Therapeutic effects and molecular mechanisms of sodium-glucose cotransporter 2 inhibitor and ezetimibe in mouse model of non-alcoholic fatty liver disease

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Directed by Professor Jun Yong Park

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ABSTRACT

Therapeutic effects and molecular mechanisms of sodium-glucose cotransporter 2 inhibitor and ezetimibe in mouse model of non-alcoholic fatty liver disease

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(Directed by Professor Jun Yong Park)

Sodium-glucose cotransporter 2 inhibitor (SGLT2i) and ezetimibe, a cholesterol-lowering drug by targeting NPC1L1, have shown therapeutic potential for non-alcoholic fatty liver disease (NAFLD). SGLT2i and ezetimibe have different pharmacological mechanism, we hypothesized the combination of empagliflozin (selective SGLT2i) and ezetimibe could improve NAFLD additively. In this study, we used the choline-deficient high fat diet (CD-HFD)-induced murine model of NAFLD that has key features of human metabolic syndrome. At experiment 1, 6-week-old C57BL/6N mice were fed a CD-HFD for 8 weeks. Then these mice were divided into four groups: vehicle, ezetimibe (10 mg/kg), empagliflozin (10 mg/kg), and ezetimibe (10 mg/kg) + empagliflozin (10 mg/kg). Drugs were given once daily by oral gavage for 8 weeks. At experiment 2, 6-week-old C57BL/6N mice were fed a CD-HFD for 30

weeks, and treatment groups received each medication for 12 weeks. In experiment 1, NAFLD model revealed mild steatosis without significant liver inflammation and fibrosis, suggesting NAFL. Significant reduction of steatosis at histological examination and serum ALT level reduction were also observed after empagliflozin and ezetimibe combination treatment group comparing vehicle group. Expression of Srebf1c and Fas (de novo lipogenic genes) and Cd11c (pro-inflammatory gene) were significantly suppressed in combination group compared to vehicle group. In experiment 2, NAFLD model revealed severe steatosis with inflammation, suggesting progressive stage of NAFLD – NASH. In this setting, any treatment group including combination therapy did not revealed significant histological improvement including steatosis and inflammation as well as blood parameters. Conclusively, combination treatment of empagliflozin and ezetimibe exerted an anti-steatotic effect on NAFL mouse model. However, combination treatment of empagliflozin and ezetimibe therapy did not have therapeutic effect on advance stage of NAFLD and did not reveal better therapeutic effects comparing monotherapy group in NAFL and NASH mouse model. Therefore, more confirmative research on therapeutic effect of combination treatment of empagliflozin and ezetimibe need to be proceeded in future.

Key words : nafld, nash, sglt2 inhibitor, ezetimibe.

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I. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an inclusive term for pathological conditions of the liver in patients without excessive alcohol consumption that range from simple fatty liver to non-alcoholic steatohepatitis (NASH).[1] According to a recent study, approximately 25% of adults are affected with NAFLD in developed countries including South Korea.[2-5] Among disease spectrum of NAFLD, NASH has gained attention due to its high progressive ratio to cirrhosis and liver-related death. Despite disease burden of NAFLD, performance of currently existing pharmacological treatment is not satisfactory, and accordingly the need for novel treatment drugs is emerging.

Sodium glucose cotransporter 2 inhibitors (SGLT2i) is orally administered, and it decreases blood levels of glucose and hemoglobin (HbA1c) and improves insulin resistance in type 2 diabetic mellitus (DM) patients.[6] Several previous

studies showed that SGLT2i improved histological hepatic steatosis and inflammation in NAFLD or NASH mice models.[7-11] Moreover, recent findings including randomized controlled trials showed that SGLT2i reduced fatty liver content and improve biological markers in NAFLD patients with type 2 DM.[12] The treatment mechanism of NAFLD associated SGLT2i therapy is summarized as decreasing of lipid production, overcoming insulin resistance and reducing oxidative stress and inflammation through blood sugar reduction and weight loss.[13]

Despite promising results, there are some unresolved issues regarding SGLT2i treatment for NAFLD. At first, the therapeutic effects of SGLT2i with other NAFLD medication is not fully evaluated. Jojima et al. demonstrated that combination therapy of SGLT2i and dipeptidyl peptidase-4 (DPP-4 inhibitor), which is also widely used in DM patients, had synergetic effect on anti-steatotic and anti-inflammatory effect on NASH mice model.[8] This study suggested that combination treatment of SGLT2i with other potential medication of NAFLD could be beneficial. Secondly, it is not well known whether SGLT2i have therapeutic effect on liver which already had advanced stages of NAFLD such as NASH. Since previous studies were cross-sectional studies, there was no observation that therapeutic effect of SGLT2i showed any change according to the progression of liver disease.

Recent studies suggested that ezetimibe, a potent inhibitor of cholesterol absorption, can lead to improvement in metabolic, biochemical, and histological abnormalities of NAFLD and might be a promising agent for treatment of NAFLD.[14] Recent studies suggested that ezetimibe ameliorates NAFLD or NASH by various mechanism such as decreasing the susceptibility of the liver to oxidative injury or by modulating autophagy and a hepatocyte-driven exosome pathway in NAFLD mouse model.[15,16]

In this study, we investigated therapeutic effect of treatment with empagliflozin, which is commonly used SGLT2i in clinical practice, and

ezetimibe in NAFLD mouse model. Secondly, we also compared efficacy of SGLT2i based treatment in advanced stage of NAFLD mouse model with those with early stage of NAFLD.

II. MATERIALS AND METHODS

1. Animal models

In this study, NAFLD mouse model using choline deficient high fat diet (CD HFD; Research Diets; D05010402) was used in this study. Previous study demonstrated that a mouse model produced by long-term feeding of CD-HFD developed NASH and have key features of human metabolic syndrome.[17] Six-week-old C57BL/6N male mice purchased from Orient Bio (Sungnam, Korea). These animals were randomly assigned to one of five groups (5 mice in each group in experiment 1, 3 mice in each group in experiment 2): basal diet; choline-deficient high fat diet (CD-HFD), vehicle-treated; or CD-HFD, ezetimibe-treated; CD-HFD, empagliflozin-treated, and ezetimibe plus empagliflozin treated; CD-HFD. The mice had free access to diet and water, with temperature maintained at 23±2°C, humidity of 60%±10%, and 12h light/dark cycles.

The experiment consisted of experiment 1 (suggesting early stage of NAFLD) and experiment 2 (suggesting progressive stage of NAFLD). Experiment design were summarized in Figure 1. Experiment 1 utilized mice fed with a CD-HFD for 8 weeks, which results in simple steatosis. After that, CD-HFD with empagliflozin 10mg/kg (empagliflozin group), ezetimibe 10mg/kg (ezetimibe group), empagliflozin 10mg/kg plus ezetimibe 10mg/kg (combination group) was given once daily by oral gavage for 8 weeks. Experiment 2 utilized mice fed with a CD-HFD for 30 weeks, which result in NASH with obesity and diabetes. After that, CD-HFD with empagliflozin 10mg/kg (empagliflozin

group), ezetimibe 10mg/kg (ezetimibe group), empagliflozin 10mg/kg plus ezetimibe 10mg/kg (combination group) was given once daily by oral gavage for 12 weeks. The chow and CD-HFD with vehicle groups received the same volume of phosphate buffered saline orally for 12 weeks. After 12 weeks, the mice were anesthetized and killed; blood was collected via heart puncture. Tissues were harvested and either snap-frozen in liquid nitrogen and stored at -70°C or fixed in formalin and embedded in paraffin. All animal studies were approved by the Animal Care and Use Committee of the Yonsei University College of Medicine.

