayer Pharma AG, Germany) at 1.6 mg/kg dosage was diluted in 0.1% dimethyl sulfoxide (DMSO) prior to administration via oral gavage daily for 6 weeks [17]. Genistein powder (Cayman chemical, MI, USA) at 16 mg/kg dosage was freshly prepared by dissolving in 0.1% DMSO and given once daily through oral gavage for 6 weeks. Rats in control, ORX + standard diet, ORX + HFHF diet groups received 1 mL of 0.1% DMSO by oral gavage once daily for 6 weeks as a vehicle control. All treatment started the day after the orchiectomy procedure in orchiectomized rats [10,16].

2.3. Bilateral orchiectomy

After the acclimatization period, bilateral orchiectomy was performed in ORX groups under anesthesia using isoflurane inhalation (2% mixed with 2.5 L/min of oxygen) by adopting the technique of Idris and his colleagues [18]. The level of sedation was monitored throughout the procedure using tail pinch technique. Briefly, a single incision was made on the ventral side of the scrotum. Testicular fat pad was identified and pulled out through the incision line with blunt forceps. Then, testicular content was exposed, and main blood vessels above the testicles were gently ligated with sterile absorbable silk to prevent bleeding. After the ligation, testis and epididymis were carefully removed with small scissors, and the remaining testicular sac content was placed back into the scrotum (Supplementary Fig. 2). Lastly, the scrotum was sutured with non-absorbable silk and swabbed with povidone-iodine solution to reduce the risk of infection. All steps were repeated on the contralateral testis. During the recovery period after the orchiectomy procedure, rats in all orchiectomy groups were individually caged for 24 h and injected with ketorolac at the dose of 0.5 mg/kg to relieve post-operative pain via subcutaneous injection.

2.4. Histopathological examination and scoring system

All fields in each liver section were examined by an experienced pathologist who was blinded to the experimental groups. The three components of NAFLD activity score (NAS), such as hepatic steatosis, lobular inflammation, and hepatocyte ballooning were graded. Each liver section was scored according to the Brunt criteria as follows [19].

Steatosis (0–3): 0 = <5% of fat deposition in the hepatocytes, 1 = <33% of fat deposition in the hepatocytes, 2 = 33–66% of fat deposition in the hepatocytes and 3 = >66% of fat deposition in the hepatocytes.

Lobular inflammation (0–3): 0 = absence of inflammation and necrosis, 1 = <2 foci per 20X field, 2 = 2–4 foci per 20X field and 3 = >4 foci per 20X field.

Hepatocyte ballooning (0–2): 0 = absence of ballooning degeneration, 1 = few ballooning hepatocytes and 2 = many prominent ballooning hepatocytes.

2.5. Hepatic lipid accumulation measurement

Oil Red O (ORO) staining is a special staining technique that is used to determine the fat droplet deposition in several tissues. Briefly, frozen liver sample was embedded in the optimal cutting temperature (OCT) compound. A 5-μm thickness of tissue section was cut and placed on adhesive microscope slide (Matsunami Japan). Then, the slides were stained with fresh Oil Red O solution (Fluka) and counterstained with hematoxylin. Subsequently, the slides were rinsed with running tap water, air dried, and mounted with mounting medium (Dako, CA, USA). Under light microscope, ten different fields of each sample at 40x magnification were randomly captured for analysis. The percentage of hepatic fat deposition was quantified using color histogram analysis on Image J software (NIH, USA) [20].

2.6. Immunoblot analysis

Tissue lysate was equally loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in which protein migrated in an electric field according to their molecular weight. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membrane by a semi-dry method. The membrane was placed on bovine serum albumin (BSA) with phosphate buffer saline with Tween 20 (PBST) at 4 °C overnight to block non-specific proteins. Subsequently, the membrane was incubated with primary antibodies; PPARδ (1:1000) and HDAC3 (1:2000) (Abcam, USA) for 1 h at a room temperature. After that, the membrane was washed with TBST again and then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies according to the manufacturer's

instructions. Levels of PPARδ and HDAC3 protein expression were presented as the ratio between band densities of each protein to cyclophilin B (CPB) which was used as a loading control. For the visualization, signals of all target proteins were generated using a chemiluminescence kit and the band density values were calculated using ChemiDocTM Touch Imaging System (BioRad laboratories, CL, USA).

