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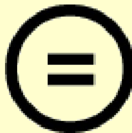

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Effect of Ezetimibe on (Hepatic/Adipose Tissue) Glucose Metabolism and Inflammatory Markers

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Effect of Ezetimibe on (Hepatic/Adipose Tissue) Glucose Metabolism and Inflammatory Markers

Directed by Professor Eun Seok Kang

Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Yongin Cho

June 2020

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor Eun Seok Kang for inspiring me and providing the purpose of this study. He generously gave me the opportunity to participate in this research, and lots of advice and guidance. I would also like to express my appreciation to Professors Beom Seok Kim, Se Eun Park, Yong-ho Lee, and Sungsoon Fang, for kindly giving their advice and helping me to complete this paper.

Most importantly, I would like to thank my wife, Hye-sun Park, who supported and helped me through difficult situations. She gave me the strength to finish my studies without giving up. I am grateful to my daughter, Yeon-seo, who always loves me unconditionally. I also give infinite thanks to my parents-in-law, who encouraged me to concentrate on my studies with confidence. Lastly, I want to convey my love and gratitude to my parents who's unwavering support gave me confidence.

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ABSTRACT

Effect of Ezetimibe on (Hepatic/Adipose Tissue) Glucose Metabolism and Inflammatory Markers

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(Directed by Professor Eun Seok Kang)

Background:

Ezetimibe is a drug that inhibits Niemann-Pick C1-Like 1 which reduces cholesterol absorption. Despite numerous investigations, the effects of ezetimibe on glucose metabolism have not yet been clearly identified. In this study, we aimed to investigate the effects of ezetimibe on glucose metabolism and inflammatory markers in a cell model, a rodent model, and in human. By identifying the molecular mechanisms of action, we aimed to determine whether ezetimibe can be safely used in patients with diabetes and hepatic steatosis.

Methods:

Changes in glucose metabolism were investigated in C57BL/6J mice and Wistar rats, using ezetimibe under high fat diet (HFD) conditions. Hepatic steatosis and inflammatory markers in liver and adipose tissues of Wistar rats were compared.

Using data from patients with hyperlipidemia, the effects of ezetimibe treatment with statin on glucose metabolism over a 1-year period were also investigated.

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Results:

In the ezetimibe groups, C57BL/6J mice and Wistar rat models both showed partial improvement in several glucose metabolism indices. Smaller fat cell size and reduced M1-polarized macrophage accumulation were also observed in the ezetimibe groups. Anti-inflammatory M2 phenotype of macrophages and fatty acid oxidation were induced within adipocytes. Free fatty acid levels were decreased in serum. However, these changes did not lead to systemic lowering of blood sugar levels, and improvement of hepatic steatosis was not significant in this group.

Clinical data analysis showed that statin monotherapy significantly increased insulin resistance. However, the use of ezetimibe in combination with statin did not increase insulin resistance, measured using the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index.

Conclusion:

Ezetimibe reduced the size of visceral fat adipocytes and serum level of free fatty acid, and induced fatty acid oxidation. It improved the inflammation of adipocytes, and partially improved glycemic indices.

Key words: ezetimibe, adipocyte, inflammation, glucose metabolism

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Effect of Ezetimibe on (Hepatic/Adipose Tissue) Glucose Metabolism and Inflammatory Markers

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I. INTRODUCTION

Ezetimibe, a hyperlipidemic drug, selectively inhibits cholesterol absorption by binding to the carrier of cholesterol, Niemann-Pick C1 like 1 (NPC1L1), which is present in the intestinal membranes. Ezetimibe lowers the concentration of blood cholesterol, by suppressing cholesterol absorption in the intestines. It can be used as an additional or alternative drug when 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (statin) provides insufficient lipid-lowering effects or is contraindicated. In a recently published large-scale clinical study (IMPROVE-IT trial), ezetimibe used in combination with statins significantly improved hypercholesterolemia and reduced cardiovascular disease, reducing the relative risk of cardiovascular event by 6.4% (compared with statin monotherapy and ezetimibe and statin combination therapy). There were no differences in liver-related side effects, muscle-related side effects, or cancer incidence between groups (1).

Replacing high-dose statin with ezetimibe (alone or in combination with statin) appears to reduce the risk of side effects such as muscle toxicity, hepatotoxicity, and hyperglycemia. However, the effects of ezetimibe on glucose metabolism have not yet been clearly identified. Although several studies have demonstrated that ezetimibe contributes to improved insulin resistance and reduction of visceral fat (2, 3), some have reported increased blood glucose levels associated with the use of ezetimibe (4). Hepatic steatosis has also been shown to be improved by

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ezetimibe in some animal model studies (5), but not in human studies (6).

There are several possible reasons for these conflicting results. Hepatic NPC1L1 is expressed in humans, but not in mice. This may have led to different effects of ezetimibe on glucose metabolism. In addition, considering the mechanism of ezetimibe inhibiting the absorption of dietary cholesterol, its effects may vary depending on the amount of dietary cholesterol. Likewise, differences in the degree of metabolic stress affected by different diets may have influenced differences in glucose metabolism.

In this study, we aimed to investigate the effects of ezetimibe on glucose metabolism in vitro and in vivo. By identifying the molecular mechanisms of action, we aimed to determine whether ezetimibe therapy can be safely used in patients with diabetes and fatty liver, and whether it improves hepatic steatosis.

II. MATERIALS AND METHODS

1. *In vivo* (Wistar rat model) study

Thirteen five-week-old male Wistar rats were housed under standard conditions (21 ± 2˚C, 60 ± 10% humidity, 12 h light/dark cycle) with *ad libitum* access to food and water. The rats were randomly assigned to either the ezetimibe group (n=6) or a control group (n=7) at 6 weeks of age. The control group was fed a high fat diet (HFD; 60 Kcal%) and the ezetimibe group was fed a HFD (60 Kcal%) containing ezetimibe (160 mg/kg). The total observation period was 14 weeks. Daily weight, dietary intake, activity patterns, and health status were monitored throughout the experiment.

All animal procedures were performed in accordance with the guidelines of the National Institutes of Health and pre-approved by the animal care and use committee of Yonsei University, College of Medicine (2017-0028).

A. Oral glucose tolerance test (OGTT) and Insulin tolerance test (ITT).

Oral glucose tolerance test (OGTT) was performed after 12 weeks of drug

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administration. After fasting for 18 hours, 2 g/kg of glucose was administered orally, and blood glucose levels were measured from caudal venous blood using a portable blood glucose meter (Boehringer-Mannheim, Indianapolis, IN, USA) at 0, 15, 30, 60, 90, and 120 minutes post glucose administration.

Insulin tolerance test (ITT) was performed after 10 weeks of drug administration. After fasting for 4 hours, 1 U/kg of insulin (Sigma-Aldrich, Cat. No. 9177C) was administered intraperitoneally, and blood glucose levels from caudal venous blood were measured by a portable blood glucose meter at 0, 15, 30, 60, 90, and 120 minutes post insulin administration.

B. Blood and tissue sampling.

Two weeks after OGTT, after 18 hours of fasting, anesthesia was performed, using a nose cone. Blood was obtained from the abdominal aorta by thoracotomy. The rat was then euthanized. The blood was centrifuged for 10 minutes and the serum was stored at -80 °C. Post euthanasia, the liver and fat tissue were excised. The liver and fat tissue samples were rapidly frozen with nitrogen solution and stored at -80 °C. Some liver and fat tissue samples were fixed in 4% paraformaldehyde (Tech & Innovation Co., Ltd., Cat. No. BPP-9004-004LR) solution for more than 48 hours and used for histological analysis.

C. Measurement of metabolic parameters and inflammation markers (blood).

Serum fasting glucose concentration, stimulated glucose, fasting insulin, stimulated insulin (Morinaga Ultra Sensitive Rat Insulin ELISA Kit (Morinaga, Cat. No. M1103), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglyceride and free fatty acid levels were determined.

D. Hematoxylin and eosin (H&E) staining.

Tissue samples were washed, dehydrated, and embedded in paraffin. Some

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were stained with hematoxylin and eosin (H&E) for observation of histological structures. Tissue samples were examined under a microscope and images were acquired using an attached digital camera. CellSens Entry software (Olympus, Tokyo, Japan) was used for image analysis.