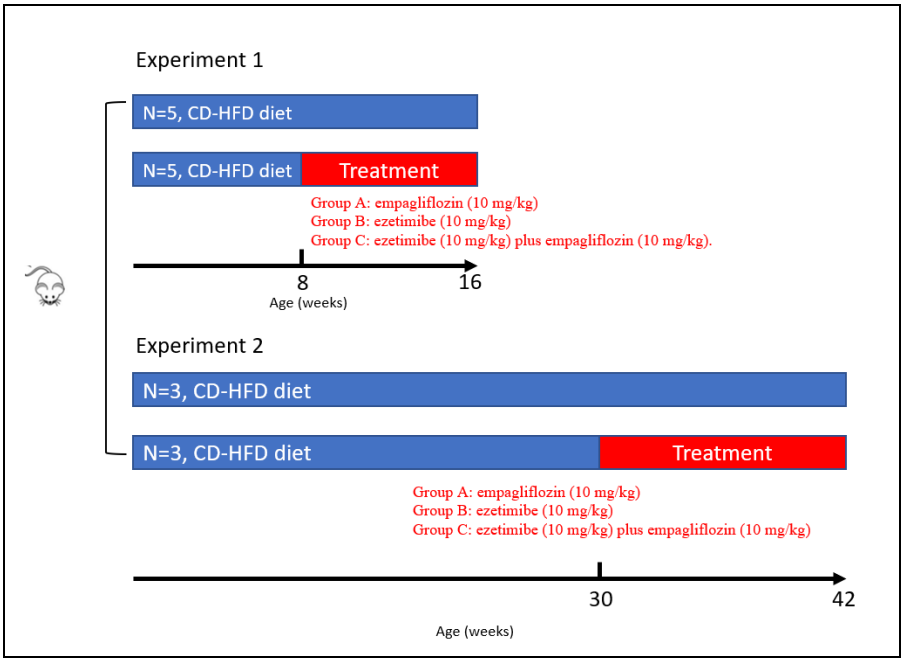

Figure 1. Study design of experiment 1 and 2.

2. Drugs and diets

Empagliflozin was purchased from Boehringer Ingelheim Pharma GmbH Co. (KG, Biberach an der Riss, Germany) and ezetimibe was purchased from Cayman Chemical (No.16331). A basal diet (BD) for control mouse model contained 22% protein, 6% fat, and 47% carbohydrate. A choline-deficient high-fat diet (CD-HFD; no added choline chloride, 22.6% protein, 23.5% fat (43% energy from fat), 5.4% fiber; Research Diets; D05010402) was used for inducing NAFLD in murine model.

3. Biochemical parameters

Right before sacrifice, body weight and liver weight were measured and liver to body weight ratio was calculated. Blood parameters including biochemical aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, fasting plasma glucose, and triglyceride (TG) were measured.

4. Measurement of hepatic triglyceride, cholesterol, and free fatty acid

After homogenization of liver tissues, triglyceride and cholesterol contents were measured using a Triglycerides Quantification Kit (Biomax, BM-TGF-100), Free Fatty Acid Quantification Kit Colorimetric/Fluorometric (Biomax, BM FFA-100) and Total Cholesterol and Cholesteryl Ester Colorimetric/Fluorometric Assay Kit (Biomax, BM-CHO–100), respectively, according to the manufacturer’s instructions.

5. Histological examination

Histological examination of liver was performed. Paraffin-embedded sections was stained with hematoxylin and eosin (H&E) and Sirius red (SR). Steatosis grade, inflammatory finding, and fibrosis grade were estimated; NAFLD activity score (NAS) were scored by pathologist in a blind manner according to the method of Kleiner et al.

6. RNA isolation and real-time polymerase chain reaction (PCR) analyses

Total RNA was extracted from liver tissues using a Hybrid-RTM (GeneAll, 305-101) and metabolism and reverse transcription was done using an iScript cDNA Synthesis Kit (BIORAD, #1708890) to get cDNA. Quantification of the complementary DNA template was performed by real-time PCR using SYBR green fluorescence on a LightCycler 480 instrument (Roche Applied Science). Primers for sterol regulatory element-binding protein 1C (Srebf1c), fatty acid synthase (Fas), acetyl-CoA carboxylase (Acc1), carnitine palmitoyltransferase 1a (Cpt1a), microsomal triglyceride transfer protein large subunit (MTTP), peroxisome proliferator-activated receptor alpha (Pparα), Adgre1, Cd11c, tumor necrosis factor (Tnf), and Acta2 are as follows. Srebf1c : forward 5'-TGACCCGGCTATTCCGTGA-3' and reverse 5'-CTGGGCTGAGCAATACAGTTC-3', Fas: forward 5'-GCGGGTTCGTGAAACTGATAA-3' and reverse 5'-GCAAAATGGGCCTCCTTGATA-3', ACC: forward 5'-CTCCCGATTCATAATTGGGTCTG-3' and reverse 5'-TCGACCTTGTTTTACTAGGTGC-3', Adgre1 : forward 5'-CTGCACCTGTAAACGAGGCTT-3' and reverse 5'-GCAGACTGAGTTAGGACCACAA-3', Acta2: forward

5'-CCCAGACATCAGGGAGTAATGG-3' and reverse 5'-TCTATCGGATACTTCAGCGTCA-3', Cpt1a: forward 5'-CCTGCATTCCTTCCCATTTG-3' and reverse 5'-TGCCCATGTCCTTGTAATGTG-3' Cd11c: forward 5'-GCCATTGAGGGCACAGAGA-3' and reverse 5'-GAAGCCCTCCTGGGACATCT-3', Tnf: forward 5'-ACCCTCACACTCAGATCATCTTC-3' and reverse 5'-TGGTGGTTTGCTACGACGT-3', Mttp: forward 5'-GAAAGAAGTGCTCCCTCAG-3' and reverse 5'-CCTTTGAACTTACTAAGGAGGG-3', Pparα: forward 5'-ACGATGCTGTCCTCCTTGATG-3' and reverse 5'-GTGTGATAAAGCCATTGCCGT-3', mouse GAPDH (NM_008084.2): forward 5'-AGAAACCTGCCAAGTATGATG-3' and reverse 5'-GGAGTTGCTGTTGAAGTCG-3'. The relative abundance of mRNA was expressed relative to the amount of the reference gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to the comparative threshold cycle (Ct) method.

7. Oral glucose tolerance test and intraperitoneal Insulin tolerance test

To evaluate insulin resistance, oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance (IPITT) were performed. OGTT procedure protocol was followings; fasting blood glucose was measured (6 h fast, blood taken from the tail vein) using glucometer. Glucose was then administrated by gavage (25% glucose solution, 1 g/kg mice) and blood glucose was measured again at time points of 15, 30, 45, 60, 75 ,90, 105 and 120 min, post gavage. IPITT procedure protocol was followings; fasting blood glucose was measured (4 h fast, blood taken from the tail vein) using a glucometer. Insulin was then

injected intraperitoneally (1 U/kg) and blood glucose was measured again at time points of 15, 30, 45, 60, 75, 90, 105 and 120 min, post injection.