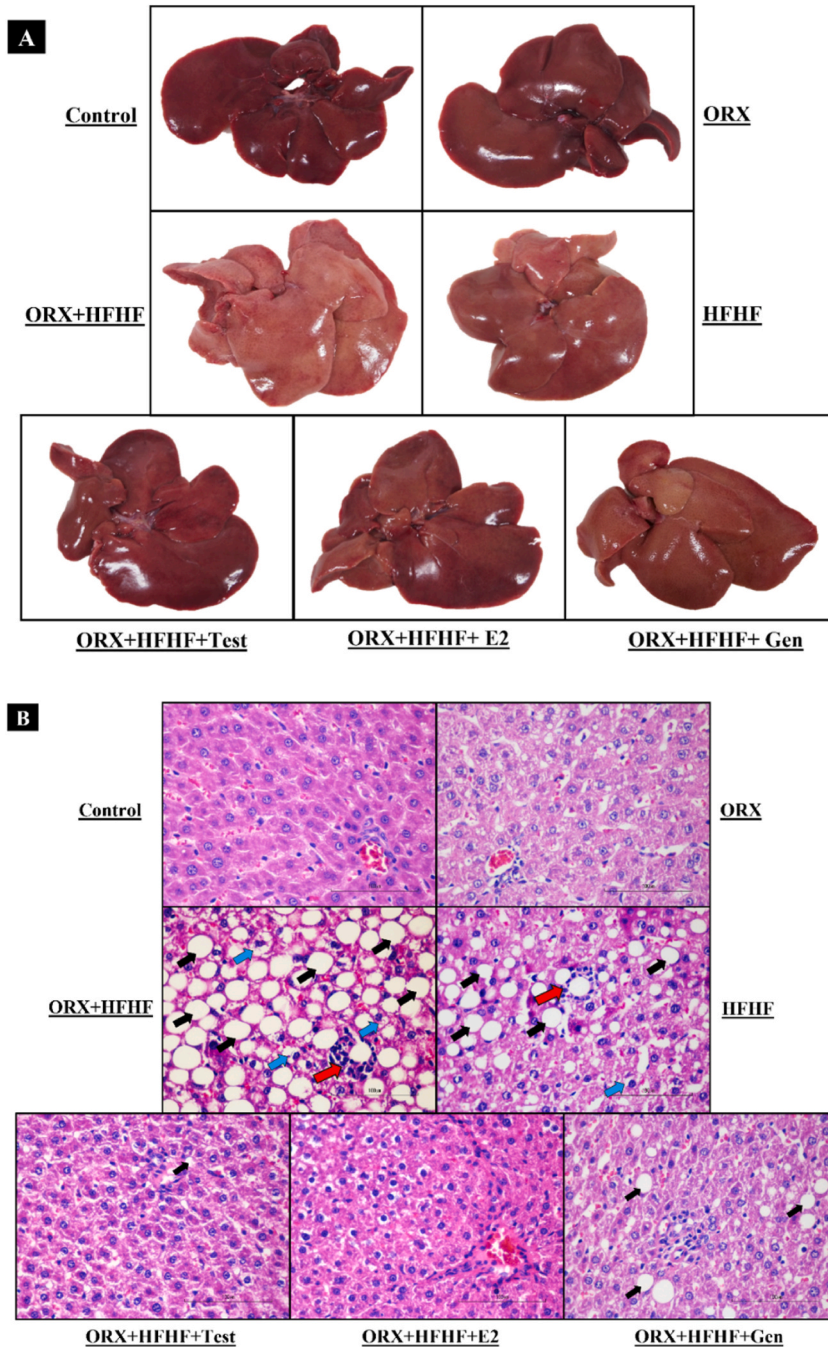

Fig. 1. General appearance and histopathological changes of the livers in all experimental groups. (A) Gross liver appearance in all experimental groups. (B) Liver histopathology in all experimental groups using hematoxylin-eosin (H&E) staining. (40X magnification; scale bar, 100 μm). Black arrows indicated macrovesicular steatosis; blue arrows indicated ballooning of hepatocytes; red arrows indicated lobular inflammation. Abbreviations: Test, Testosterone; E2, estradiol; Gen, Genistein. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.7. Immunohistochemistry

The immunohistochemistry was performed to evaluate the levels of hepatic NF-κB expression in all experimental groups. In brief, liver sections were deparaffinized and rehydrated using a different concentration of xylene and ethanol. Next, antigen retrieval was performed using citrate buffer pH 6.0 in a microwave. To prevent the misinterpretation from a false positive staining, 3% hydrogen peroxide (H_2O_2) was used to block endogenous peroxidase activity. Slides were incubated with the primary antibody of NF-kB p65 at a concentration of 1:150 (Abcam, Cambridge, USA) for 1 h at a room temperature, followed by the incubation with a secondary antibody for 30 min. Positive brown color was developed with diaminobenzidine incubation and counterstained with hematoxylin. Digital images of the protein expression were captured under a light microscope and analyzed with Aperio ImageScope software program (Leica Biosystems Imaging, Inc., MD, USA). Hepatocytes with positive brown-stained nuclei were counted in ten random fields from each sample at high magnification and expressed as the percentage of immunoreactive cells using Positive pixel count V9 algorithm [16].

2.8. Statistical analysis

Statistical analysis was performed using the Statistics Package for the Social Science (SPSS) software version 22.0 for Windows. Data were presented as mean ± standard deviation (SD). Mean differences among all experimental groups were compared using one-way analysis of variance (One-way ANOVA) and Tukey *post-hoc* test. Descriptive statistics was used for the histological examination. Differences were considered statistically significant at p-value of less than 0.05 ($p < 0.05$).

