Therapeutic effect of the α-glucosidase inhibitor on diet-induced fatty liver disease in mice

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ABSTRACT

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Non-alcoholic fatty liver disease (NAFLD) is a chronic metabolic disease associated with unhealthy lifestyle, especially unhealthy eating. Increased intake of glucose, fructose, or saturated fat increases hepatic *de novo* lipogenesis, a major mechanism of NAFLD development. α -glucosidase inhibitors (AGIs) are a class of antidiabetic agents that inhibit the conversion of polysaccharides to monosaccharides in the intestinal lumen. This function of AGIs is considered to be effective in the prevention and treatment of NAFLD. Although studies have reported that AGIs effectively prevent NAFLD in animal models, studies on the effects of AGIs in nondiabetic NAFLD models are still lacking. Furthermore, to the best of our knowledge, there are no published studies on the effects of AGIs in NAFLD model induced by a high-fat, high-fructose (HFHF) diet. Therefore, we examined the therapeutic effect of voglibose (an orally active AGI) on a HFHF diet-induced nondiabetic NAFLD mouse model. We also assessed the effectiveness of voglibose in combination with pioglitazone, a proven therapeutic agent for NAFLD.

Seven-week-old male C57BL/6J mice were randomly divided into two groups: a normal chow diet and a HFHF diet groups. After 10 weeks, HFHF diet group was subdivided into four groups: HFHF-vehicle treated (HFHF), HFHF-voglibose treated (HFHF-V), HFHF-pioglitazone treated (HFHF-P), and HFHF-combination therapy (HFHF-C) groups. Each treatment was administered daily via oral gavage for 10 weeks. The normal chow diet group maintained the same diet with vehicle administration for the 10 weeks.

After 20 weeks, HFHF group became obese and developed NAFLD, including hepatic steatosis, inflammation and fibrosis. Voglibose administration attenuated HFHF-induced hepatic steatosis and reduced proinflammatory and fibrotic markers in the liver. These effects were comparable to those of pioglitazone treatment. Combination therapy with voglibose and pioglitazone showed therapeutic effects similar to each monotherapy group but without any additive effects. In the HFHF-V group, gene expression and protein levels of hepatic lipogenesis markers, including sterol regulatory element-binding transcription factor-1 and carbohydrate response element binding protein, were significantly downregulated; HFHF-C group showed similar results. These findings indicate that voglibose ameliorated NAFLD by reducing hepatic *de novo* lipogenesis in the HFHF diet-induced nondiabetic NAFLD model. In the *in vitro* experiment, reducing the influx of glucose into hepatocytes significantly reduced hepatic steatosis and *de novo* lipogenesis even in the presence of sufficient fructose and fat, demonstrating the potential benefits of voglibose in HFHF diet-induced NAFLD. Hepatic expression of autophagy markers was improved in voglibose treated mice, which may also be beneficial for the treatment of NAFLD.

In conclusion, voglibose robustly improved HFHF diet-induced NAFLD in mice, by suppressing hepatic *de novo* lipogenesis. Although combination therapy with voglibose and pioglitazone showed significant therapeutic effects, there were no additive or synergistic effects due to the combination therapy in this experiment.

Key words : non-alcoholic fatty liver disease, α-glucosidase inhibitor

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I. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic metabolic disease in which lipid is abnormally accumulated in the liver, and its prevalence is increasing worldwide. Approximately 25% of the global population is affected by this disease[1], and increasing prevalence of it is considered to be associated with unhealthy lifestyle, especially unhealthy eating[2]. Increased intake of glucose, fructose and saturated fat increases hepatic *de novo* lipogenesis. In addition, they also induce systemic insulin resistance and low grade inflammation, which promote hepatitis as well as hepatic steatosis. Prolonged non-alcoholic steatohepatitis (NASH) could eventually progress to cirrhosis or hepatocellular carcinoma[3,4].