8. Statistical analyses

Data are expressed as the median (IQR), or no.(%) as appropriate. Differences among the continuous and categorical variables were examined for statistical significance using Student's t-test (or Mann-Whitney test, if appropriate) and chi-squared test (or Fisher's exact test, if appropriate), respectively. A P-value <0.05, based on two-tailed test, was considered statistically significant. Data analysis was conducted using SAS software, version 9.2 (SAS Institute).

III. RESULTS

1. Baseline characteristics of NAFLD model of experiment 1 and 2

In the present study, CD-HFD was used to induce NAFLD in mice. In experiment 1, control and NAFLD groups were fed with BD and CD-HFD, respectively, for 16 weeks. After 16 weeks of feeding, body weight and liver weight were significantly higher in NAFLD group than control group. The liver histology of NAFLD group revealed steatosis but, neither significant liver inflammation nor fibrosis was not found and NAS were not significantly different between two groups (Fig 2). Serum level of AST, ALT, and cholesterol and hepatic lipid contents were significantly higher in NAFLD group than control group in experiment 1. The mRNA expression of Srebf1c and Fas in NAFLD group were higher than control group. Among inflammatory markers, only the mRNA expression level of Tnf was significantly higher in NAFLD group than control group (Fig 9).

In experiment 2, BD or CD-HFD was fed for 42 weeks. After 42 weeks, body weight, liver weight, and liver to weight ratio were significantly higher in NAFLD group than control group. Inflammatory cell infiltration and hepatocyte ballooning, which were known as the hallmark of NASH, were observed in NAFLD group, and NAS was significantly higher in NAFLD group than control group (Fig 2). The serum level of AST, ALT, cholesterol, and TG and hepatic lipid contents were significantly higher in NAFLD group than control group. (Fig 6). The hepatic mRNA levels of Srebf1c, Fas, Adgre1, Cd11c and Tnf were significantly higher in NAFLD group than control group, whereas the expression level of MTTP, which is marker of lipid outflow in the liver, is significantly lower in NAFLD group than control group. Acta2 tend to be higher in NAFLD group, but it was not statistically significant (Fig 10). Overall, these findings suggested that the NAFL model induced in Experiment 1 might

reflect an early stage of NAFLD with simple steatosis, whereas the model derived in Experiment 2 might reflect advanced stage of NAFLD - NASH.

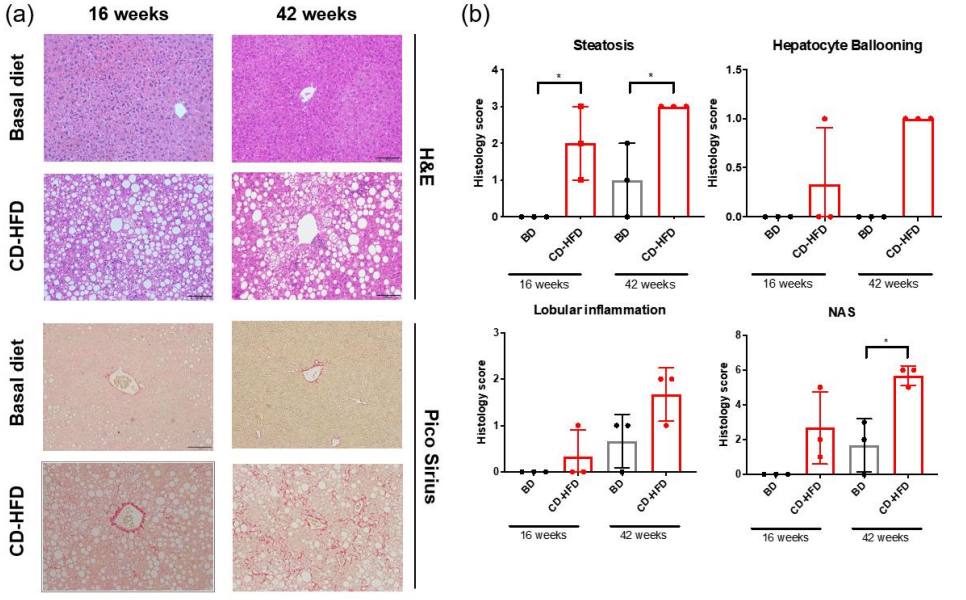

Figure 2. Representative microphotographs of liver sections stained with hematoxylin eosin and NAFLD activity score (NAS) in control group and vehicle group. Original magnification ×100. CD-HFD mice sequentially develop a fatty liver, steatohepatitis, advanced fibrosis and insulin resistance. Data are median ± range *P < 0.05 vs. vehicle.

2. Effect of empagliflozin and ezetimibe on the liver body weight ratio and hepatic lipid content

In experiment 1, body weight, liver weight and liver to weight ratio were significantly lower in empagliflozin group, ezetimibe group, and empagliflozin plus ezetimibe group than in vehicle group, but there was no significant difference among treatment groups (Fig 3). Similarly, hepatic cholesterol, hepatic TG, and hepatic FFA were significantly lower in empagliflozin group, ezetimibe group, and empagliflozin plus ezetimibe group than in vehicle group. (Fig 5).

In experiment 2, body weight, liver weight and liver to weight ratio were significantly lower in empagliflozin group and ezetimibe plus empagliflozin group than in vehicle group, but there was no significant difference between ezetimibe group and vehicle group (Fig 4). Hepatic FFA was significantly lower in empagliflozin group, ezetimibe group, and empagliflozin plus ezetimibe group than vehicle group, but hepatic cholesterol level was significantly lower only in empagliflozin group. (Fig 6)

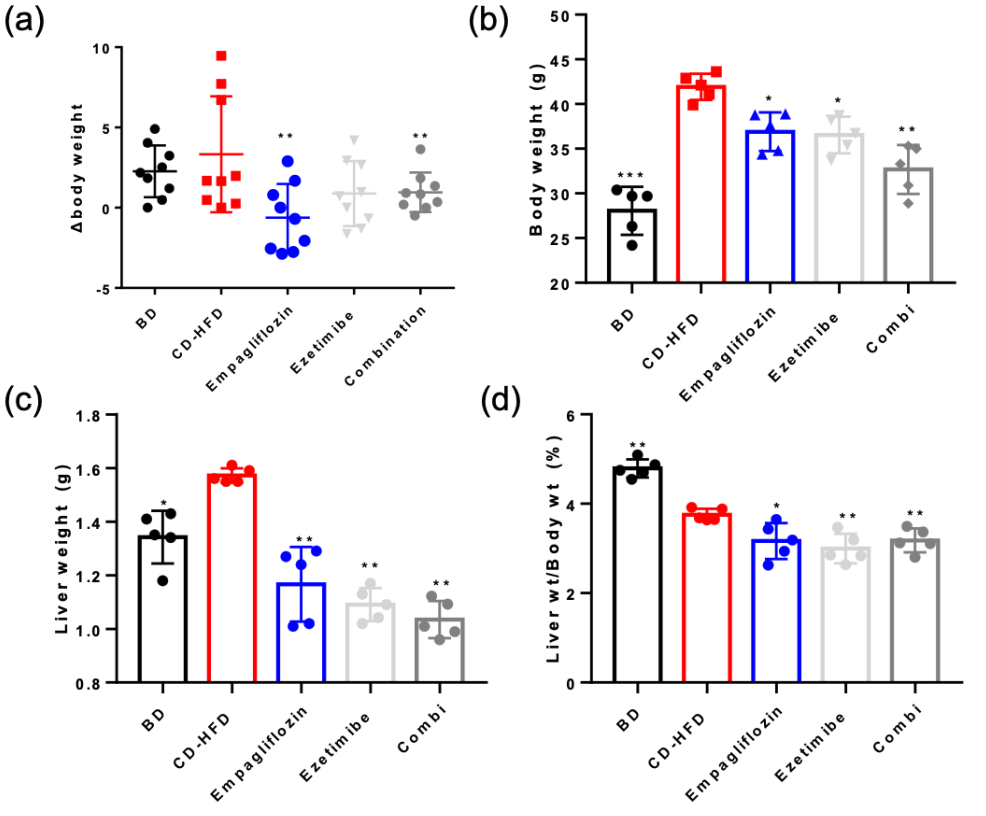

Figure 3. Change in body weight (a), body weight (b), liver weight (c), liver to body weight ratio (d) in the five groups in experiment 1. Data are median (range). *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle.