3. Results

3.1. Effects of genistein and sex hormone treatment on liver general appearance and NASH histological features

Gross examination showed soft and pale yellowish livers in the ORX + HFHF group when compared to dark brown ones in control and ORX + standard diet groups. Livers of rats in the HFHF group showed a light-yellow color when compared to those of the ORX + HFHF group. Treatment with testosterone, estradiol and genistein could normalize liver appearance (Fig. 1A).

NASH histological features and the severity of hepatic steatosis, lobular inflammation and hepatocellular ballooning were demonstrated in Fig. 1B–Table 1, and Fig. 2(A-D). In the control group, liver histology was normal. In the ORX + standard diet group, some degree of lobular inflammation and hepatocyte ballooning was observed. Histological scores of steatosis, inflammation and hepatocyte ballooning were higher in the HFHF group than in control and ORX + standard diet groups. All NASH histological features were most severe in the ORX + HFHF group as compared with other groups. Testosterone, estradiol and genistein administration could attenuate the severity of NASH in all histological components when compared with the ORX + HFHF group.

3.2. Effects of genistein and sex hormone treatment on hepatic lipid accumulation

As shown in Fig. 3A, significant hepatic fat deposition was observed in HFHF and ORX + HFHF groups when compared with control and ORX groups. However, in the HFHF group, fat droplets were mostly microvesicular, whereas in the ORX + HFHF group, they are macrovesicular. After treatment with testosterone, estradiol and genistein, the degree of fat deposition was significantly reduced from that of the ORX + HFHF group.

The ratio of red-stained pixels to total pixels in each section was used to calculate the percentage of ORO positive area. Ten different fields were used in each rat for the calculation. Percentages of ORO positive area in both ORX + HFHF and HFHF groups were significantly higher than those in control and ORX groups (54.19 ± 1.88 vs. 26.90 ± 4.20 vs. 2.80 ± 0.98 vs. 6.68 ± 1.30, respectively, $p < 0.05$). Testosterone, estradiol and genistein treatment resulted in a marked decrease in positive ORO staining area when compared with the ORX + HFHF group (2.86 ± 0.36 vs. 2.03 ± 0.70 vs. 10.77 ± 2.99 vs. 54.19 ± 1.88, respectively, $p < 0.05$) (Fig. 3B).

Table 1
NAFLD histopathological scores in each group.

Group	N	Steatosis				Inflammation				Hepatocyte ballooning		
		0	1	2	3	0	1	2	3	0	1	2
Control	6	6	–	–	–	4	2	–	–	3	3	–
ORX	6	6	–	–	–	–	5	1	–	2	3	1
ORX + HFHF	6	–	1	3	2	–	2	4	–	–	1	5
HFHF	5	1	2	2	–	–	3	2	–	–	2	3
ORX + HFHF + Test	6	4	2	–	–	5	1	–	–	1	3	2
ORX + HFHF + E2	6	5	1	–	–	6	–	–	–	–	1	5
ORX + HFHF + Gen	6	1	3	1	1	2	4	–	–	–	3	3

Data indicated the number of rats with that histopathological grade in each group. According to the Brunt's criteria, steatosis was graded as 0 = < 5%, 1 = < 33%, 2 = 33–66%, 3 = > 66%. Inflammation was graded as 0 = normal, 1 = mild, 2 = moderate, 3 = severe. Hepatocyte ballooning was graded as 0 = no ballooning, 1 = few balloon cells, 2 = many cells/prominent ballooning.