To improve or treat NAFLD, weight loss through lifestyle modification is preferentially recommended[5,6], and in severely obese cases, bariatric surgery could be also considered[7]. However, pharmacological interventions for the treatment of NAFLD is still lacking in options and evidence. The use of vitamin E, an antioxidant, and the antidiabetic agent pioglitazone are generally introduced in the guidelines[2,7,8], but they were not widely used due to limited efficacy or side effects. Recently, therapeutic or preventive effects of glucagon-like peptide-1 (GLP-1) agonists or sodium glucose cotransporter-2 (SGLT-2) inhibitors, which are relatively novel antidiabetic agents, for NAFLD have been actively studied[9-12], but they have not yet been recognized as a formal option for NAFLD

treatment.

Since increased intake of glucose is the important factor in NAFLD pathogenesis, α-glucosidase inhibitors (AGIs), which delay the absorption of glucose by competitively inhibiting the enzyme that converts complex carbohydrate into monosaccharide in the lumen of small intestine, could be an attractive candidate for NAFLD management. By slowing glucose absorption from the intestine, AGIs suppress the surge of postprandial blood glucose levels and the amount of hepatic glucose intake[13]. There were a few of previous studies which evaluated the preventive effects of AGIs for NAFLD in animal models[14-16]. In addition, there was a clinical, single-arm study that showed therapeutic effects of the AGI in histologically-confirmed NAFLD patients with diabetes[17].

However, studies on the effects of AGIs in the nondiabetic NAFLD model, especially the therapeutic effects rather than preventive effects for NAFLD, are still lacking. Furthermore, to the best of our knowledge, there are no published studies on the effects of AGIs in NAFLD model induced by high-fat, high-fructose (HFHF) diet, which is considered as a major factor of NAFLD pathogenesis.

Therefore, in this study, we examined the therapeutic effect of the AGI (voglibose) on the HFHF diet-induced nondiabetic NAFLD mouse model. A pioglitazone group was created as a positive comparator group, and a combination group was also created to determine the effect of combination therapy with AGI and pioglitazone.

II. MATERIALS AND METHODS

1. Animal experiments

Seven-week-old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). After 1-week acclimatization period, the mice were randomly divided into two groups depending on the diet; a chow diet (N=6) and a HFHF diet group (N= 40). After 10 weeks of the diet period, mice in the HFHF group were randomly subdivided into four groups; HFHF diet with vehicle (HFHF group, N=10), HFHF diet with voglibose (HFHF-V group, N=10), HFHF diet with pioglitazone (HFHF-P group,

N=10), and HFHF diet with combination therapy (HFHF-C, N=10) groups. Mice in chow diet group maintained the same diet with daily vehicle administration. Each treatment was administered daily via oral gavage for 10 weeks. The doses of voglibose and pioglitazone were determined to be 1.0 mg/kg/day and 10 mg/kg/day, taking into account human doses and previous experiments[18-21]. The mice were maintained at a temperature of 23℃ ± 2℃ and humidity level of 60% ± 10% under a 12-hour light/dark cycle.

Regular chow diet (PicoLab Rodent Diet 20 [5053]) contained 23.6% protein, 64.5% carbohydrate, and 11.9% fat (% of total kcal), and the HFHF diet (Catalog number D17010102; Research Diets, New Brunswick, NJ, USA) contained 20% protein, 40% fat (of these 22.7% trans-fat), 20% fructose (% of total kcal), and high cholesterol (2% by weight.). This HFHF diet was selected to induce NAFLD in mice because composition of the diet was similar to the Amylin liver NASH diet which showed significant NAFLD induction in mice in previous studies[22,23].

After the 20-week experiment, consisting of the NAFLD-induction period for the first 10 weeks and the treatment period for 10 weeks thereafter, animals were anesthetized and sacrificed 24 hours after the final administration. All animal procedures were approved by the Animal Care and Use Committee at the Yonsei University College of Medicine (2018-0180) and all experiments were performed in accordance with the relevant guidelines and regulations.

2. Biochemical measurement and tissue preparation

Random blood glucose concentrations were assessed weekly by sampling tail vein. Body weight was also measured weekly over the entire treatment period. The amounts of food intake per cage were measured weekly during the treatment period, from 11th to 20th week. At sacrifice, blood was collected via heart puncture and tissues were harvested. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, insulin, total cholesterol, triglyceride (TG), free fatty acids were measured. Insulin resistance was assessed by using homeostasis model assessment (HOMA-IR)[24].