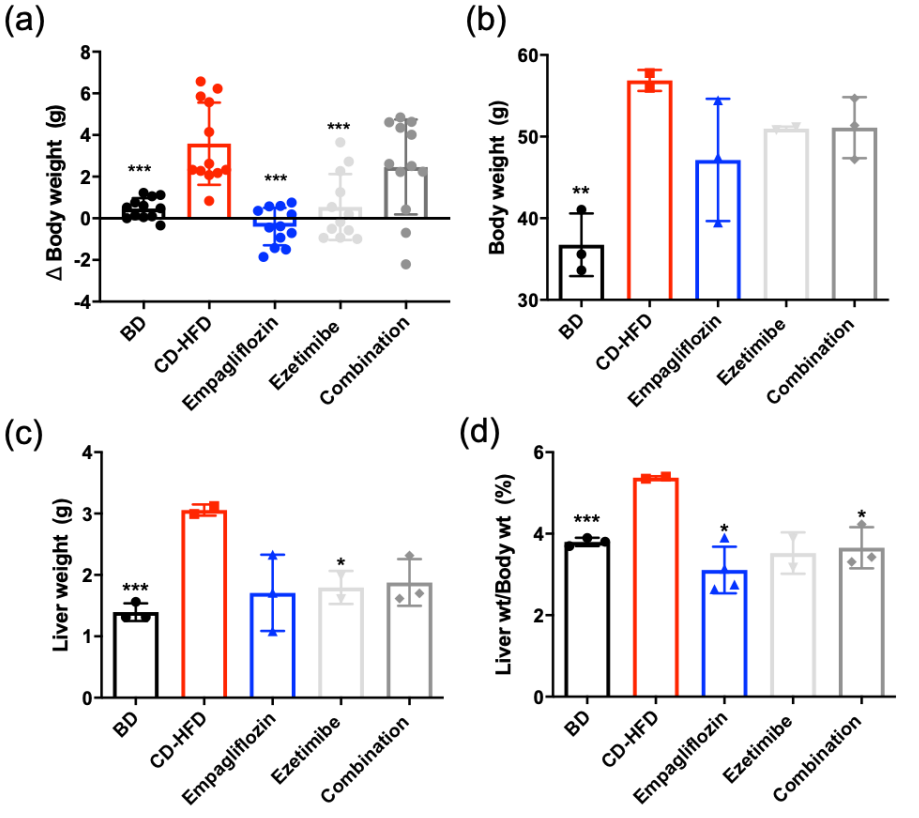

Figure 4 Change in body weight (a), body weight (b), liver weight (c), liver to body weight ratio (d) in the five groups in experiment 2. Data are median (range). *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle.

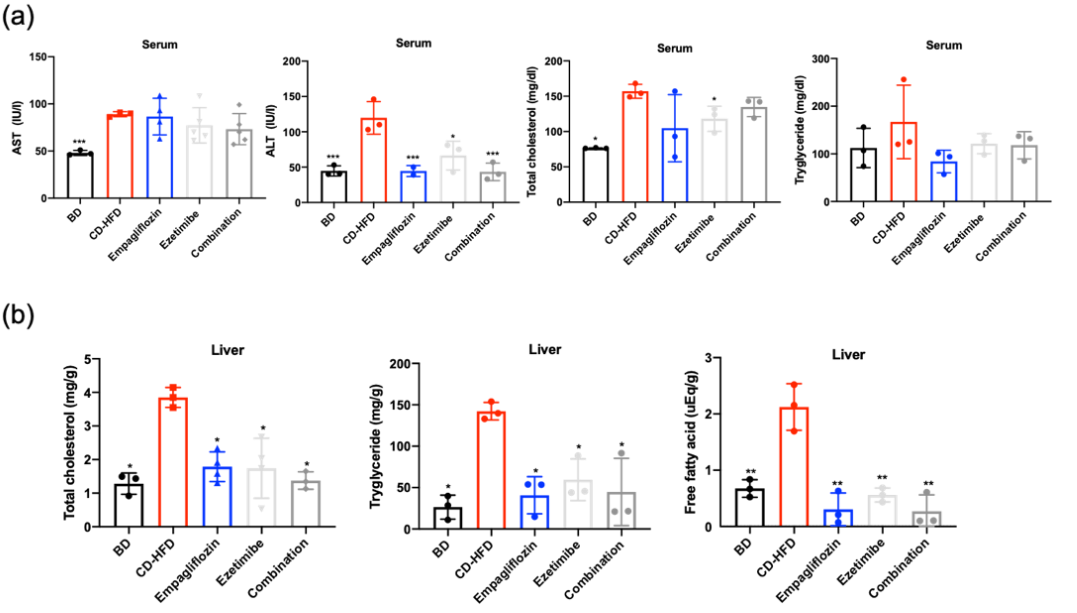

Figure 5. Blood parameter (A) and hepatic lipid contents (B) in five group in experiment 1. Data are median (range). *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle. Abbreviations: AST, Alanine aminotransferase; ALT, alanine aminotransferase; TG, Triglycerides.

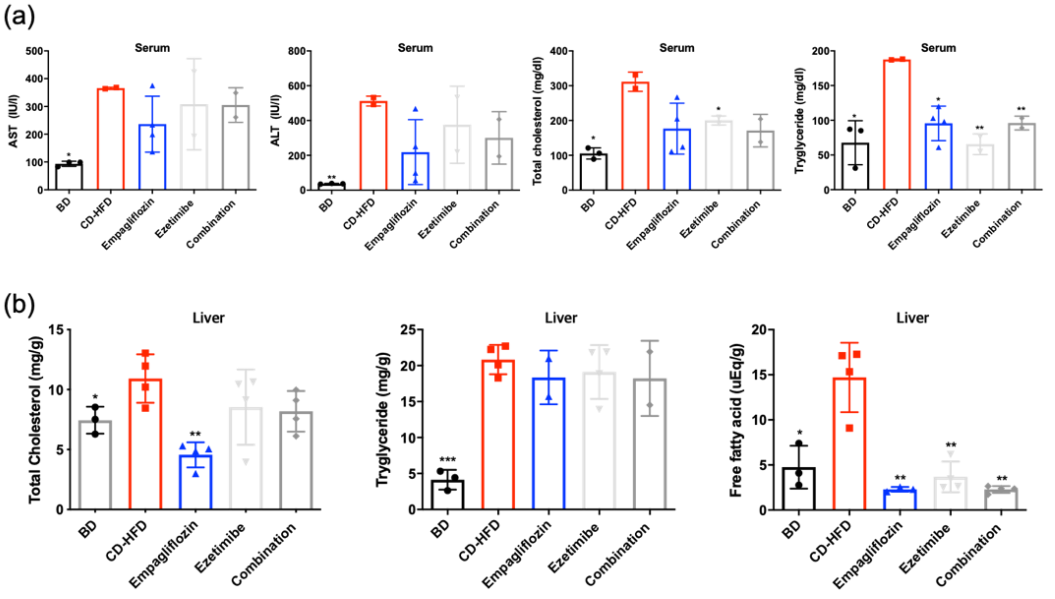

Figure 6. Blood parameter (A) and hepatic lipid contents (B) in five group in experiment 2. Data are median (range). *P < 0.05, **P < 0.01 vs. vehicle. Abbreviations: AST, Alanine aminotransferase; ALT, alanine aminotransferase; TG, Triglycerides.