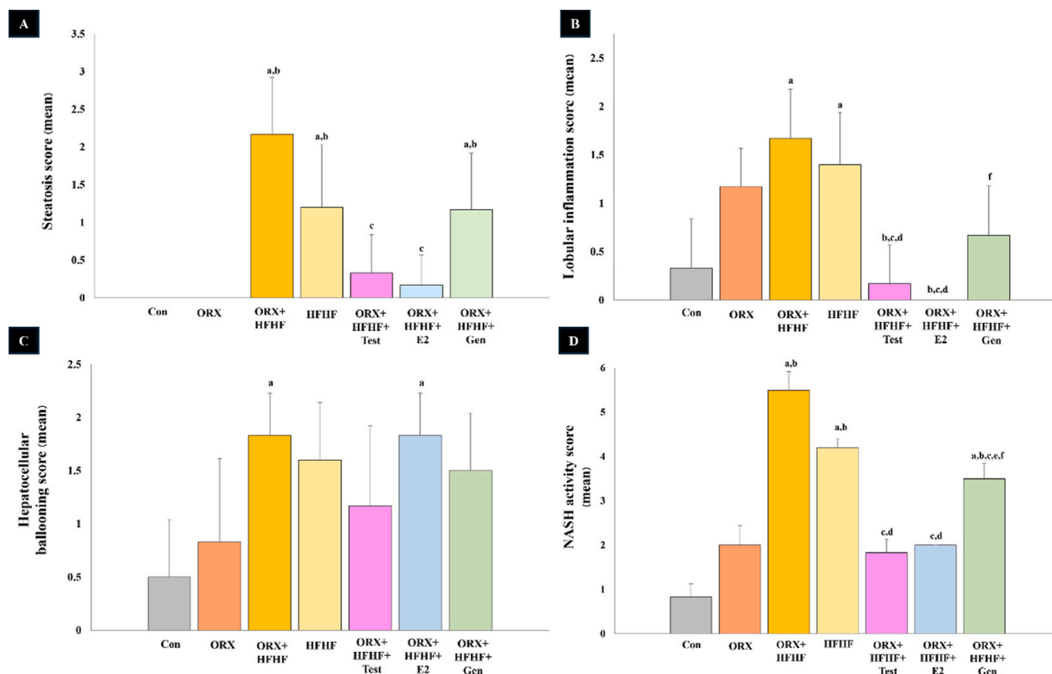

Fig. 2. Histological scores according to the Brunt's criteria in all experimental groups. (A) Steatosis score, (B) Lobular inflammation score, (C) Hepatocyte ballooning score and (D) NASH activity score in all experimental groups. Data were expressed as mean ± standard deviation (SD). $^{a}p<0.05$ when compared with the control group, $^{b}p < 0.05$ when compared with the ORX group, $^{c}p < 0.05$ when compared with the ORX + HFHF group, $^{e}p < 0.05$ when compared with the ORX + HFHF + Test group and $^{f}p < 0.05$ when compared with the ORX + HFHF + E2 group.

3.3. Effects of genistein and sex hormone treatment on HDAC3 and PPARδ protein expression

The expression patterns of HDAC3 and PPARδ were shown in Fig. 4A and B, respectively. A significant decrease in HDAC3 protein expression was observed in the ORX + HFHF group when compared with that in the control and ORX + standard diet groups (0.54 ± 0.12 vs. 1.07 ± 0.04 vs. 1.06 ± 0.10, respectively, $p < 0.05$). Hepatic HDAC3 expression was also significantly lower in the ORX + HFHF group than in the ORX group (0.54 ± 0.12 vs. 0.88 ± 0.13, $p < 0.05$). Testosterone, estradiol and genistein treatment significantly increased hepatic HDAC3 expression as compared to the ORX + HFHF group (1.14 ± 0.02 vs. 1.16 ± 0.09 vs. 0.89 ± 0.08 vs. 0.54 ± 0.12, respectively, $p < 0.05$). In contrast with testosterone and estradiol which could restore HDAC3 expression to the level of controls, the effect of genistein on HDAC3 expression was inferior to the estradiol therapy (0.89 ± 0.08 vs. 1.16 ± 0.09, $p < 0.05$).

PPARδ expression was significantly downregulated in the ORX + HFHF group as compared with control and ORX + standard groups (0.48 ± 0.03 vs. 0.61 ± 0.01 vs. 0.64 ± 0.01, respectively, $p < 0.05$). The protein level of PPARδ also significantly decreased in the ORX + HFHF group when compared with that of HFHF group (0.48 ± 0.03 vs. 0.57 ± 0.02, respectively, $p < 0.05$). After treatment with testosterone, estradiol and genistein, the reversal of PPARδ protein expression was observed in all 3 groups as compared with the ORX + HFHF group (0.65 ± 0.02 vs. 0.61 ± 0.02 vs. 0.59 ± 0.02 vs. 0.48 ± 0.03, respectively, $p < 0.05$).

3.4. Effects of genistein and sex hormone treatment on hepatic NF-kB protein expression

As shown in Fig. 5A and B, the percentage of NF-kB immunoreactive cells were significantly elevated in ORX, ORX + HFHF and HFHF groups when compared with the control group (6.60 ± 0.43 vs. 21.08 ± 1.90 vs. 12.76 ± 1.33 vs. 1.30 ± 0.43, respectively, $p < 0.05$). Moreover, a significant increase in NF-kB positive cells was observed in the ORX + HFHF group as compared with the HFHF group (21.08 ± 1.90 vs. 12.76 ± 1.33, respectively, $p < 0.05$). The significantly decreased expression of NF-kB was detected in testosterone, estradiol and genistein treated groups when compared with ORX + HFHF and HFHF groups (3.23 ± 0.94 vs. 3.53 ± 1.11 vs. 2.10 ± 0.58 vs. 21.08 ± 1.90 vs. 12.76 ± 1.33, respectively, $p < 0.05$). Of note, genistein treatment could decrease the level of NF-kB expression more than estradiol treatment could.

4. Discussion

In this study, we used a testosterone deficient rat model fed to mimic the condition of NASH in transwomen. Previous studies showed that testosterone deficiency is associated with the increased prevalence of diabetes, obesity, and NAFLD [21]. A previous transcriptomic analysis demonstrated that testosterone deficiency heightened the severity of HFD induced NAFLD through the impairment of lipid metabolism, and the increment of inflammatory activity, oxidative stress, and apoptosis which was alleviated by