Harvested tissues were liver and jejunum. For the harvest of jejunum, entire small intestine was flushed with iso-osmotic phosphate-buffered solution to remove intraluminal content. The jejunum specimens were collected at the most proximal 1/6 portion between the end of duodenum and the beginning of cecum.

3. Histological analysis

Harvested tissue specimens were snap-frozen in liquid nitrogen and maintained at -80℃ until analysis. Hepatic TG levels were determined using the TG quantification kit (K622; Biovision) according to the manufacturer's instructions.

For histological assessment of liver sections under a light microscope (Olympus BX40, Olympus Optical Co. Ltd., Tokyo, Japan), 5mm × 5mm sections were fixed in 4% paraformaldehyde for 48 hours, and embedded in paraffin. Tissue Sections (4μm) were prepared using a microtome (Reichert Scientific Instruments, Buffalo, NY) and placed on glass slides. Paraffin-embedded liver specimens were stained with hematoxylin and eosin (H&E). Liver fibrosis was assessed using the Masson's trichrome (MT) staining.

4. Ribonucleic acid (RNA) isolation and real-time polymerase chain reaction analysis

Total RNA was extracted using a Hybrid-R RNA purification kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, 4368814) and 2.5μM random primers. Quantitative real-time polymerase chain reaction (qPCR) was performed in 10μl reactions containing 1.0μl cDNA, 5pmol of each oligonucleotide primer and 5.0μL of Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659). qPCR reactions were performed using the 2-ΔΔCt method and a StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA,USA) in a 96-well plate. The expression of target genes was normalized to that of reference gene, 18S ribosomal RNA (18S). Expression of

18S, interleukin-1 beta (*Il-1β*), monocyte chemoattractant protein-1 (*Mcp-1*), transforming growth factor-β (*Tgf-β*), α-smooth muscle actin (*α-Sma*), collagen type 1α1 chain (*Col1a1*), sterol regulatory element-binding transcription factor-1 (*Srebp-1*), carbohydrate response element binding protein (*Chrebp*), acetyl-CoA carboxylase (*Acc*), fatty acid synthase (*Fas*), autophagy-related gene 7 (*Atg7*), beclin 1 (*Becn1*), glucose transporter 2 (*Glut2*), glucose transporter 5 (*Glut5*) and sodium glucose cotransporter-1 (*Sglt-1*) were assessed using specific primers, of which sequences are listed in Table 1.

Table 1. Primer sequences used in this study

Primer		Sequence
18S	Forward	5’ -GATGTGAAGGAAGTACAG-3’
	Reverse	5’ -CTTCTTGGATACACCCACAGTTC-3’
Il-1β	Forward	5’ -CTGGTGTGTGACGTTCCCATTA -3’
	Reverse	5’ -CCGACAGCACGAGGCTTT -3’
Mcp-1	Forward	5’ -ATCCCAATGAGTAGGGTGGAGAGG -3’
	Reverse	5’ -CAGAAGTGCTTGAGGTGGTTGTG -3’
Tgf-β	Forward	5’ - AAGAAGTCACCCGCGTGCTA -3’
	Reverse	5’ -TGTGTGATGTCTTTGGTTTTGTCA -3’
α-Sma	Forward	5’ -CGTGGCTATTCCTTCGTTAC-3’
	Reverse	5’ -TGCCAGCATGACTCCATCC-3’
Col1a1	Forward	5’ -CCTGGTAAAGATGGTGCC -3’
	Reverse	5’ -CACCAGGTTCACCTTCGACC -3’
Srebp-1	Forward	5’ -CGCAAGCTGTCGGGGTAG -3’
	Reverse	5’ -GTTGTTGATGAGCTGGAGCA -3’