2. *In vivo* (mouse model, pilot study) study

Thirty-nine male four-week-old C57BL/6J mice were fed a standard diet (5% wt/wt fat) to adapt to the environment. At 5 weeks of age, mice were randomly assigned to receive HFD with ezetimibe (45 Kcal% diet containing 0.004% w/w ezetimibe) in the ezetimibe group (n=21) or a HFD (45 Kcal%) in the control group (n=18). The total observation period was 19 weeks. Daily weight, dietary intake, activity patterns, and health status were monitored throughout the experiment. Fasting blood glucose levels were measured at baseline and after 19 weeks of drug administration.

Oral glucose tolerance test was performed after 17 weeks of drug administration, when diabetes was developed. After fasting for 12 hours, 2 g/kg of glucose was administered orally, and blood glucose was measured from caudal venous blood by portable blood glucose meter at 0, 15, 30, 60, 90, and 120 minutes post glucose administration.

Insulin tolerance test was performed one week after OGTT (after 18 weeks of drug administration). After fasting for 4 hours, 1 U/kg of insulin (Sigma-Aldrich, Cat. No. 9177C) was administered intraperitoneally, and blood glucose was measured from caudal venous blood by portable blood glucose meter at 0, 15, 30, 60, 90, and 120 minutes post insulin administration.

3. *In vitro* study

The effect of ezetimibe on the glucose metabolism of HepG2 cells was compared assuming a direct action of ezetimibe on hepatic NPC1L1. Hepatocellular carcinoma (HepG2) cell lines were cultured in Dulbecco's

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modified Eagle's medium (Thermo Scientific, SH30243.01) containing 10% fetal bovine serum (Thermo Scientific, SH30071.03), 100 U/ml penicillin, and 100 mg/ml streptomycin (Thermo scientific, SV30010) in a 5% CO_2 incubator at 37°C.

Ezetimibe was dissolved in dimethyl sulfoxide before dilution in culture medium. In all experiments, the final ezetimibe concentration was 25 uM and final dimethyl sulfoxide concentration was ≤0.1%.

A. Western blot.

Cells and tissues were lysed in radioimmunoprecipitation assay buffer, containing of 20 mM tris (hydroxymethyl) aminomethane hydrochloride (pH 7.5), 150 mM sodium chloride (NaCl), 1 mM disodium ethylenediaminetetraacetate dihydrate, 1 mM ethylene glycol bis(-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 % Nonidet P-40, 1 % sodium deoxycholate, 2.5mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM sodium orthovanadate, 1 µg/ml leupeptin (BRI-9010-010M, Tech & Innovation Co., Ltd), and protease inhibitor and phosphatase cocktail (Thermo Scientific, 78440). Protein samples were separated using 10 % polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, PVH00010). The membranes were incubated with the primary antibodies to measure the expression of the following proteins: G6Pase (Santacruz, Cat. No. sc-25840), phosphoenolpyruvate carboxykinase (PEPCK, santacruz, Cat. No. sc-32879), NPC1L1 (Invitrogen, Cat. No. PA1-16800), Total Akt (Cell signaling, Cat. No. 4691S), p-Akt (Ser473, Cell signaling, Cat. No. 4060S). Anti-mouse IgG, horseradish peroxidase (HRP)-linked Antibody (Santacruz, Cat. No. sc-516102) and anti-rabbit IgG, HRP-linked Antibody (Cell signaling, 7074S) were used as secondary antibodies. NPC1L1 expression in liver tissue was also confirmed.

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B. Quantitative real time polymerase chain reaction.

Total RNA was isolated from tissues and cells with TRIzol reagent (15596–018, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and 2 mg of total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (4368814, Applied Biosystems, Foster City, CA, USA). The cDNA was amplified in the ABI 7500 sequence detection system (4350584, Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix (4367659, Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: 40 cycles of 95°C for 5 sec, 58°C for 10 sec, and 72°C for 20 sec. Target gene expression was normalized to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase, and quantitative analyses were conducted using the △△cycle threshold method and StepOne Software version 2.2.2.

4. Human study

Patient data were reviewed to investigate the effect of ezetimibe on insulin resistance in human. Electronic medical records were reviewed from patients at Severance Hospital (a tertiary university hospital in Seoul, Korea) aged ≥19 years with dyslipidemia who newly started medication for dyslipidemia and had undergone both fasting insulin and fasting glucose level tests before and after 1 year of pharmacotherapy between January 2006 and December 2018. Patients were excluded if their anti-dyslipidemic or anti-diabetic medications were changed during the observation period. The Ethics Committee of the Yonsei University College of Medicine approved this study (4-2020-0514).

Age, sex, weight, height, diabetes status, and current medications were recorded. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2). Following an overnight fast (≥ 8 hours), blood samples before (0 minutes, designated as 'fasting') and after (120 minutes, designated as 'stimulated') meal were obtained to measure HbA1c, basal and stimulated

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glucose, fasting insulin, and other chemistry profiles. Insulin sensitivity was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR) index (7).

5. Statistical analysis

All categorical variables were expressed as number (proportion) and compared by χ^2 analysis. Outcomes were compared using the Wilcoxon-Mann-Whitney U test to determine the differences between continuous variables. An intention-to-treat analysis was performed for human data. Wilcoxon signed-rank test was used for additional analysis, when indicated. To compare continuous variables, patient characteristics were analyzed using the Kruskal-Wallis test, and categorical variables were compared using the $\chi 2$ test, followed by post hoc analyses using the Dunn procedure for Kruskal-Wallis test.

Statistical analyses were performed using IBM SPSS statistical software for Windows, version 25.0 (IBM, Armonk, NY, USA). A p value < 0.05 was considered statistically significant.

III. RESULTS

1. Effect of ezetimibe on glucose metabolism without direct effect on liver.

The OGTT and ITT results of the pilot study using a mice model were analyzed. As shown in figure 1, the use of ezetimibe did not significantly affect the overall glycemic pattern. However, on ITT, significant decreases in blood glucose levels were observed in the ezetimibe group at 30 and 60 minutes. Through this pilot study, we hypothesized that use of ezetimibe would contribute to improved insulin resistance.

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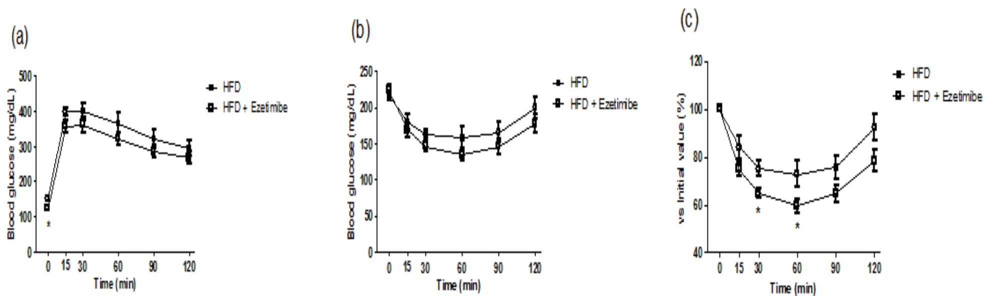

Figure 1. **Systemic glucose metabolism evaluated with oral glucose tolerance test and insulin tolerance test** (a) Oral glucose tolerance test, (b) insulin tolerance test, (c) insulin tolerance test (vs initial value %). Error bars represent standard error of mean.

*$p < 0.05$ versus corresponding HFD value.

HFD, high fat diet

2. Expression of hepatic NPC1L1 on mice liver.

To investigate whether ezetimibe has a direct effect on mice liver, the expression of NPC1L1 was tested in both mice liver and HepG2 cells. Cellular lysates were immunoblotted and probed with anti-NPC1L1 antibody. As shown in Figure 2, there was no evidence of expression of NPC1L1 in mice liver. On the other hand, hepatic NPC1L1 expression in HepG2 cells was confirmed. This suggests that ezetimibe is likely to affect glucose metabolism without direct action, in the mice liver model.

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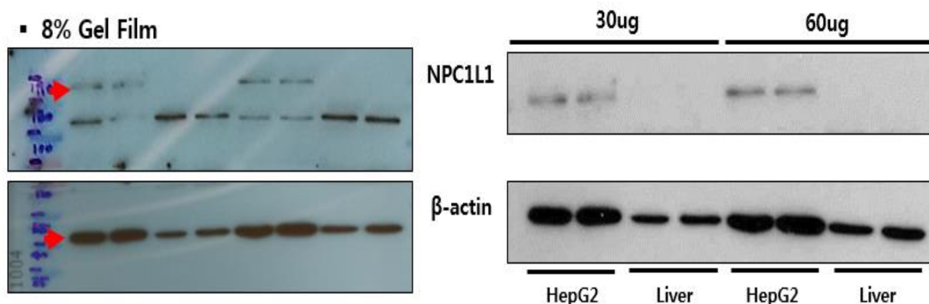

Figure 2. The expression of hepatic NPC1L1 on mice liver

Mice liver and HepG2 cells were separated, homogenized with lysis buffer, and gel electrophoresis was performed. Western blotting was performed using an anti-NPC1L1 antibody against the antigen and actin (control) to determine the expression of NPC1L1 in mice liver.