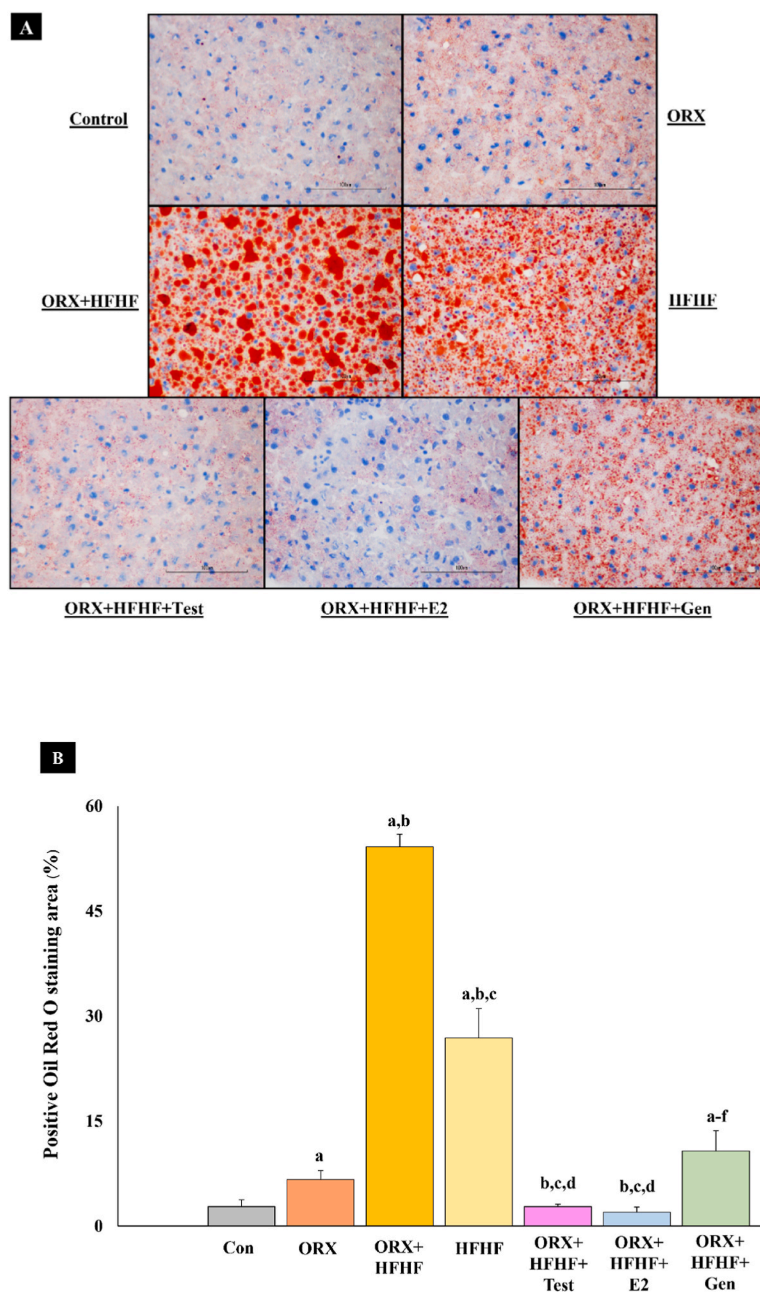

Fig. 3. Oil Red O staining and the percentage of hepatic lipid droplet deposition in all experimental groups. (A) Representative photomicrographs of Oil Red O staining in all experimental groups (40X magnification; scale bar 100 μm), (B) Bar graphs representing positive Oil Red O staining area in each group. $^{a}p<0.05$ when compared with the control group, $^{b}p < 0.05$ when compared with the ORX group, $^{c}p < 0.05$ when compared with the ORX + HFHF group, $^{d}p < 0.05$ when compared with the HFHF group, $^{e}p < 0.05$ when compared with the ORX + HFHF + Test group and $^{f}p < 0.05$ when compared with the ORX + HFHF + E2 group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

testosterone replacement [4]. Our results were in accordance with other reports that testosterone deficiency induced by bilateral orchiectomy caused a higher degree of hepatocyte injury and inflammation than in normal rats. These changes were particularly more severe in the presence of HFHF diet. These findings were not surprising as the high fructose consumption has been shown to promote NAFLD development. A previous study showed that mice fed with fructose had a higher degree of hepatic lipid accumulation, plasma endotoxin levels and TNF-α protein expression than mice fed with other sugar types [22]. Moreover, fructose products could directly activate lipogenic genes, such as sterol-regulatory element binding protein (SREBP)-1c and carbohydrate-response element binding protein (ChREBP) and promote reactive oxygen species (ROS) overproduction and liver inflammation [23,24].