Table 1. Body weight, liver weight and biochemical parameters on week 16 in NAFLD mice (vehicle, empagliflozin (10 mg/kg), empagliflozin (10 mg/kg), ezetimibe (10 mg/kg) plus ezetimibe (10mg/kg)

	Basal Diet (N=5)	Vehicle (N=5)	Ezetimibe (N=5)	Empagliflozin (N=5)	Ezetimibe plus Empagliflozin (N=5)
Body weight (g)	29.93±0.40	42.86±0.75	37.43±1.77	36 ± 2.43	31.7±3.27
Liver weight (g)	1.39±0.04	1.57±0.02	1.08±0.05	1.1±0.14	0.98±0.02
Serum Biochemistry					
AST (IU/I)	48.5±3.53	102.25±22.29	77.2±18.72	86.5±19.41	73.2±16.48
ALT (IU/I)	47±8.48	140.25±38.15	71.2±19.31	73.25±35.14	46.2±10.32
Total cholesterol(mg/dl)	71.6±8.38	160.6±9.23	132.6±25.74	127.2±45.78	135.4±13.08
TG (mg/dl)	128.2±41.75	116±82.38	120±47.01	66.8±29.43	72.75±45.49

Notes: Data expressed as mean ± standard deviation, Abbreviations: AST, Alanine aminotransferase; ALT, alanine aminotransferase; TG, Triglycerides.

Table 2. Body weight, liver weight and biochemical parameters on week 42 in NAFLD mice (vehicle, empagliflozin (10 mg/kg), empagliflozin (10 mg/kg), ezetimibe (10 mg/kg) plus ezetimibe (10mg/kg)

	Basal Diet (N=3)	Vehicle (N=3)	Ezetimibe (N=3)	Empagliflozin (N=3)	Ezetimibe plus Empagliflozin (N=3)
Body weight (g)	36.75±3.84	56.89±1.28	50.96±0.25	47.14 ± 7.48	51.09±3.74
Liver weight (g)	1.39±0.14	3.05±0.09	1.79±0.26	1.70±0.62	1.87±0.38
Serum Biochemistry					
AST (IU/I)	93.66±9.23	366±2.83	308±164.04	237±123.5	305±62.22
ALT(IU/I)	37±2	512±28.28	375.5±221.32	207.66±226.47	300.5±150.61
Total cholesterol (mg/dl)	67.7±31.7	87.5±0.71	65.5±14.84	94.6±30.36	96±9.89
TG (mg/dl)	105.33±16.01	311.5±27.58	200±12.72	167.3±86.55	171±46.66

Notes: Data expressed as mean ± standard deviation, Abbreviations: AST, Alanine aminotransferase; ALT, alanine aminotransferase; TG, Triglycerides.

3. Effect of empagliflozin and ezetimibe on the histological examination

In experiment 1, steatosis grade was significantly lower in empagliflozin plus ezetimibe group than vehicle group. However, there is no significant difference of lobular inflammation grade and NAS between two groups. (Fig 7) Other treatment group did not reveal significant histological improvement than vehicle group. In experiment 2, steatosis grade and lobular inflammation grade, and NAS were not significantly different between any treatment groups and vehicle group, and there was no difference among treatment groups. (Fig 8)

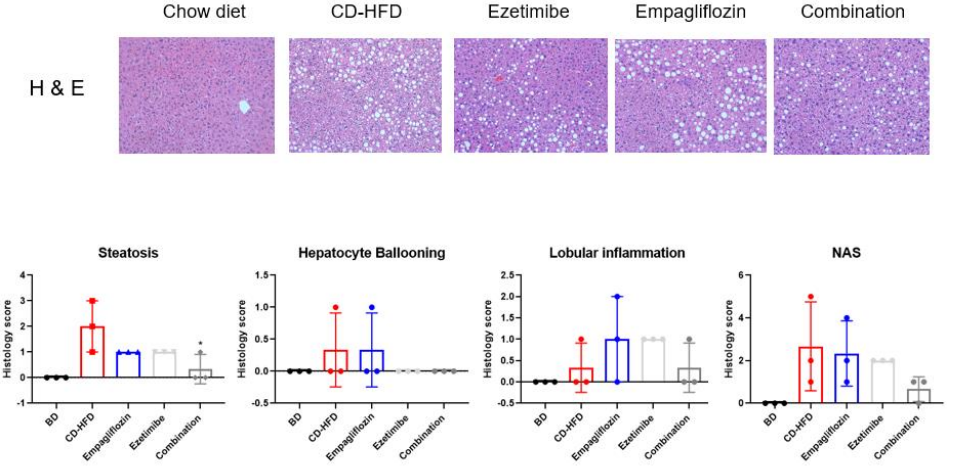

Figure 7. Representative microphotographs of liver sections stained with hematoxylin eosin and NAFLD activity score (NAS) in the five groups in experiment 1. Original magnification ×100. Data are median (range) *P < 0.05 vs. vehicle.

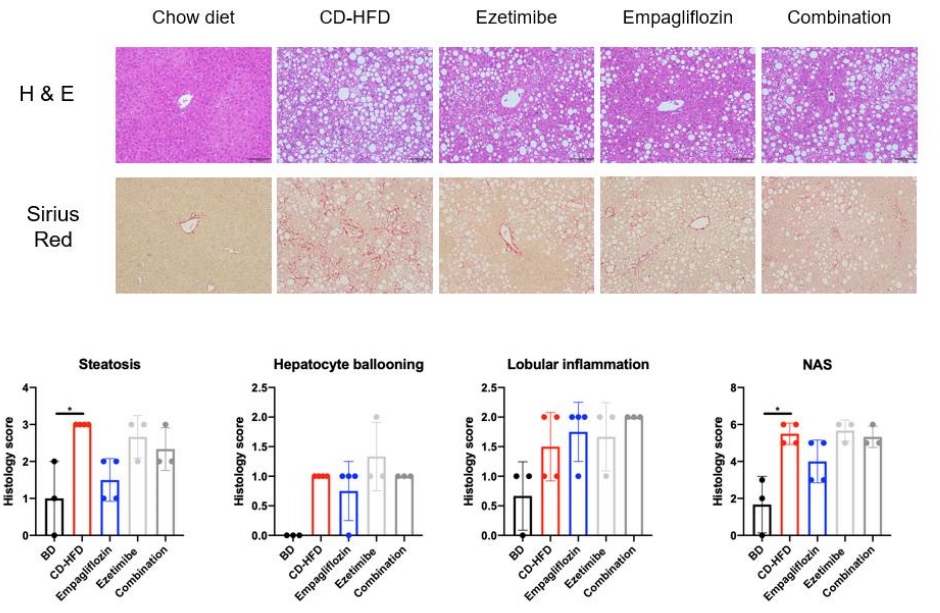

Figure 8. Representative microphotographs of liver sections stained with hematoxylin eosin and sirius red staining and NAFLD activity score (NAS) in the five groups in experiment 2. Original magnification ×20. Data are median (range) *P < 0.05 vs. vehicle.