3. Effect of ezetimibe on glucose metabolic indicators in rat model.

Previous studies established the presence of hepatic NPC1L1 in humans (8). However, its role in glucose metabolism remains unclear. We aimed to investigate the effect of ezetimibe on glucose metabolism under HFD conditions in a rat model with hepatic NPC1L1 expression which is similar to human hepatic NPC1L1 expression.

The characteristics of the rat model are described in Table 1. There was no difference in baseline body weight between groups. At the end of the treatment period (14 weeks), no difference was observed in body weight or amount of weight gain between groups. Liver weight was significantly decreased in the ezetimibe group compared to the control group (13.0 ± 1.4 g vs. 11.2 ± 1.0 g, $p = 0.015$), but there was no significant difference in liver weight or total body weight (%) or perigonadal fat weight between groups (all $p > 0.05$).

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Table 1. Characteristics of rat control group (HFD) and ezetimibe group (HFD + ezetimibe)

	HFD (n=7)	HFD + Ezetimibe (n=6)	*p value*
Baseline (6 weeks)			
Body weight (g)	195.6 ± 7.9	195.3 ± 8.2	0.830
At the end of treatment (20 weeks)			
Body weight (g)	588.0 ± 46.7	559.0 ± 33.1	0.174
Weight gain (g)	392.1 ± 43.0	363.5 ± 29.0	0.153
Liver weight (g)	13.0 ± 1.4	11.2 ± 1.0	0.015
Liver weight / Body weight (%)	3.3 ± 0.5	3.0 ± 0.0	0.171
Perigonadal Fat weight (g)	14.0 ± 1.7	12.8 ± 2.9	0.388
Perigonadal Fat weight / Body weight (%)	3.6 ± 0.5	3.7 ± 0.8	0.937

Data are expressed as mean ± SD and compared by Mann-Whitney U test Dunnett's post-test. *$p < 0.05$ versus HFD group. HFD; high fat diet

Over the 14-week study period, no difference in body weight gain was observed between groups (Figure 3a). No statistical difference was observed in overall food intake between groups (Figure 3b).

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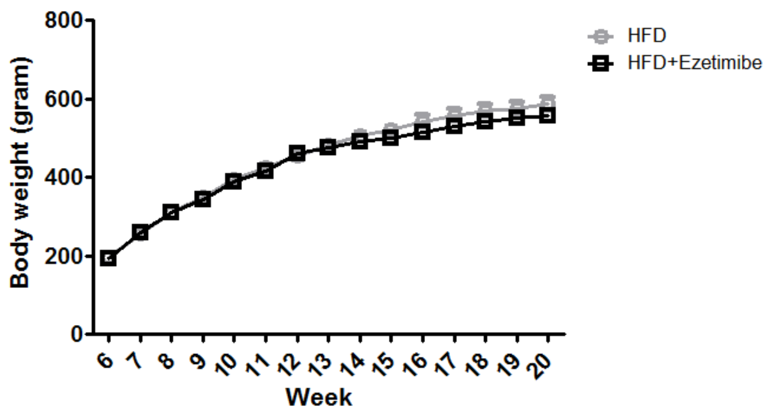
(a)



(b)

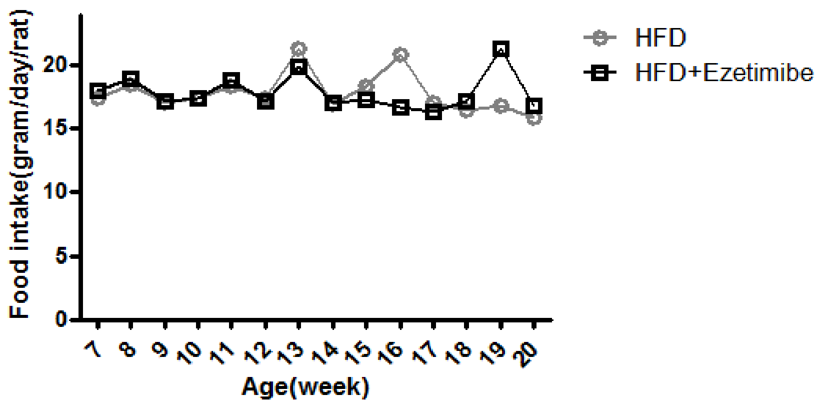


Figure 3. Body weight gain and food intake of rats

(a) body weight gain, (b) food intake (gram/day-rat)

4. Effects of ezetimibe on glucose and insulin tolerance.

In the rat model, OGTT results showed that the ezetimibe group exhibited significant improvement in glucose tolerance 15 minutes after glucose

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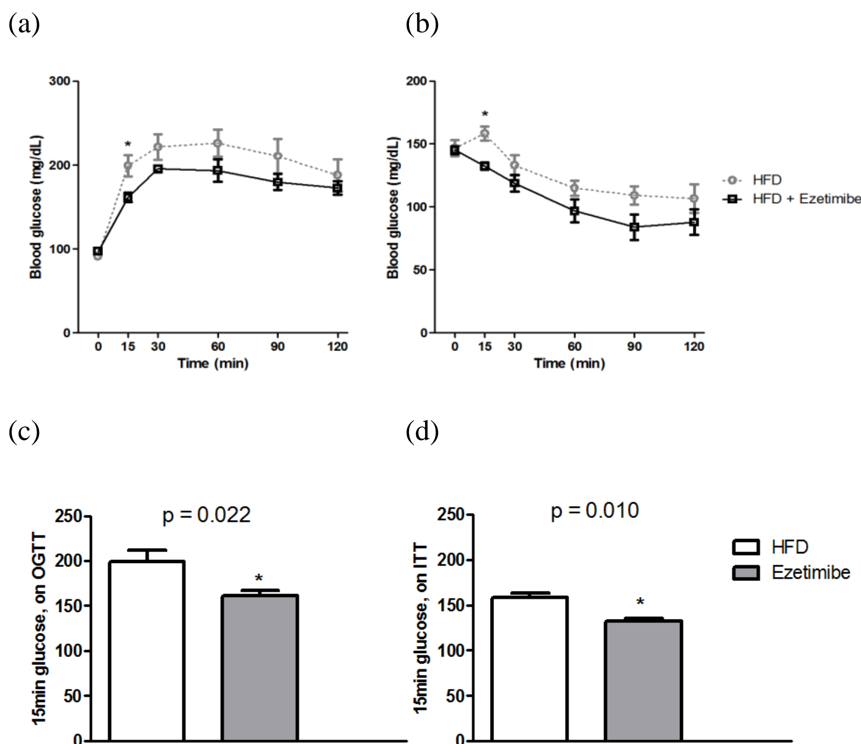
administration, compared with the control group ($p < 0.022$, Figures 4a and 4c). The AUC value of OGTT was lower for the ezetimibe group than the control group, however, the difference was not statistically significant (Figure 4e, $p = 0.063$).

Insulin tolerance improved in the ezetimibe group 15 minutes after insulin administration, compared with the control group ($p = 0.010$, Figures 4b and 4d). The AUC value of ITT was lower for the ezetimibe group than the control group, however, the difference was not statistically significant (Figure 4f, $p = 0.063$).



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(e) (f)

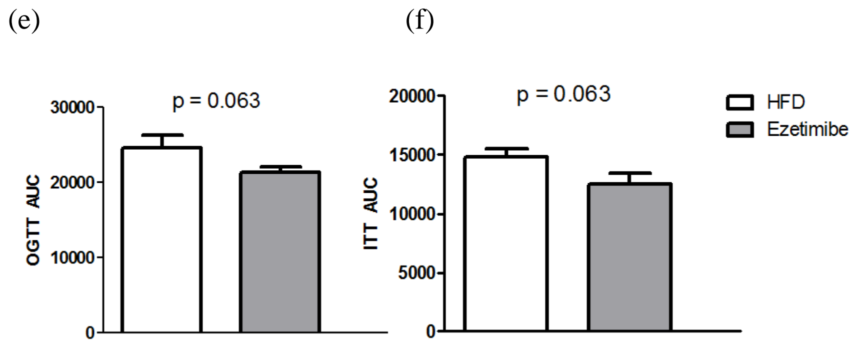

Figure 4. Effects of ezetimibe on glucose and insulin tolerance test (a) Oral glucose tolerance test, (b) insulin tolerance test, (c) 15-minute glucose levels in OGTT, (d) 15-minute glucose in ITT, (e) AUC of OGTT, (f) AUC of ITT.
Error bars represent standard error of mean. Compared by the Wilcoxon-Mann-Whitney U test. * $p < 0.05$

Triglyceride and free fatty acid values were significantly lower in the ezetimibe group than the control group (all $p < 0.01$, Figure 5).