Chrebp	Forward	5’ -CCACAGCGGACACTTCATGG -3’
	Reverse	5’ -AGGCTCTCCAGATGGCGTTG -3’
Acc	Forward	5’ -ATGGGCGGAATGGTCTCTTTC -3’
	Reverse	5’ -TGGGGACCTTGTCTTCATCAT -3’
Fas	Forward	5’ -GGAGGTGGTGATAGCCGGTAT -3’
	Reverse	5’ -TGGGTAATCCATAGAGCCCAG -3’
Atg7	Forward	5’ -CAGAAGAAGTTGAACGAGTA -3’
	Reverse	5’ -CAGAGTCACCATTGTAGTAAT -3’
Becn1	Forward	5’ -GCGGGAGTATAGTGAGTT -3’
	Reverse	5’ -GGTGGCATTGAAGACATT -3’
Glut2	Forward	5’ -CACCACTCTCACCTGGTTCTC -3’
	Reverse	5’ -GCCTGATAAGCAGCAGACCT -3’
Glut5	Forward	5’ -CCAGGAAGCACATTCTGCGA -3’
	Reverse	5’ -TCCACAATCTCTAGCTCCCGA -3’
Sglt-1	Forward	5’ -CCAGTGGGCTGTACCAACAT -3’
	Reverse	5’ -GAGAGTACTGGCGCTGTTGA -3’

Abbreviation. 18S, 18S ribosomal RNA; Il-1β, interleukin-1 beta; Mcp-1, monocyte chemoattractant protein-1; Tgf-β, transforming growth factor-β; α-Sma, α-smooth muscle actin; Col1a1, collagen type 1α1 chain; Srebp-1, sterol regulatory element-binding transcription factor-1; Chrebp, carbohydrate response element binding protein; Acc, acetyl-CoA carboxylase; Fas, fatty acid synthase; Atg7, autophagy-related gene 7; Becn1, beclin 1; Glut2, glucose transporter 2; Glut5, glucose transporter 5; Sglt-1, sodium glucose cotransporter-1

5. Western blot analysis

Protein concentration was determined from clear supernatants by Bradford reagent (Sigma Aldrich) using bovine serum albumin (BSA) as a control. Equal amounts (20μg) of total protein were electrophoresed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 10% gradient gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Samples were immunoblotted overnight with the indicated primary antibodies (typically 1:1,000 dilution) followed by secondary antibody conjugated with horseradish peroxidase (1:5,000 dilution). The supersignal west pico plus kit (Thermo) was used for detection. Specific antibodies against SREBP (PA1-46142, Thermo Fisher Scientific) and ChREBP (NB400-135, Novus Biologicals) were used.

6. Cell culture and treatment

The human hepatoma cell line HepG2 (American Type Culture Collection [ATCC], Manassas, VA, USA) was cultured in low glucose (5.5mM) Dulbecco's modified Eagle's medium (DMEM; SH30021.01, HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin in a 5% CO_2 incubator at 37°C. Cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 and cultured for 3 days to achieve 70% confluence before treatment. HepG2 cells were treated with oleic acid (OA, Sigma-Aldrich, 1.0mM) and fructose (20mM) for 48 hours with different concentrations of glucose, 5mM or 20mM. After treatment, hepatic TG quantification and qPCR for SREBP-1 and ChREBP were conducted.