4. Effect of empagliflozin and ezetimibe on biochemical parameters

In experiment 1, ALT was significantly lower in empagliflozin group, ezetimibe group, and empagliflozin plus ezetimibe group than in vehicle group. Serum cholesterol was significantly lower in ezetimibe group than in vehicle group. There was no significant difference of AST and serum TG levels between treatment groups and vehicle group. (Fig 5)

In experiment 2, there was no significant difference of AST and ALT between treatment groups and vehicle group. Serum cholesterol was significantly lower in ezetimibe group than vehicle group and serum TG level was significantly lower in empagliflozin group, ezetimibe group, and empagliflozin plus

ezetimibe group than in vehicle group. (Fig 6)

5. Effect of empagliflozin and ezetimibe on hepatic fat metabolism, hepatic inflammation, and hepatic fibrosis

In experiment 1, the mRNA expression of Srebf1c and Fas were significantly suppressed in empagliflozin plus ezetimibe group than in vehicle group. The mRNA expressions of MTTP, which is marker of lipid outflow in the liver, were significantly higher in ezetimibe group comparing in vehicle group (Fig 8). The mRNA expressions of Cd11c which is one of inflammatory markers, was significantly lower in ezetimibe group, and empagliflozin plus ezetimibe group than in vehicle group. There was no significant difference of the expressions of Acta2 between treatment groups and vehicle group.

In experiment 2, the CPT1a was significantly lower in empagliflozin group than in vehicle group. Except that, there was no significant difference of the mRNA expression level of Srebf1c, Fas, Acc1, MTTP, Pparα, Adgre1, Cd11c, TNF, and Acta2 between any treatment groups and vehicle group (Fig 10).

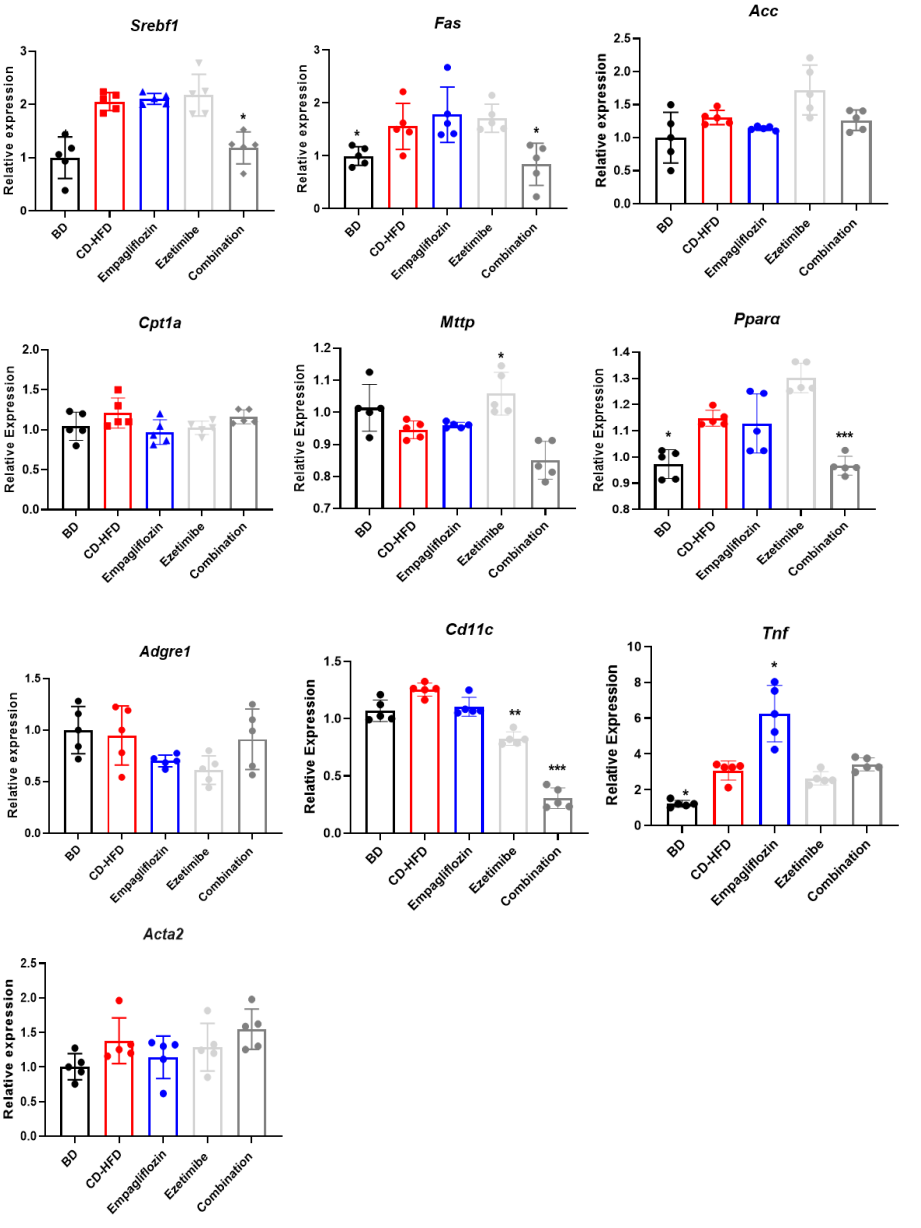

Figure 9. Gene expression of lipogenesis, inflammation and fibrosis in the liver of the five groups in experiment 1. Data are median (range). *P < 0.05 vs. vehicle.