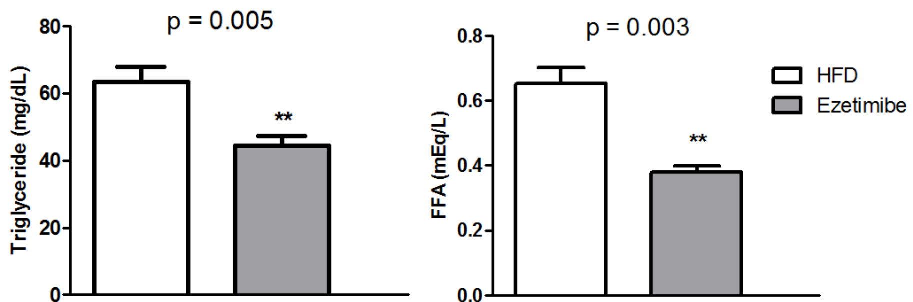

Figure 5. Effects of ezetimibe on triglyceride and free fatty acid levels compared using the Wilcoxon-Mann-Whitney U test
** $p < 0.01$

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5. Effects of ezetimibe on visceral adipose tissue.

The size of adipocyte (visceral) was measured. The cell size in the adipose tissue of the ezetimibe treatment group was significantly smaller, compared to the control group (112.2 ± 25.1 μm control group vs. 99.8 ± 23.4 μm of ezetimibe group, $p < 0.001$, figure 6).

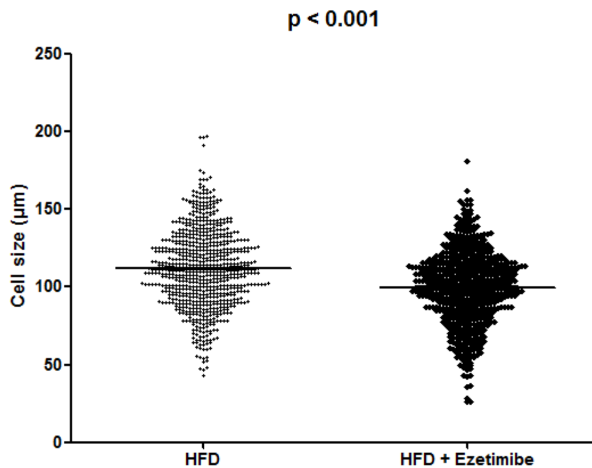

Figure 6. Effects of ezetimibe on the size of adipocytes in a total of 1,300 adipocytes (n=100 per animal). Compared using the two-sample t-test. Markedly smaller adipocyte size was observed in the ezetimibe group compared to the control group ($p<0.001$).

M1 markers IL-1 beta, MCP1, and IL-6 were significantly decreased in the ezetimibe group (all $p <0.05$, figure 7). Conversely, M2 markers IL-10 and Arg-1 were significantly increased in the ezetimibe group (all $p < 0.05$).

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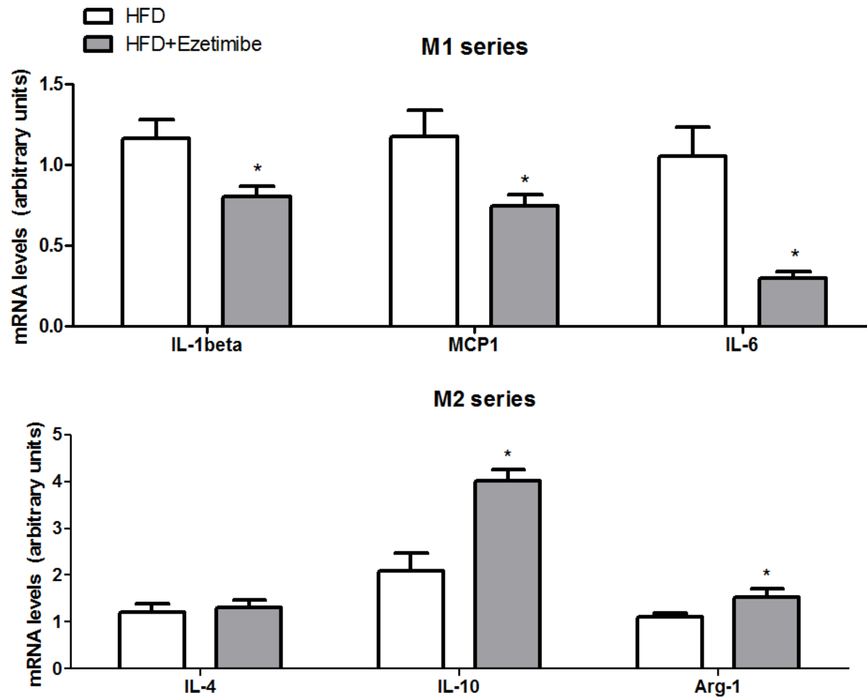

Figure 7. Effects of ezetimibe on inflammation markers, compared using the Wilcoxon-Mann-Whitney U test

* $p < 0.05$

The mRNA levels of the genes associated with lipogenesis (Figures 8a to 8c) or lipolysis (Figures 8d to 8e) showed no significant change after ezetimibe treatment. Whereas, the mRNA levels of pyruvate dehydrogenase kinase-2 which is associated with fatty acid oxidation significantly increased in ezetimibe treatment group (Figure 8f). Expression of long-chain acyl-CoA dehydrogenase, which plays an important role in β-oxidation, also showed increased tendency without statistically significance (Figure 8g).

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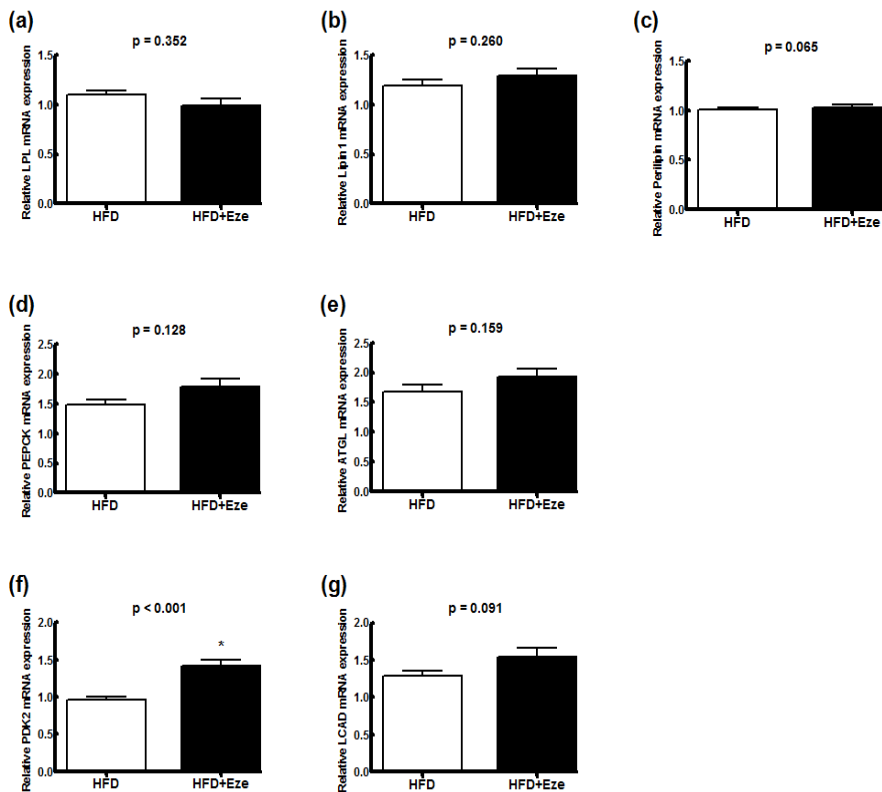

Figure 8. Effects of ezetimibe on the genes involved in lipogenesis, lipolysis and β-oxidation

(a) LPL, (b) Lipin1, (c) Perilipin, (d) PEPCK, (e) ATGL, (f) PDK2, (g) LCAD. Error bars represent standard error of mean. Compared using the Wilcoxon-Mann-Whitney U test. * $p < 0.05$

LPL; lipoprotein lipase, PEPCK; phosphoenolpyruvate carboxykinase, ATGL; adipose triglyceride lipase, PDK2; pyruvate dehydrogenase kinase-2, LCAD; long-chain acyl-CoA dehydrogenase

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6. Effects of ezetimibe on liver.