7. Statistical analysis

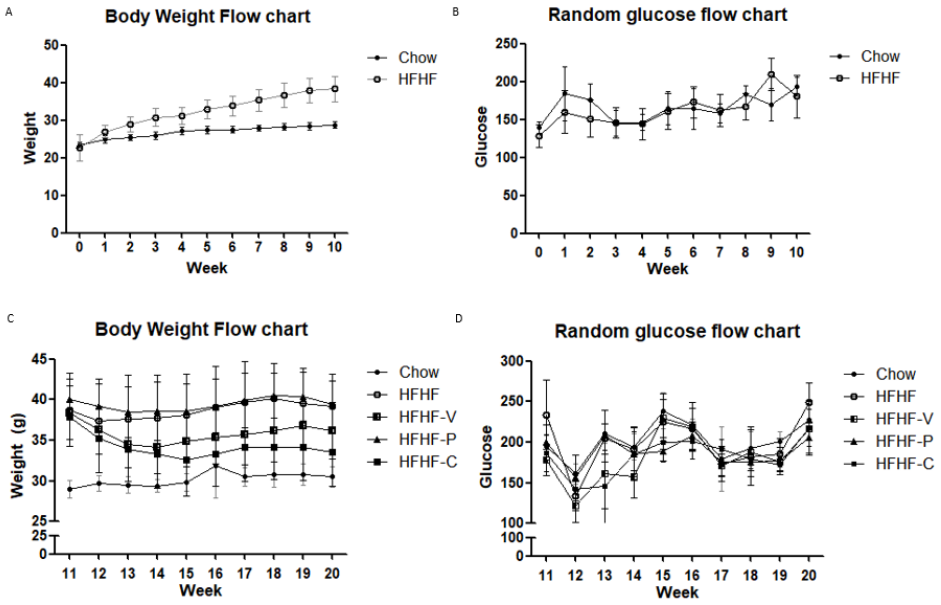
Data are expressed as the mean ± standard deviation (SD). Student's t test was used to compare variables between the each two groups. P-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 21.0

for Windows (IBM Corp.,Armonk, NY, USA).

III. RESULTS

1. Biochemical characteristics

The graphs showing changes in body weight, food intake, and random blood glucose of mice during the experiment period were presented in Figure 1. During the NAFLD-induction period for the first 10 weeks, the weight of mice under HFHF diet increased significantly higher than chow diet group, whereas random blood glucose levels were not different between the two groups. During the latter 10 weeks of treatment period, mice in HFHF-V group and HFHF-C group were significantly less weight gain than mice in HFHF group and HFHF-P group. Random blood glucose levels did not show any differences or trends between the five groups. The amount of food intake was increased in all HFHF-diet groups, and there was no significant difference between the HFHF-V and HFHF-P group. However, it was slightly decreased in the HFHF-C group.



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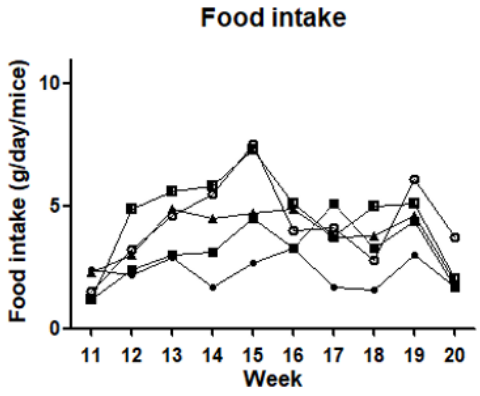


	11wk	12wk	13wk	14wk	15wk	16wk	17wk	18wk	19wk	20wk
Chow	2.4	2.2	2.9	1.7	2.7	3.3	1.7	1.6	3.0	1.7
HFHF	1.5	3.2	4.6	5.5	7.5	4.0	4.1	2.8	6.1	3.7
HFHF-V	1.2	4.9	5.6	5.8	7.3	5.1	3.8	5.0	5.1	2.0
HFHF-P	2.3	3.0	4.9	4.5	4.7	4.9	3.7	3.8	4.6	1.9
HFHF-C	1.2	2.4	3.0	3.1	4.5	3.3	5.1	3.3	4.4	1.7

Figure 1. Changes in body weight, random blood glucose levels, and food intake. During the first 10 weeks of diet, high-fat, high fructose (HFHF) diet group showed marked increase in body weight (A), but random blood glucose was not different between HFHF group and chow group (B). Since the administration of drugs or vehicle started at 11th week, voglibose or combination therapy of voglibose plus pioglitazone resulted in less weight gain compared to the HFHF group (C). There was no significant difference in blood glucose levels between the five groups (D). The amount of food intake was increased in all HFHF-diet groups, and there was no significant difference between the HFHF-V and HFHF-P group (E).

Abbreviation. HFHF, high-fat, high-fructose diet group; HFHF-V, HFHF diet with voglibose group; HFHF-P, HFHF diet with pioglitazone group; HFHF-C, HFHF diet with combination (voglibose plus pioglitazone) group.