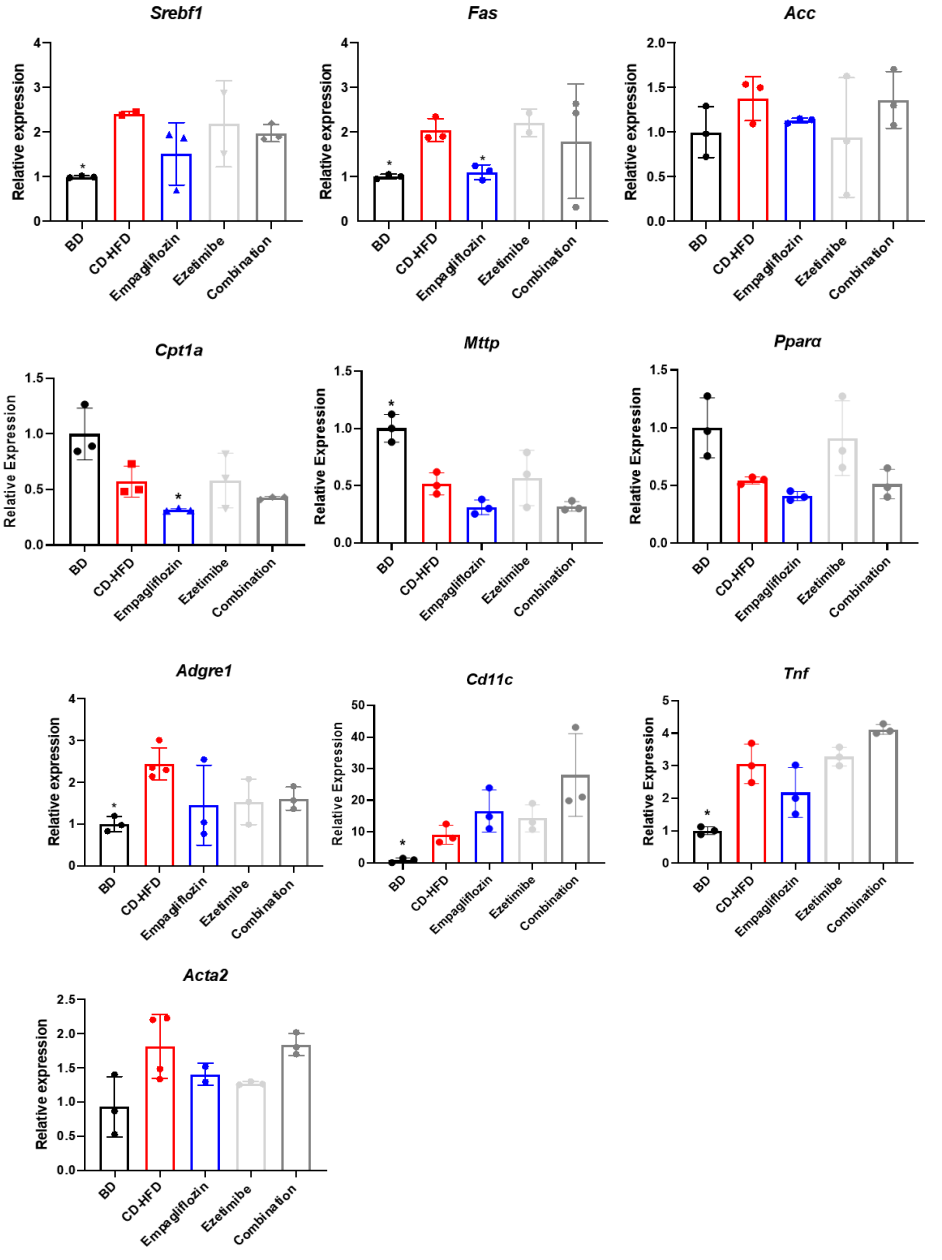

Figure 10. Gene expression of lipogenesis, inflammation and fibrosis in the liver of the five groups in experiment 2. Data are median (range). *P < 0.05 vs. vehicle.

6. Effect of empagliflozin and ezetimibe on insulin resistance

OGTT and IPITT tests were performed at 16 (Experiment 1) and 42 (Experiment 2) weeks to estimate improvement insulin resistance by treatment. In the OGTT of experiment 1, the plasma glucose increased to a maximum after 30 min of oral administration of glucose in all groups, but this maximum was not significantly different between vehicle group and treatment groups. The time-course of glucose clearance in vehicle group was delayed compared to treatment groups, remaining elevated for 120 min after glucose administration, qualifying for characterization as glucose intolerance. IPITT revealed higher decline in plasma glucose after 15 min of insulin administration in treatment groups than in vehicle group, and plasma glucose remained higher in vehicle group than in treatment groups at all time-points up to 120 min, suggesting that treatment improved insulin resistant.

In experiment 2, IPITT revealed that significant decline of plasma glucose after insulin administration were observed in empagliflozin group and empagliflozin plus ezetimibe group, suggesting insulin resistance was improved in SGLT2i-based treatment in this condition.

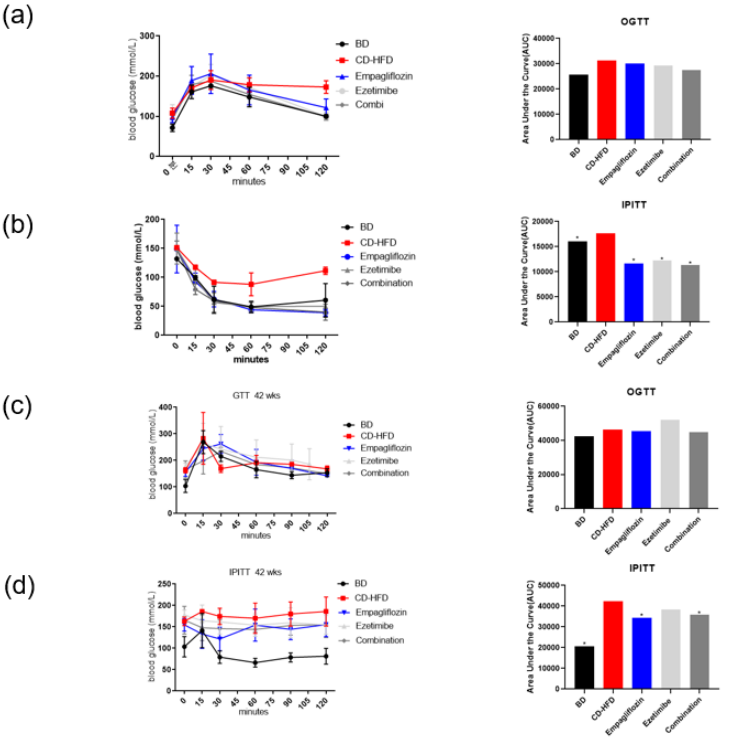

Figure 11. Oral glucose tolerance test and intraperitoneal insulin tolerance test. (A) (B) Comparing OGTT and IPITT at 16 weeks at control group between vehicles. (C), (D) Comparing OGTT and IPITT at 42 weeks at control group between vehicles. *$P < 0.05$ vs. vehicle.

IV. DISCUSSION

Previous studies have shown that SGLT2i reduced hepatic steatosis or steatohepatitis in obese type 2 diabetic mice or rats[7, 9, 11, 18] Additionally, recent study demonstrated that empagliflozin significantly reducing liver fat and improves ALT levels in patients with type 2 diabetes and NAFLD.[12] These study suggested that SGLT2i could be useful option for treating NAFLD. Consistent with previous study, our study revealed that empagliflozin treatment prevented development of NASH in early stage NAFLD mouse model. Histological improvement of steatosis and significant ALT reduction were found in empagliflozin groups. Reduction of intrahepatic lipid contents was also found in empagliflozin group. Among known mechanisms of developing NAFLD, the previous study suggested that the main mechanisms of SGLT2i for improving NAFLD is decrease of lipid production (de novo lipogenesis).[8] As preventing intrahepatic lipid accumulation is important with development of steatosis in NASH, we hypothesized that combination of empagliflozin and lipid lowering agent might have synergetic effect on NAFLD. Among variable lipid lowering agent, ezetimibe is widely used as lipid lowering agent in clinical practice and is generally well tolerated in combination with other medications such as statins or insulin-sensitizing agents.[14] There were several evidences from preclinical models to supporting the use of ezetimibe for NAFLD. Wang et al. demonstrated that administration of ezetimibe significantly reduced liver steatosis and fibrosis in NAFLD mouse model induced high fat diet and ezetimibe significantly reduced hepatic mRNA gene expression, which are involved in hepatic fatty acid synthesis such as ACC1 and Scd1.[19] Moreover, they revealed that ezetimibe could upregulate of Cd36 gene expression, which is significantly associated with insulin resistance and hyperinsulinemia. Other recent study also suggested that ezetimibe decreased the susceptibility of the liver to oxidative injury by preventing apoptotic cell death through