Aspartate aminotransferase and ALT levels were decreased in the ezetimibe group (all $p < 0.05$, Figures 9a and 9b). However, no statistically significant difference in hepatic triglyceride level was observed ($p = 0.886$, Figure 9c).

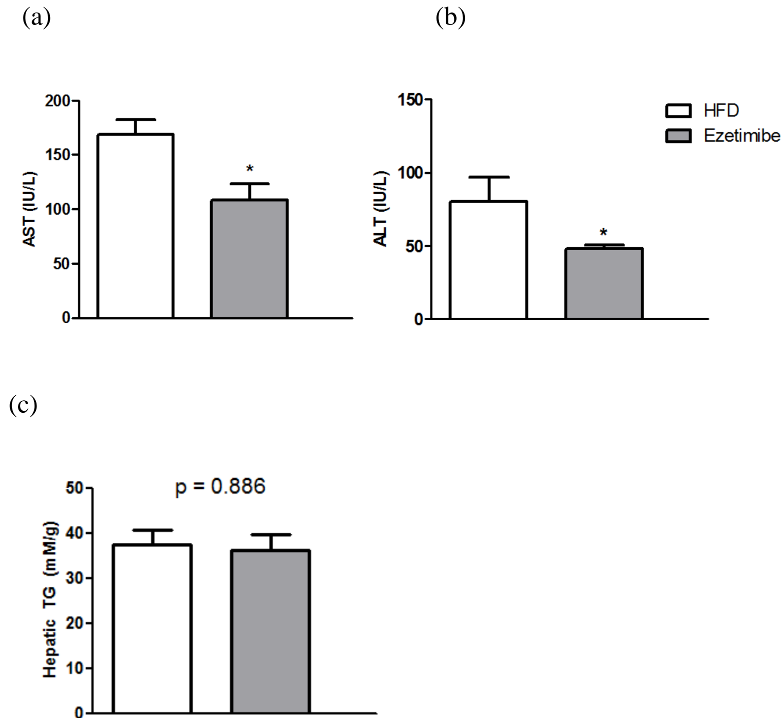

Figure 9. Effects of ezetimibe on aspartate aminotransferase and alanine aminotransferase levels and hepatic triglyceride level

(a) AST level (IU/L), (b) ALT level (IU/L), (c) hepatic triglyceride (mM/g). Hepatic triglyceride level was measured using triglyceride quantification kit (Abcam, Cat. No. ab65336). Error bars represent standard error of mean. Compared using the Wilcoxon-Mann-Whitney U test. * $p < 0.05$

AST; aspartate aminotransferase, IU/L; international units per liter, ALT; alanine aminotransferase

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7. Direct effects of ezetimibe on liver (*in vitro*).

The phosphorylation level of Akt 5 and 15 minutes after the stimulation of HepG2 cells with insulin was evaluated to analyze the direct effect of ezetimibe on insulin response in the liver. No significant difference in the level of Akt phosphorylation in HepG2 cells was observed between groups.

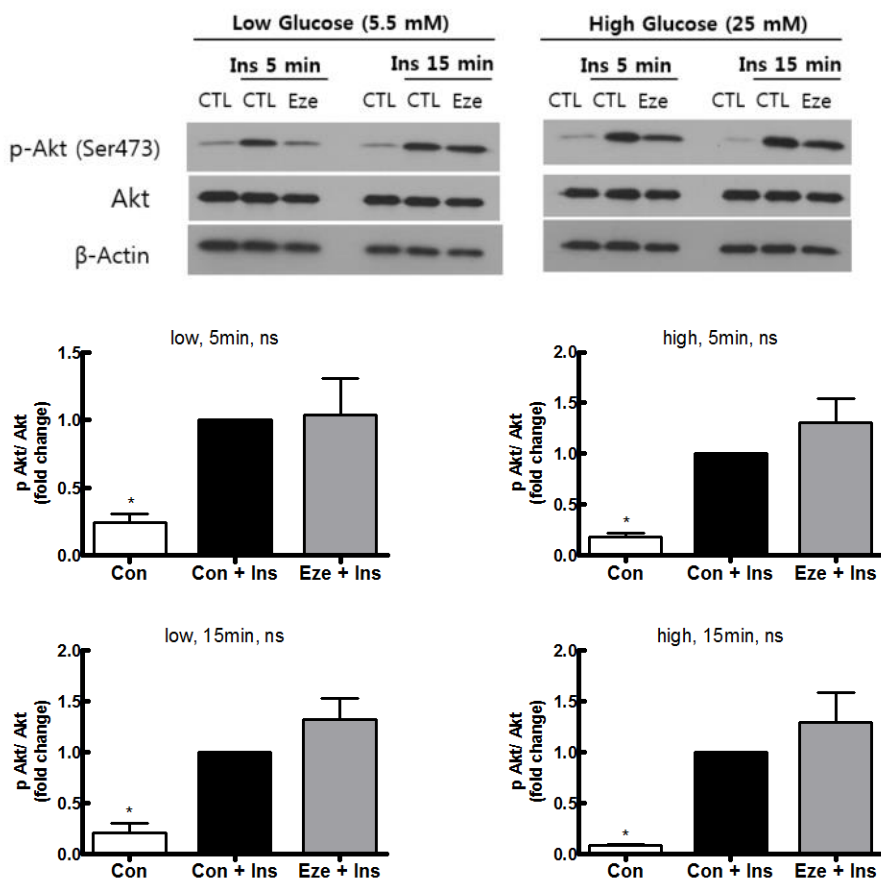

Figure 10. Effects of ezetimibe on phosphorylation levels of Akt in HepG2 cells

Above: Representative western blots. Below: Plots of densitometric analysis of

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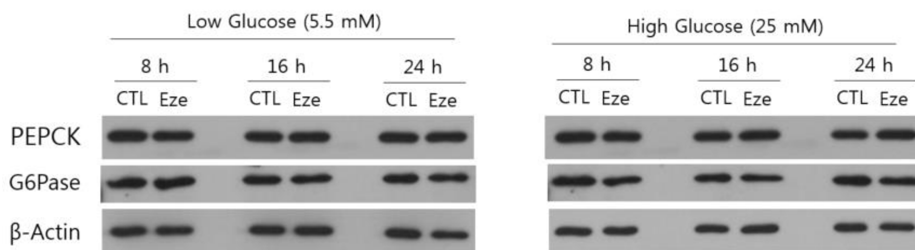
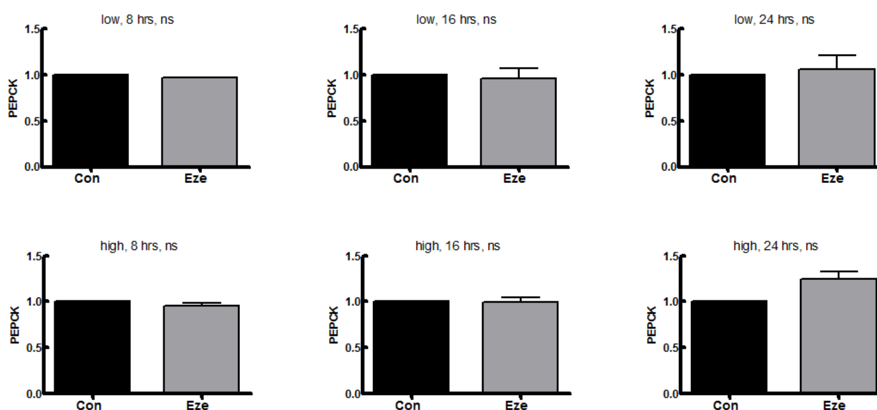
p-Akt/Akt ratio in relation to insulin-stimulated control cells (means ±SE, n=3). Error bars represent standard error of mean. Compared by the Wilcoxon-Mann-Whitney U test. * $p < 0.05$

The G6pase and PEPCK level stimulation with low (5.5 mM) and high (25 mM) concentrations of glucose were evaluated to analyze the direct effect of ezetimibe on gluconeogenesis. No significant difference was observed in the levels of G6pase or PEPCK in HepG2 cells between groups.