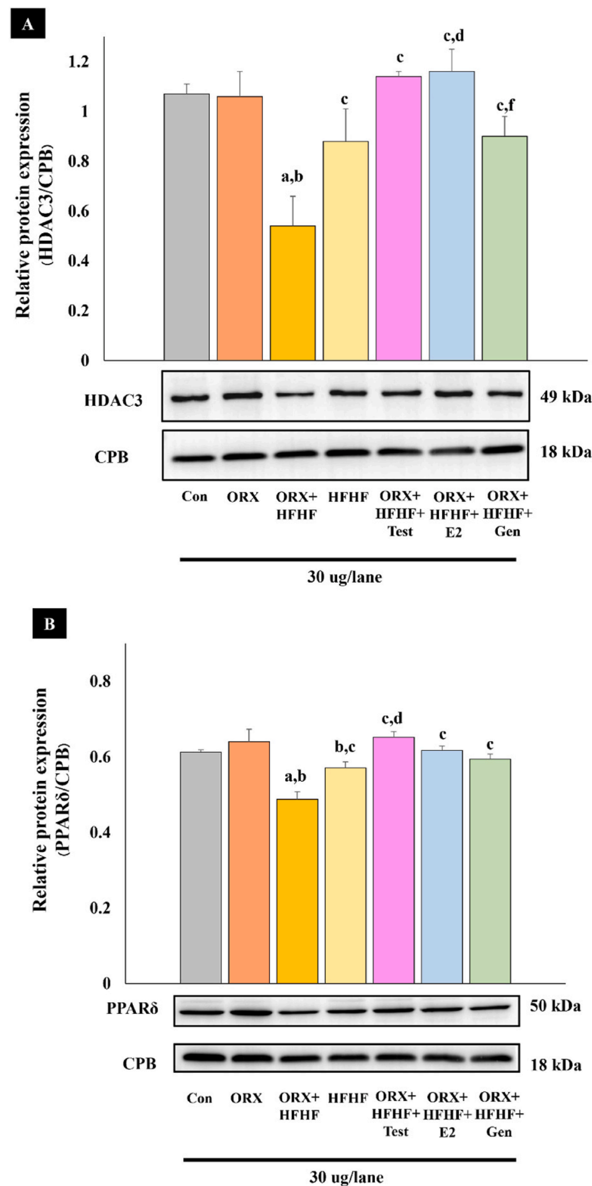

Fig. 4. Immunoblot analyses of HDAC3 and PPARδ protein expression in liver tissue of all experimental groups. Cyclophilin B (CPB) was used as loading control. The ratio of HDAC3 (A) to CPB and the ratio of PPARδ (B) to CPB were expressed as mean ± SD. $^{a}p<0.05$ when compared with the control group, $^{b}p < 0.05$ when compared with the ORX group, $^{c}p < 0.05$ when compared with the ORX + HFHF group, $^{d}p < 0.05$ when compared with the HFHF group and $^{f}p < 0.05$ when compared with the ORX + HFHF + E2 group. Full versions of all immunoblot images are exhibited in Supplementary Figs. 3 and 4.

HDAC3 is a class I histone deacetylase that forms a complex with the nuclear hormone receptor co-repressors to regulate gene transcriptional repression. In our study, we found a lower level of HDAC3 expression in the ORX + HFHF group. Previous studies showed that the loss of hepatic HDAC3 promoted liver steatosis through the upregulation of lipogenic genes, such as fatty acid synthase (FAS) and SREBP, and PPARγ expression [12,25]. Moreover, altered lipid homeostasis and NAFLD might occur in the setting of hepatic HDAC3 deficiency due to the defect in a circadian rhythm of histone acetylation and gene expression [26]. We could infer from these data that HDAC3 is an important modulator of lipid homeostasis in the liver and might be one of the key players in NASH pathogenesis.

Due to its being the least known subtype, PPARδ has increasingly become a focus of much research in the recent years. PPARδ is implicated in energy homeostasis through the enhancement of fatty acid transport and oxidation, the improvement of insulin sensitivity and the modulation of inflammatory responses [27,28]. A preclinical study demonstrated that testosterone deficiency in pigs fed with HFD could markedly reduce the expression of PPARδ, which was likely contributed to the development of hepatic steatosis and liver inflammation in these pigs [4]. Similarly, in this study, the hepatic expression of PPARδ was downregulated in ORX + HFHF rats.