p62-dependent Nrf2 activation.[16] To date, there is no data regarding that combination of SGLT2i and lipid lowering agent might have synergetic effect to preventing NAFLD or not, our study tried to demonstrate the hypothesis that SGLT2i and ezetimibe might have additive effect. In experiment 1, we observed that combination treatment of empagliflozin and ezetimibe treatment induced significant histological steatosis improvement, whereas empagliflozin group or ezetimibe group did not. Expression of both Srebf1c and Fas, which is involved in de novo lipogenesis, were significantly only lower in empagliflozin and ezetimibe group than in vehicle group. Our results might suggest that combination of empagliflozin and ezetimibe might have a better effect for preventing NAFLD in early stage than single use of empagliflozin or ezetimibe. However, our data did not provide a conclusive evidence about the effect of combination with empagliflozin and ezetimibe, because the empagliflozin plus ezetimibe group failed to show statistically significant differences in NAS and intrahepatic lipid contents compared to monotreatment group of empagliflozin or ezetimibe. Therefore, it requires further investigation to draw a conclusion about the additive effect of SGLT2i and ezetimibe on NAFLD.

This study also investigated whether the combination of empagliflozin and ezetimibe could have therapeutic effects in advanced stage of NAFLD. In this study, model derived in experiment 2 reflects advanced stage of NAFLD with inflammation. Unexpectedly, our data revealed that empagliflozin treatment as well as empagliflozin plus ezetimibe combination treatment did not show significant reduction of histological steatosis and inflammation in experiment 2. Although liver to weight to ratio was significantly lower in both empagliflozin group and empagliflozin plus ezetimibe group, hepatic cholesterol and TG were not significantly different between these two groups and vehicle group. Additionally, reduction of ALT and mRNA expression of proinflammatory gene and fibrosis were not different between two groups. Our data suggested that therapeutic effect of SGLT2i could be attenuated in NASH, which resulted

from persistent hyperalimentation state.

However, contrary with our results, some studies suggested that SGLT2i based treatment could ameliorate hepatic fibrosis in the advanced stage of NAFLD. Jojima et al. demonstrated that empagliflozin significantly reduced collagen deposition and hepatic expression of inflammatory genes, suggesting SGLT2i might have anti-fibrotic and anti-inflammatory effects on NASH.[8] It is not easy to compare directly our findings to previous results, because NAFLD mouse models were not identical between two studies. However, considering histological ALT level in vehicle group (42 IU/L vs 512 IU/L), the degree of fatty liver progression seemed to be more severe in our study. Therefore, it needs more investigation to conclude that the effect of SGLT2i-based therapy might be influenced according to the degree of NAFLD progression.

Our study has several limitations. At first, although the NAFLD mouse model generated using CD-HFD is known to have obesity and insulin resistance, which are associated with NASH in humans, liver damage due to prolonged CD-HFD administration might be so severe that medication effect might be affected by impaired liver function or cirrhotic status. Secondly, our data did not provide research results on the mechanism that SGLT2i and ezetimibe combination therapy may have a synergy effect.

V. CONCLUSION

In conclusion, combination treatment of empagliflozin and ezetimibe showed anti-steatotic effect on early stage of NAFLD. However, the therapeutic effect of the drug seemed to be attenuated in the case of advanced NAFLD, and synergetic effect of combination treatment over monotherapy was not demonstrated in both early and late NAFLD. Therefore, more confirmative research on therapeutic effect of combination treatment of empagliflozin and ezetimibe need to be proceeded in future.

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ABSTRACT(IN KOREAN)

SGLT2억제제와 Ezetimibe 치료요법의 비알코올성간질환 mice model에서의 치료 효과 및 분자 메카니즘 규명 연구

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정규식

비알코올성지방간 (NAFLD)은 현재 국내외에서 유병율이 늘어날 뿐 아니라, 그에 따른 질병 부담이 늘어나는 중요한 질환이라고 할 수 있다. 하지만, 현재 존재하는 약물치료의 성적이 만족스럽지 않으므로, 새로운 치료제에 대한 연구가 지속되고 있다. 최근의 연구결과는 당뇨약제인 SGLT2 저해제 (SGLT2i) 및 고지혈증약제인 Ezetimbie가 NAFLD의 치료효과가 있음이 보고되었다. 이 연구에서는 NAFLD Mouse 모델에서 SGLT2i와 Ezetimibe를 병용투여하였을 때 치료효과 및 그에 따른 메커니즘을 조사하였다. 본 연구에서는 choline deficient high fat diet (CD-HFD)를 이용한 NAFLD mouse model을 사용하였으며, 경구 SGLT2 저해제로는 empagliflozin을 사용하였다. 실험은 기간에 따라 실험1과 2로 나누었다. 실험 1에서, 6주령 수컷 C57BL/6N mouse에 8주동안 CD-HFD를 투여하였으며, 이후 4군으로 나누어 각각 vehicle group, empagliflozin, ezetimibe, empagliflozin와 ezetimibe 병합요법으로 나누어 CD-HFD와 약제를 동시에 투여하였다. 실험 2는 실험1과 동일한 조건에서, vehicle group은 42 주 동안 CD-HFD식이를

공급받았고, 약물투여군은 CD-HFD를 30 주 동안 공급 한 후 CD-HFD를 약물과 함께 12 주 동안 투여받았다. 이후 sacrifice 하여 결과를 분석하였다. 실험 1에서, 16주 CD-HFD 투여를 통해 NAFLD 모델은 조직학적으로 지방증발생을 보였으나, 염증반응 및 섬유화발생은 거의 일어나지 않았다. Vehicle group과 비교하여 empagliflozin와 ezetimibe 병합요법투여 그룹에서 조직학적검사상 지방간 축척이 감소하였으며, NAS가 감소하였다. 또 한, 간내 콜레스테롤 및 중성지방 축척이 감소되었으며, 혈청 ALT 수준도 유의미하게 감소하였다. 또 한 인슐린 저항성이 호전되었다. 또 한, empagliflozin와 ezetimibe 병합요법투여 그룹에서 지방 생성과 연관이 있는 유전자인 SREBP 및 FAS의 발현과 염증 발현과 연관된 Cd11c의 유전자 발현은 유의하게 억제되었다. 실험 2에서, 42주의 CD-HFD 투여를 통하여, NAFLD 모델은 염증 및 섬유화를 동반한에 의한 진행된 NAFLD소견을 보였다. 이 실험에서는 병용 요법을 포함한 모든 치료 그룹에서 Vehicle group과 비교하여 지방간 축척 및 염증성 변화에 대한 조직학적 개선 및 ALT 개선 등이 관찰되지 않았으며, 지방생성, 염증, 섬유화 와 연관된 gene의 expression에도 유의한 차이가 관찰되지 않았다.

결론: 본 연구에서 empagliflozin와 ezetimibe 병합요법투여 병용치료는 NAFLD mouse model의 초기단계에서 효과를 나타냈다. 그러나, 진행된 NAFLD의 경우, 약물의 치료 효과가 약화된 것으로 관찰되었으며, 병합요법투여가 단독치료군에 비해 명확하게 개선된 치료효과를 보이지는 않았다. 따라서, empagliflozin와 ezetimibe 병합요법투여의 효능에 대한 추가적인 연구가 필요할 것으로 생각된다.

지방간, 지방간염, SGLT2 저해제, 에제티미브

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