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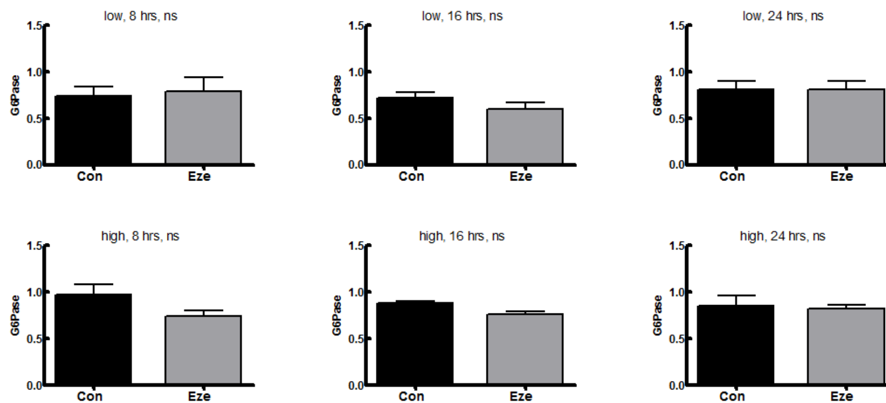

Figure 11. Effects of ezetimibe on markers of gluconeogensis in HepG2 cells, evaluated by western blot

Above: Representative western blots. Below: plots of densitometric analysis of PEPCK and G6Pase in relation to control cells (means ±SE, n=3). Error bars represent standard error of mean. Compared by the Wilcoxon-Mann-Whitney U test. * $p < 0.05$

The G6pase and PEPCK level stimulation with low (5.5 mM) and high (25 mM) concentrations of glucose were also evaluated by PCR. G6pase and PEPCK levels were significantly increased in several conditions with the ezetimibe treatment (Figures 12).

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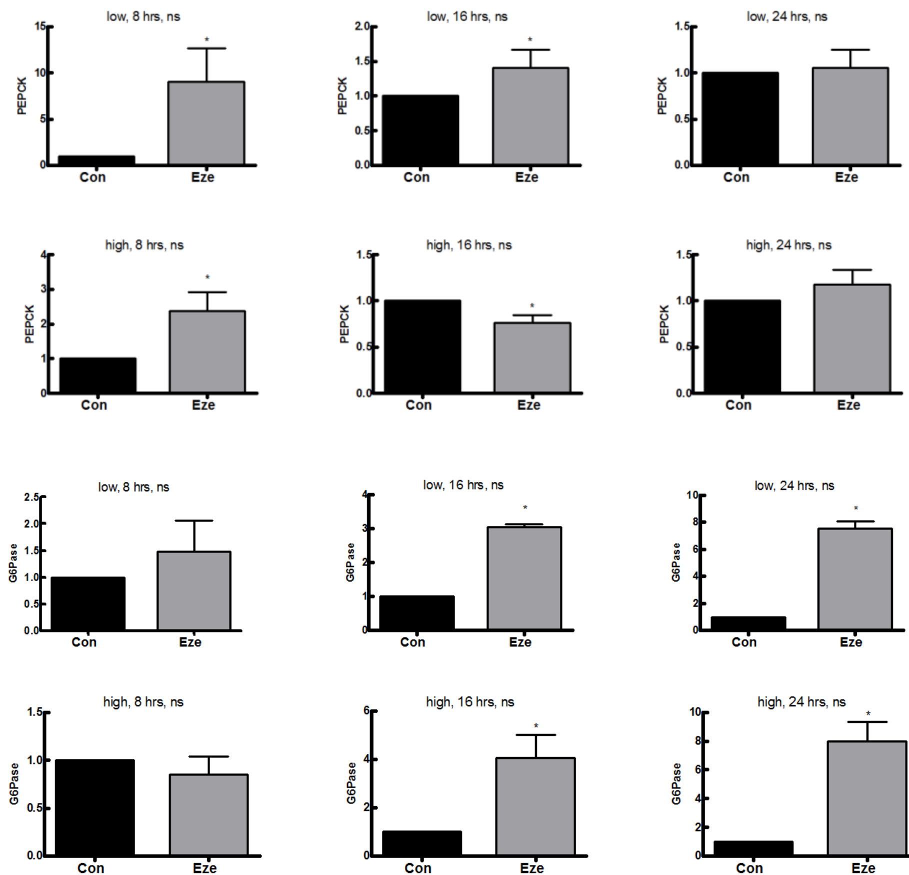

Figure 12. Effects of ezetimibe on markers of gluconeogenesis in HepG2 cells, evaluated by mRNA levels

Representative mRNA levels. Plots of densitometric analysis of (above) PEPCK and (below) G6Pase in relation to control cells (means ±SE, n=3). Error bars represent standard error of mean. Compared by the Wilcoxon-Mann-Whitney U test. * $p < 0.05$

Hepatic glucose outflow was significantly increased with the ezetimibe treatment (Figures 13).

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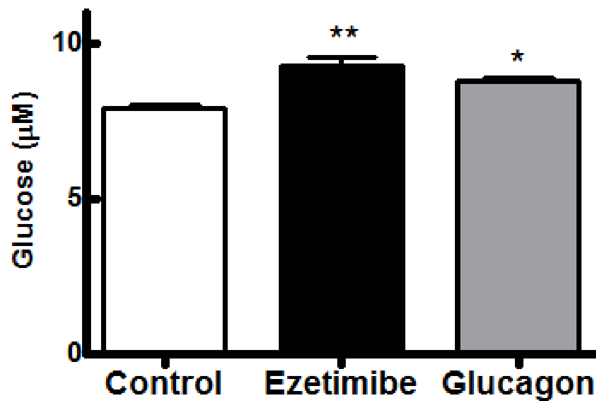

Figure 13. Effects of ezetimibe on glucose outflow in HepG2 cells

Plots of glucose outflow in HepG2 cells (means ±SE, n=12). Error bars represent standard error of mean. Compared by the ANOVA test. * $p < 0.05$, ** $p < 0.01$

8. Effects of ezetimibe on glycemic indicators (human study).

In total, 133 patients with dyslipidemia who had newly started medication for dyslipidemia and undergone both fasting insulin and fasting glucose level tests for before and after 1 year of pharmacotherapy were included in this study. Patients were classified the into the statin monotherapy group (n=90, 67.7%) and the ezetimibe combination group (n=43, 32.3%) which was sub-classified into ezetimibe add on statin (n=13, 9.8% of total) and ezetimibe start with statin (n=30, 22.6% of total). Baseline characteristics are summarized in table 2. The study population included 66.9% women and 11.3% patients with type 2 diabetes. All the participants were Asian. The groups had similar baseline characteristics except fasting glucose, total cholesterol, and low density lipoprotein (LDL)

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cholesterol levels. There were differences in cholesterol levels at the beginning of each treatment, possibly due to the different indications for each drug. The fasting glucose level was higher in the ezetimibe add on statin group (all $p < 0.05$, Table 2).

Table 2. Baseline characteristics of study population

	Combination therapy		Monotherapy	
	Ezetimibe add on statin (n=13)	Ezetimibe start with statin (n=30)	Statin monotherapy (n=90)	p value
Age (years)	61.0 (16.5)	58.5 (11.8)	58.0 (10.0)	0.467
Female (%)	9 (69.2)	17 (56.7)	63 (70.0)	0.398
Diabetes (%)	2 (15.4)	3 (10.0)	10 (11.1)	0.873
BMI (kg/m^2)	24.0 (6.9)	24.7 (3.9)	24.0 (3.7)	0.658
Glucose, fasting (mg/dl)	109.0 (27.0)	105.0 (15.3)	99.0 (17.0)*	0.008
Glucose, stimulated (mg/dl)	123.5 (79.0)	118.0 (40.0)	111.0 (30.0)	0.492
Insulin, fasting (uU/ml)	7.3 (9.2)	6.1 (3.1)	6.2 (3.6)	0.126
HOMA-IR	2.0 (2.5)	1.6 (0.9)	1.5 (0.9)	0.060
HbA1c (%)	6.1 (1.1)	5.8 (1.0)	5.9 (0.4)	0.110
AST (IU/L)	22.0 (5.5)	19.5 (6.3)	21.0 (8.0)	0.401
ALT (IU/L)	18.0 (17.5)	18.5 (7.8)	18.0 (9.5)	0.743
Total cholesterol (mg/dl)	184.0 (38.5)	259.0 (40.5) *	232.0 (47.5) *†	<0.001
Triglycerides (mg/dl)	113.0 (73.5)	125.0 (96.5)	132.0 (68.0)	0.989
HDL cholesterol (mg/dl)	50.0 (14.0)	54.0 (13.5)	52.0 (15.0)	0.429
LDL cholesterol (mg/dl)	112.6 (29.6)	169.6 (36.2) *	150.2 (52.0) *†	<0.001

Abbreviations: BMI, body mass index; HOMA, homeostatic model assessment; IR, insulin resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein

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Kruskal-Wallis test, Dunn procedure

NOTE. Bold text indicates p values < 0.05

* p values < 0.05 vs. ezetimibe add on statin group, by post hoc analyses (Dunn procedure)

† p values < 0.05 vs. ezetimibe start with statin group, by post hoc analyses (Dunn procedure)

‡ Significant chi-square tests, p values < 0.05

A significant increase in insulin resistance was observed after one year in the statin monotherapy group (+ 0.33, p = 0.002), however, no significant increase was observed in the ezetimibe combination group (+ 0.14, p = 0.530). In addition, the ezetimibe add on statin group showed a tendency of decreased insulin resistance, calculated using the HOMA-IR (-0.24, p = 0.382, figure 14).