After a total of 20 weeks of experiment, mice in the HFHF group showed significantly increased body weight, liver weight and liver enzymes (Table 2), corresponding to NAFLD. HFHF-V and HFHF-C group showed a trend of lower body weight and liver weight compared to HFHF diet group. Blood liver enzymes showed a trend of

improvement in all three-treatment groups, but significant improvement compared to HFHF group was observed only in HFHF-V for AST levels and only in voglibose-treated groups (HFHF-V and HFHF-C) for ALT levels. There were no significant differences in fasting blood glucose levels and insulin resistance assessed by HOMA-IR between the four HFHF-fed groups. Total cholesterol and TG levels were also similar between the HFHF-fed groups, whereas free fatty acid levels were significantly lower in all three treatment groups than the HFHF diet group.

Table 2. Body weight and biochemical measurements at 20th week

	Chow	HFHF	HFHF-V	HFHF-P	HFHF-C
Body weight (g)	27.0 ± 0.9	38.2 ± 3.4$^{\#}$	33.0 ± 2.8$^{\#\dagger}$	35.0 ± 2.0$^{\#}$	32.3 ± 3.0$^{\#\dagger}$
Liver weight (g)	1.1 ± 0.1	3.0 ± 0.7$^{\#}$	1.8 ± 0.4$^{\#\dagger}$	2.6 ± 0.4$^{\#*}$	2.3 ± 0.6$^{\#}$
Liver to body weight (%)	4.2 ± 0.1	7.9 ± 1.2$^{\#}$	5.3 ± 0.7$^{\#\dagger}$	7.4 ± 0.8$^{\#*}$	7.2 ± 1.0$^{\#*}$
AST (IU/L)	82.6 ± 17.4	463.8 ± 250.8$^{\#}$	154.2 ± 66.1†	288.4 ± 96.8$^{\#}$	185.0 ± 112.1
ALT (IU/L)	26.0 ± 6.4	487.0 ± 303.1$^{\#}$	114.6 ± 88.8†	255.6 ± 99.7$^{\#}$	92.4 ± 75.7†
Fasting glucose (mg/dL)	133.6 ± 27.8	235.8 ± 118.4	177.0 ± 75.5	184.1 ± 31.8$^{\#}$	181.0 ± 45.8$^{\#}$
Fasting insulin (ng/dL)	0.34 ± 0.02	0.25 ± 0.004$^{\#}$	0.25 ± 0.01$^{\#}$	0.26 ± 0.004$^{\#}$	0.25 ± 0.01$^{\#}$
HOMA-IR	2.8 ± 0.6	3.7 ± 1.9	2.4 ± 0.9	2.9 ± 0.5	2.8 ± 0.7
TC	73.2 ± 5.1	168.0 ± 47.8$^{\#}$	121.0 ± 20.1$^{\#}$	172.8 ± 29.5$^{\#}$	147.6 ± 48.0$^{\#}$

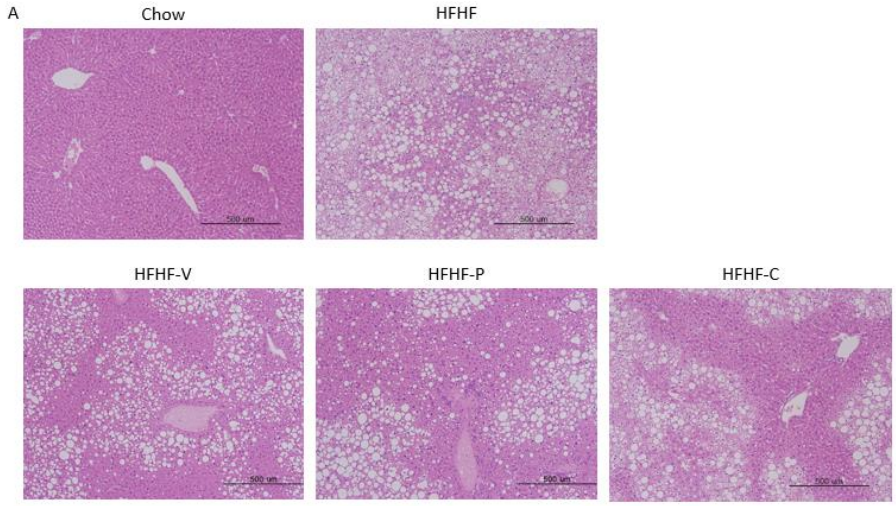
(mg/dL)					
TG (mg/dL)	45.7 ± 9.4	24.5 ± 6.0$^{\#}$	28.7 ± 7.6$^{\#}$	21.1 ± 5.9$^{\#}$	20.9 ± 3.4$^{\#}$
Free fatty acid (mM)	0.17 ± 0.006	0.6 ± 0.03$^{\#}$	0.5 ± 0.01$^{\#\dagger}$	0.5 ± 0.01$^{\#\dagger}$	0.5 ± 0.03$^{\#\dagger *}$