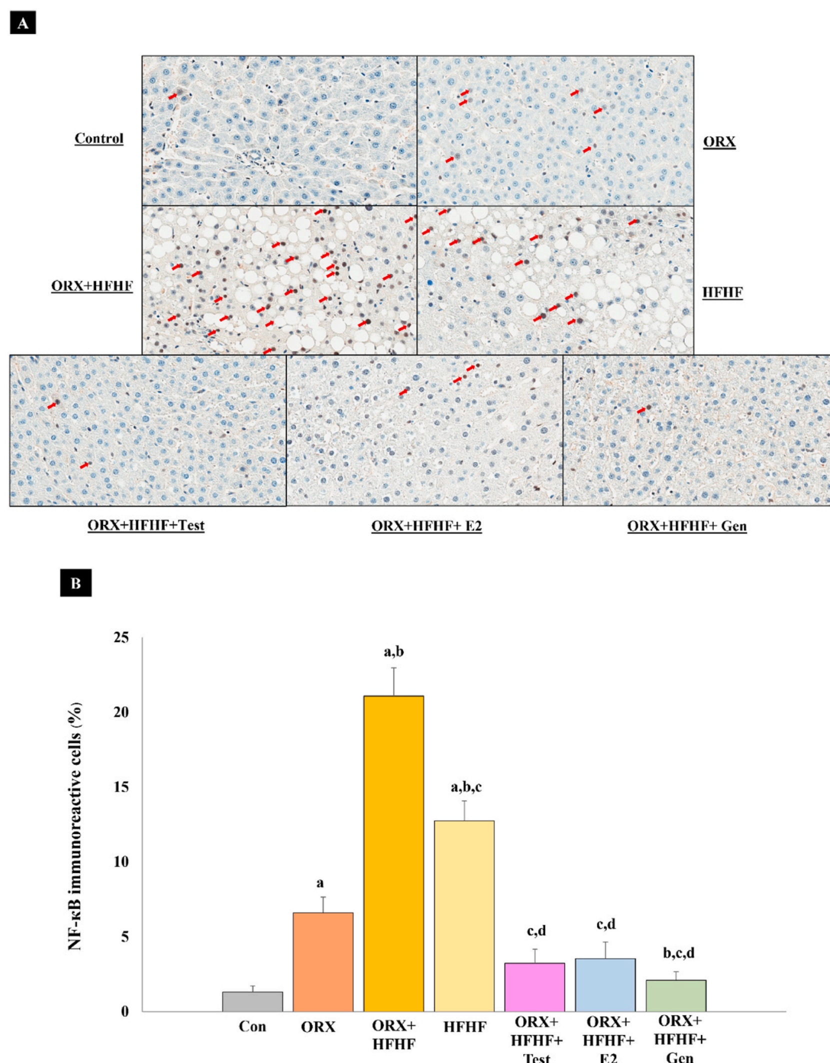

Fig. 5. Immunohistochemistry of hepatic NF-κB in rat liver tissue of all experimental groups. (A) Representative images of immunohistochemical study of NF-κB (40X magnification). (B) Bar graphs representing percentage of NF-κB -positive cells. Red arrows indicated NF-κB-positive cells. $^{a}p<0.05$ when compared with the control group, $^{b}p < 0.05$ when compared with the ORX group, $^{c}p < 0.05$ when compared with the ORX + HFHF group and $^{d}p < 0.05$ when compared with the HFHF group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

These results suggested that PPARδ could play a role in metabolic homeostasis and inflammatory responses in NAFLD. However, since protein levels of PPARδ were similar between control and ORX groups, testosterone deficiency alone might not have a significant impact on hepatic PPARδ expression.

NF-κB is recognized as a key mediator in the regulation of chronic inflammatory response and is activated in chronic liver diseases [29]. Previous research has shown that NF-κB signaling pathway played essential roles in testosterone deficiency and HFD induced NAFLD in pigs [4]. Moreover, PPARδ potentially exert its anti-inflammatory properties through the inhibition of NF-κB activation by displacing its p65 subunits in the liver [30,31]. Wang and colleagues reported that high expression levels of HDAC3 in arterial tissue could reduce inflammation via NF-κB inactivation in an atherosclerotic mouse model [32]. In line with prior studies, our results showed that PPARδ and HDAC3 down-regulation in ORX + HFHF rats might trigger the hepatic inflammation through NF-κB mediated pathway as evidenced by increased NF-κB expression in these rats.

Male and female sex hormones have previously been shown to improve NAFLD [4,33]. A previous cross-sectional study reported that low levels of circulating testosterone were closely associated with hepatic steatosis in male subjects [25]. Cai and his team also demonstrated that testosterone deficiency in pigs fed with HFD induced hepatic steatosis and liver inflammation [4]. Furthermore, estradiol replacement could also decrease fatty acid synthesis and liver steatosis in the pericentral area of the liver in orchiectomized rats fed with HFD [33]. These observations were in accordance with our findings that testosterone and estradiol treatment normalized NASH pathological features, restored HDAC3 and PPARδ expression, and reduced NF-κB protein levels comparing with the ORX +

HFHF group. Although there were no studies that directly determined the effect of sex hormone treatment on HDAC3 expression in NAFLD, we hypothesized that sex hormone ligand binding on its nuclear hormone receptors interacted with HDAC3 and thereby repressing certain genes involved in lipid metabolism and inflammation [34].

Genistein is a potential alternative for a hormone replacement therapy in transwomen because of its estrogen-like structure [35]. We found that genistein not only ameliorated hepatic steatosis, lobular inflammation, and ballooning degeneration, but also increased HDAC3 and PPARδ protein levels, and inhibited NF-κB expression. Even though the effect of genistein on hepatic fat accumulation was inferior to estradiol, its effect on hepatic inflammation was superior. Genistein exerts its action through estrogen receptors (ERs); however, its affinity to estrogen receptors is weaker than estradiol, which might explain its inferior effect on hepatic steatosis [36]. Nevertheless, genistein has anti-inflammatory effects that act through the inhibition of NF- κB leading to reduced hepatic inflammation in this study [7,37]. Due to its anti-lipogenic, anti-inflammatory, and sex hormone-modulating effects, genistein could potentially alleviate NASH in men with sex hormone deficiency. Clinical studies are needed to confirm this observation.