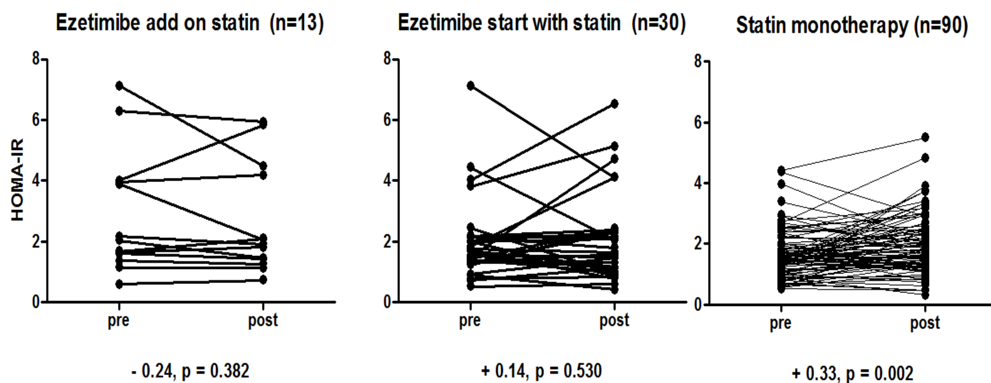

Figure 14. Changes of homeostatic model assessment of insulin resistance in patients of each group

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Table 3. Changes in glycemic indicators and cholesterol levels before and after treatment

Before	Glucose, fasting (mg/dl)	Insulin, fasting (uU/ml)	HOMA-IR	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL cholesterol (mg/dl)
Ezetimibe combination (n=43)	106.0 (97.0-115.0)	7.0 (4.8-8.6)	1.7 (1.3-2.2)	241.0 (205.0-266.0)	125.0 (93.0-177.0)	154.0 (119.4-179.8)
ezetimibe add on statin (n=13)	109.0 (101.0-128.0)	7.3 (5.6-14.8)	2.0 (1.5-4.0)	184.0 (168.5-207.0)	113.0 (98.0-171.5)	112.6 (93.8-123.4)
ezetimibe start with statin (n=30)	105.0 (96.5-111.8)	6.1 (4.8-7.9)	1.6 (1.3-2.1)	259.0 (233.0-273.5)	125.0 (90.0-186.5)	169.6 (150.8-186.9)
Statin monotherapy (n=90)	99.0 (91.0-108.0)	6.2 (4.4-8.0)	1.5 (1.1-2.0)	232.0 (203.0-250.5)	132.0 (99.0-167.0)	150.2 (116.2-168.2)
After 1 year						
Ezetimibe combination (n=43)	105.0 (97.0-117.0)	7.3 (4.7-9.3)	1.8 (1.1-2.4)	167.0 (152.0-199.0)	116.5 (88.8-159.8)	82.9 (72.4-111.2)
ezetimibe add on statin (n=13)	105.0 (98.0-120.0)	8.0 (4.8-13.9)	1.9 (1.3-4.3)	167.0 (147.5-194.0)	112.0 (84.0-141.5)	90.8 (72.8-113.9)
ezetimibe start with statin (n=30)	106.5 (96.8-114.3)	7.0 (4.3-8.9)	1.7 (1.1-2.4)	167.5 (151.3-207.3)	119.0 (89.0-168.0)	82.2 (72.0-104.4)
Statin monotherapy (n=90)	101.0 (93.0-110.3)	7.3 (5.1-9.2)	1.8 (1.3-2.4)	168.5 (150.0-190.3)	110.5 (82.3-159.0)	90.0 (76.4-106.6)

Data are expressed as median (IQR).

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Table 4. Changes in parameters after 1 year of treatment between ezetimibe combination and station-treated patients

	Ezetimibe combination (n=43)			Statin monotherapy (n=90)			Difference between groups
	Baseline	Post-treatment	p value	Baseline	Post-treatment	p value	p value
Glucose, fasting (mg/dl)	106.0 (18.0)	105.0 (20.0)	0.296	99.0 (17.0)	101.0 (17.3)	0.021	0.996
Glucose, stimulated (mg/dl)	118.0 (58.0)	127.0 (52.5)	0.946	111.0 (30.0)	132.0 (54.0)	0.016	0.117
Insulin, fasting (uU/ml)	7.0 (3.8)	7.3 (4.6)	0.828	6.2 (3.6)	7.3 (4.1)	0.003	0.066
HOMA-IR	1.7 (0.9)	1.8 (1.3)	0.923	1.5 (0.9)	1.8 (1.1)	0.002	0.109
HbA1c (%)	6.0 (0.9)	5.9 (0.8)	0.793	5.9 (0.4)	5.9 (0.3)	0.758	0.865
AST (IU/L)	21.0 (6.0)	23.0 (9.0)	0.003	21.0 (8.0)	23.0 (7.0)	0.009	0.825
ALT (IU/L)	18.0 (9.0)	23.0 (21.0)	0.012	18.0 (9.5)	21.0 (13.0)	0.003	0.694
Total cholesterol (mg/dl)	241.0 (61.0)	167.0 (47.0)	<0.001	232.0 (47.5)	168.5 (40.3)	<0.001	0.850
Triglycerides (mg/dl)	125.0 (84.0)	116.5 (71.0)	0.347	132.0 (68.0)	110.5 (76.8)	0.014	0.629
HDL cholesterol (mg/dl)	53.0 (15.0)	51.5 (18.3)	0.175	52.0 (15.0)	52.0 (16.3)	0.077	0.830
LDL cholesterol (mg/dl)	154.0 (60.4)	82.9 (38.8)	<0.001	150.2 (52.0)	90.0 (30.2)	<0.001	0.566

Data are expressed as median (IQR) with p values from Wilcoxon signed-rank test. P value-difference was determined using intention-to-treat analysis.

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Compared to baseline, there were significant reductions in total cholesterol and LDL cholesterol levels in both groups (all $p < 0.05$, Table 4). There were significant increases in fasting glucose, stimulated glucose, fasting insulin, and HOMA-IR in the statin monotherapy group, only (all $p < 0.05$). Intention-to-treat analysis showed no difference between groups (all $p > 0.05$).

IV. DISCUSSION

The present study evaluated the effects of ezetimibe on glucose metabolism in HFD-fed mice and rat models. In both models, in comparison with the control groups, the ezetimibe groups showed no significant difference in food intake or weight gain. However, both models showed partial improvement in several glucose metabolism indices. In the rat model, the ezetimibe group showed smaller fat cell size and reduced M1-polarized macrophage accumulation. Also, anti-inflammatory M2 phenotype of macrophages were induced within adipocytes and decreased free fatty acid levels were observed in serum. These differences did not lead to systemic lowering of blood sugar levels, and the improvement of hepatic steatosis was not significant. In human, clinical data analysis showed statin monotherapy significantly increased insulin resistance. On the other hand, the use of ezetimibe in combination with statin did not increase insulin resistance.