Abbreviation. HFHF, high-fat, high-fructose diet group; HFHF-V, HFHF diet with voglibose group; HFHF-P, HFHF diet with pioglitazone group; HFHF-C, HFHF diet with combination (voglibose plus pioglitazone) group; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triglyceride

$^{\#}$P<0.05 vs. chow, †P<0.05 vs. HFHF, *P<0.05 vs. HFHF-V, §P<0.05 vs. HFHF-P

2. Voglibose ameliorates hepatic steatosis, inflammation and fibrosis

Histological assessment of hepatic steatosis or fibrosis was performed on the H&E- or MT-stained liver tissue sections (Figure 2). Voglibose significantly improved both hepatic steatosis and fibrosis. Those effects were comparable to pioglitazone. Combination therapy showed similar degree of improvement of steatosis and fibrosis to the monotherapy groups.



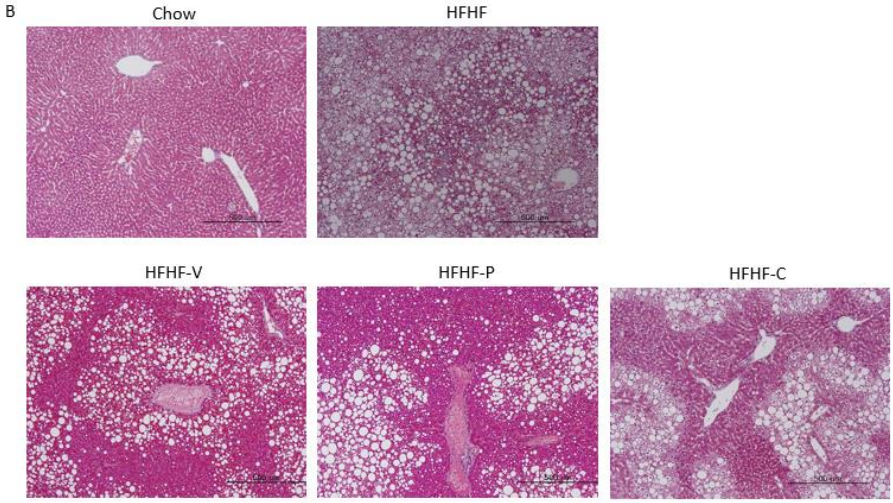


Figure 2. Histopathological phenotypes of the five groups. In the H&E-stained liver tissue sections (A), administration of voglibose (HFHF-V), pioglitazone (HFHF-P), or combination therapy (HFHF-C) showed marked improvement in hepatic steatosis. Liver fibrosis was also improved in all three treatment groups, assessed in the MT-stained liver tissue sections (B).

Abbreviation. HFHF, high-fat, high-fructose diet group; HFHF-V, HFHF diet with voglibose group; HFHF-P, HFHF diet with pioglitazone group; HFHF-C, HFHF diet with combination (voglibose plus pioglitazone) group.