While hormones and genistein were found to be helpful in alleviating the severity of NASH, they should be used with caution in individuals at risk or with hormone-dependent and receptor-positive cancers. While studies have indicated that soy isoflavone intake may be associated with a reduced risk of breast cancer in pre-menopausal women, there were several confounders present in those studies regarding dosage [38]. Moreover, Susan and colleagues demonstrated that the high consumption of dietary phytoestrogens (lignan) could decrease the risk of ER positive/PR positive breast cancer in postmenopausal women [39]. In contrast, another study showed that soy protein isolate in a high concentration increased the growth of androgen-dependent prostate cancer model [40]. Interestingly, moderate consumption of phytoestrogens has been associated with beneficial effects, while excessive intake might have adverse effects, especially for individuals with hormone-dependent cancers [41]. Hormone therapy has widely been used for various indications including in transgenders. In transgender population, there have been reported instances of hormone-sensitive tumor development in individuals undergoing male-to-female transition with estradiol therapy; however, data remain conflicting due to the lack of sufficient long-term studies. For example, a recent systematic review demonstrated that the risk of hormone dependent tumor development was similar between transgender individuals and general population [42]. Moreover, a long-term study showed that increased mortality in transgender individuals was not due to hormone-related causes [43]. Until there is more data to confirm or dispute the risk of hormone-dependent tumors in transwomen, it is recommended to pursue a cautious approach by applying the minimum dose of hormone treatment required to achieve feminization.

NAFLD and/or NASH represent the most prevalent types of chronic liver disease globally, with a confirmed association with lipid metabolism imbalance, oxidative stress, mitochondrial dysfunction, and inflammation. Due to their similar properties to genistein, various antioxidative agents, including plant-derived and synthetic antioxidants, along with antioxidant vitamins, have been extensively studied in the management of NAFLD. For example, silybin, a natural product extracted from the *Silybum marianum* plant, acts as a PPARα agonist, regulating lipid metabolism and mitigating oxidative stress in NAFLD mice induced by HFD [44]. A previous *in vivo* NASH model reported that silybin exhibited antioxidant and anti-inflammatory properties by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) and suppressing NF-κB signaling pathways [45]. Similarly, resveratrol, a polyphenol compound, has been used as an antioxidant in the treatment of NAFLD. Resveratrol administration in animal models receiving a high-glucose and high-fat diet led to a reduction in hepatic triglyceride levels, together with a decrease in the expression of FAS and SREBP1c, and the induction of methylation at the promoter region of Nrf2 [46]. These results indicated that resveratrol had a potential to alleviate NAFLD through the epigenetic modification. Additionally, antioxidants vitamins (i.e. A, C, and E) have demonstrated positive effects on NAFLD. Prior rodent studies showed that antioxidant vitamins could protect against NAFLD by reducing lipogenesis, oxidative stress, inflammation, and fibrosis markers [47–49]. To summarize, acquiring a more comprehensive understanding of the biochemical mechanisms responsible for the hepatoprotective roles of these antioxidative compounds would facilitate the development of more precise and effective treatments for NAFLD.

Lastly, it is important to acknowledge both the strengths and limitations of this study. To the best of our knowledge, this was the first study to evaluate the treatment effects of genistein in an animal model that resembled transgender women with NASH. We found that genistein at the dose of 16 mg/kg BW showed the beneficial effects in ameliorating fat deposition and liver inflammation in this setting. However, due to the study design, this study only proved that genistein could prevent NASH in testosterone deficient rats that received genistein simultaneously with a HFHF diet. Further study is needed to evaluate whether genistein would be effective in improving liver histology in rats that have already developed NASH. Another limitation was that we did not measure the amount of food consumed by each rat so we could not say with certainty that the caloric intake was equal for all rats. Moreover, clinical studies are warranted to determine the appropriate dose and safety of genistein in transgender women with NASH.

5. Conclusion

Testosterone deficiency induced by orchiectomy increased the severity of NASH in the HFHF diet model. Treatment with testosterone, estradiol and genistein could attenuate hepatic fat accumulation and liver inflammation through the activation of HDAC3 and PPARδ expression, and the suppression of NF-κB expression. These findings suggested a link between HDAC3, PPARδ and NF-κB related pathways in the pathogenesis of NASH, and that genistein might be useful as a hormone replacement therapy to prevent NASH in transgender women.

Ethics declaration

The experimental procedures were conducted in accordance with the guidelines for experimental animals by the National Research

Council of Thailand, and approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Chulalongkorn University (License No.006-2564).

Consent for publication

Not applicable.

Data and material availability

All data generated or analyzed during this study are included in this published article and its supplementary file.

Funding

This work was supported by the 100th Anniversary Fund of Chulalongkorn University, the 90th Anniversary Fund of Chulalongkorn University (Ratchadaphiseksomphot Endowment Fund) (grant number GCUGR1125642007D) and the Grant of Ratchadaphiseksomphot, Faculty of Medicine, Chulalongkorn University, Thailand (grant number 2565-23).

CRediT authorship contribution statement

Fatist Okrit: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Maneerat Chayanupatkul:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Natcha Wanpiyarat:** Writing – review & editing, Methodology, Investigation. **Prasong Siriviriyakul:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Duangporn Werawatganon:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26055.

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