Cholesterol, which is absorbed from bile or the intestines, is transferred in the form of chylomicron to the blood via lymphatic circulation, and transmitted to the body through systemic circulation. Especially after meals, chylomicron, which contains more cholesterol, increases and contributes to the development of atherosclerosis. This reduces LDL-receptor expression in the liver and increases LDL cholesterol (9). Lipid overload in obesity is associated with adipocyte dysfunction, inflammation, macrophage infiltration, and decreased fatty acid oxidation (10). In an animal study using monkeys, an increase in the size and

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inflammation of visceral adipocytes was reported when cholesterol ingested as food was increased (11). Therefore, by limiting dietary cholesterol by inhibiting intestinal cholesterol absorption, ezetimibe may contribute to a fatty acid oxidation and relief of the inflammatory reaction in peripheral tissues (e.g., visceral adipose tissue). Moreover, previous studies report that an accumulation of cholesterol in adipocytes correlates with increased risk of metabolic syndrome and cardiovascular disease (12). The IMPROVE-IT study revealed that the use of ezetimibe contributes to reduced cardiovascular risk (1). While LDL cholesterol lowering is the main mechanism of the cardiovascular benefits of ezetimibe (1), the results of the current study suggest that the reduction of adipocyte cholesterol accumulation may also contribute to reduced cardiovascular risk.

Hypertrophy of adipocytes is associated with increased inflammation, which is accompanied by increased insulin resistance (13). Free fatty acid also induces muscle insulin resistance (14). Previous studies have shown that ezetimibe may contribute to the inhibition of cholesterol absorption as well as dietary free fatty acid (15). The changes in adipocyte size, inflammation, and levels of free fatty acid observed in this study probably contributed to the noted improvement in insulin resistance. A meta-analysis by Hong et al. reported that ezetimibe-statin combination therapy is associated with greater cardiovascular benefits in patients with diabetes, compared to those without diabetes (16). However, although some improvements in insulin resistance were found in this study, the overall effect of ezetimibe on dysglycemia was not significant. Also in one monkey-model study, despite different adipocyte size, due to dietary cholesterol differences, improvement of glucose metabolism was not clearly evident (11). Considering the relatively short duration of both this study (14 weeks) and the monkey study (10 weeks), further research is needed to determine whether longer-term use of ezetimibe may contribute to improvement of insulin resistance and dysglycemia.

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Ezetimibe has been demonstrated to increase intestinal Glucagon-like peptide-1 (GLP-1)(17), and improve insulin resistance in rodents (5). However, in studies conducted on human subjects, the effect of ezetimibe on glucose metabolism was not clearly reported. Some randomized controlled trials using ezetimibe were discontinued due to increased blood glucose during the study period (4). Several ezetimibe studies reported that, unlike statin, the use of ezetimibe does not contribute to worsening blood glucose levels, but rather improves insulin resistance (18). Although there was no significant difference in the analysis results between groups, clinical data used in the present study indicated that glucose levels and insulin resistance indices worsened in the statin monotherapy patients. However, this phenomenon was not observed to a significant degree in patients taking ezetimibe and statin in combination. Because relatively high doses of statins were used in the statin monotherapy group, it is unclear whether this effect was caused by ezetimibe treatment. However, it may be clinically valuable that the combination of ezetimibe in the real world may have similar LDL cholesterol lowering effects without the use of high doses of statins and may reduce statins' adverse effects on glucose metabolism.

Non-alcoholic fatty liver disease has features which are closely related to metabolic syndrome, including obesity and insulin resistance. Improvement of insulin resistance results in inhibition of the sterol regulatory element binding protein-1c and blockade of fatty acid synthase (19) which contributes to improved hepatic steatosis. In animal studies, ezetimibe was shown to improve fatty liver through the activation of nuclear factor erythroid 2-related factor 2, an antioxidant transcription factor. Improvement of fatty liver also has been reported in small studies with human subjects (4, 20). In recent studies, ezetimibe improved steatohepatitis by inhibiting NOD-like receptor family, pyrin domain containing 3 inflammasome and activating autophagy (21). However, in the current study, significant improvement of fatty liver in the ezetimibe group was not identified.

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Similarly, some human studies investigating fatty liver improvement associated with ezetimibe also report unclear findings. Loomba et al. report no significant improvement of hepatic steatosis when liver fat was measured by magnetic resonance imaging proton density fat fraction in an ezetimibe treatment group (6). The experimental period of above study (14 weeks) may not have been long enough to induce hepatic steatosis, further studies with a longer study period may be needed.

In the current study, *in vitro* experiments revealed a lack of significant difference in insulin signaling and gluconeogenesis in hepatocytes in the ezetimibe treatment groups. Rather, gluconeogenesis in human hepatocytes was tended to increase in the ezetimibe treatment group. Not only is NPC1L1 present in the intestinal membrane but also in hepatocytes. It is involved in the reabsorption of cholesterol released into the bile (22), but the role and mechanisms of NPC1L1 are not yet fully understood. Previous studies using rodent models report that while NPC1L1 is not expressed in hepatocytes in mice, it is expressed in both rats and humans. These differences probably affect blood glucose levels and lipid metabolism. Considering that the ezetimibe-induced inhibition of hepatic NPC1L1 did not lead to improved insulin resistance or reduced gluconeogenesis, the effect of ezetimibe observed in this study may be caused by inhibition of intestinal NPC1L1.

There are several limitations of this study. First, an insulin secretion function evaluation through a pancreatic beta cell study was not performed. Second, the rat and human study populations were not large enough for several analyses. For this reason, we cannot exclude the possibility that the statistically insignificant results in this study may still have clinical significance. In the case of human study, it was retrospectively designed study. In this reason, important data such as body weight or waist circumference were not measured, so they could not be

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reflected in the study results. Third, it was difficult to explain how ezetimibe indirectly affected adipocytes due to the limited expression of NPC1L1 in specific organs.

V. CONCLUSION

The use of ezetimibe reduced the size of visceral fat adipocytes. Furthermore, ezetimibe reduced M1-polarized macrophage accumulation and induced the anti-inflammatory M2 phenotype of macrophages within adipocytes leading to a fatty acid oxidation, reduction of free fatty acid and improved insulin resistance. Considering the result of the current study, ezetimibe would be safely used in patients with diabetes and hepatic steatosis. .

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ABSTRACT (IN KOREAN)

Niemann-Pick C1 like 1 (NPC1L1) 억제가
(간/지방조직에서) 혈당 및 염증 지표에 미치는 영향 연구

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서론:

에제티미브는 Niemann-Pick C1 Like 1(NPC1L1)을 억제하여 주로 장에서 콜레스테롤 흡수를 감소시킨다. NPC1L1은 장 뿐 아니라 간에서도 발현되는 것으로 알려져 있으며 에제티미브의 포도당 대사에의 영향에 대해서는 아직 연구가 많이 이루어져 있지 않다. 본 연구는 에제티미브의 포도당 대사에 대한 영향을 간세포주, 설치류 (마우스와 렛트)에서의 실험과 후향적 임상데이터 분석을 통해 알라보고자 하였다. 또한 지방세포에서의 염증지표에 대해여서도 연구를 진행하였다.

재료 및 방법:

C57BL/6J 마우스와 Wistar 렛트에 고지방 식이를 투여하고 에제티미브 투여에 따른 포도당 대사의 변화를 확인하였다. 특히 Wistar 렛트의 간 및 지방 조직에서의 지방간, 염증 관련 지표를 각기 비교하였다. 후향적 임상연구로 세브란스병원에서

고지질혈증으로 치료를 받은 환자 데이터를 사용하여 스타틴 단독 투여군과 스타틴에 에제티미브를 병합하여 투여한 군에서 포도당 대사에 영향을 확인하였다.

결과:

마우스와 렛트연구에서 에제티미브 사용으로 일부 인슐린 저항성 지표들의 개선을 확인할 수 있었으며, 에제티미브 사용은 지방세포의 크기 감소, M1 대식세포 지표의 감소와 M2 대식세포 지표의 증가, 지방산의 산화, 혈중 유리지방산 농도의 감소와 동반되어 있었다. 그러나 이러한 차이가 전신적인 혈당 개선으로 이어지지는 않았으며, 지방간의 유의한 개선 또한 명확하지 않아 전반적인 포도당 대사에 에제티미브가 미치는 영향은 크지 않았다. 임상 데이터를 통한 분석에서, 에제티미브 복합사용군에서 스타틴제제 단독 사용군에 비해 HOMA-IR로 대변되는 인슐린 저항성 증가가 현저하지 않았다.

결론:

동물실험에서 에제티미브는 지방세포의 크기를 감소시켰으며, 지방세포내의 염증 지표 개선 소견을 보였다. 이는 유리지방산 농도의 감소와, 일부의 혈당 지표 개선으로 이어져서 인슐린 저항성 개선효과를 보인 반면 전신적인(systemic) 포도당 대사에는 큰 영향은 없었으며 이 결과는 역행적 임상연구 결과로 재확인되었다.

핵심되는 말: 에제티미브, 지방세포, 염증, 당대사