To quantify those effects, we used a TG quantification kit and analyzed the messenger RNA (mRNA) expression of fibrotic markers including TGF-β, α-SMA and Col1a1 (Figure 3). In addition, we also analyzed the mRNA expression of proinflammatory markers such as IL-1β and MCP-1. Mice with HFHF diet showed markedly increased TG content, increased proinflammatory and fibrotic markers, which showed their severe NAFLD status including hepatic steatosis, inflammation, and fibrosis. Administration of voglibose significantly reduced hepatic TG contents and improved both proinflammatory and fibrotic markers. These effects were comparable to those of pioglitazone, in line with the histological findings. Combination therapy with voglibose and pioglitazone also

showed marked improvements in terms of steatosis, inflammation and fibrosis, but there were no additive effects compared to the monotherapy groups.

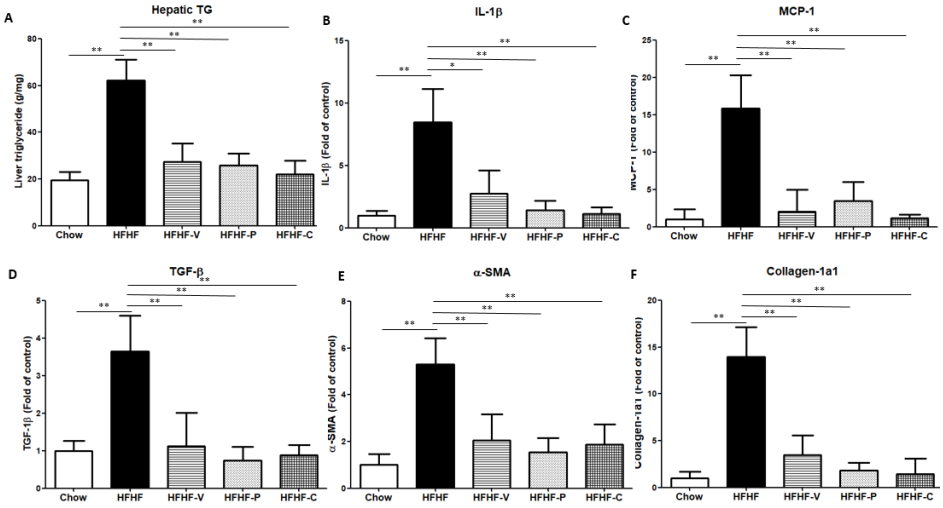


Figure 3. Hepatic TG contents, mRNA expression of proinflammatory markers and fibrotic markers of the five groups. Administration of voglibose (HFHF-V) reduced liver TG (A), expression of proinflammatory markers such as IL-1β (B), MCP-1 (C), and expression of fibrotic markers including TGF-β (D), α-SMA (E), and col1a1 (F). These effects were comparable to those of pioglitazone (HFHF-P). Combination therapy (HFHF-C) also showed significant improvements, but there was no additive effect compared to the monotherapy groups.

Abbreviation. TG, triglyceride; IL-1β, interleukin-1 beta; MCP-1, monocyte chemoattractant protein-1; TGF-β, transforming growth factor-β; α-SMA, α-smooth muscle actin; Col1a1, collagen type 1α1 chain; HFHF, high-fat, high-fructose diet group; HFHF-V, HFHF diet with voglibose group; HFHF-P, HFHF diet with pioglitazone group; HFHF-C, HFHF diet with combination (voglibose plus pioglitazone) group.

$^{*}P<0.05$, $^{**}P<0.01$

3. Voglibose reduces hepatic *de novo* lipogenesis

Voglibose is well known to reduce glucose absorption by inhibiting α-glucosidase in intestinal lumen, leading to the reduction in hepatic inflow of glucose, an important material for *de novo* lipogenesis in the liver. To evaluate whether voglibose treatment reduced hepatic *de novo* lipogenesis, we measured the mRNA expression and protein levels of SREBP-1 and ChREBP, key regulators of hepatic *de novo* lipogenesis[25], and the mRNA expression of hepatic lipogenic enzymes such as ACC and FAS (Figure 4). As a result, voglibose robustly reduced hepatic *de novo* lipogenesis, showing significantly lower expression levels of SREBP-1, ChREBP, ACC and FAS. Combination therapy of voglibose plus pioglitazone showed similar degree of reduction in lipogenic markers, compared to the monotherapies.

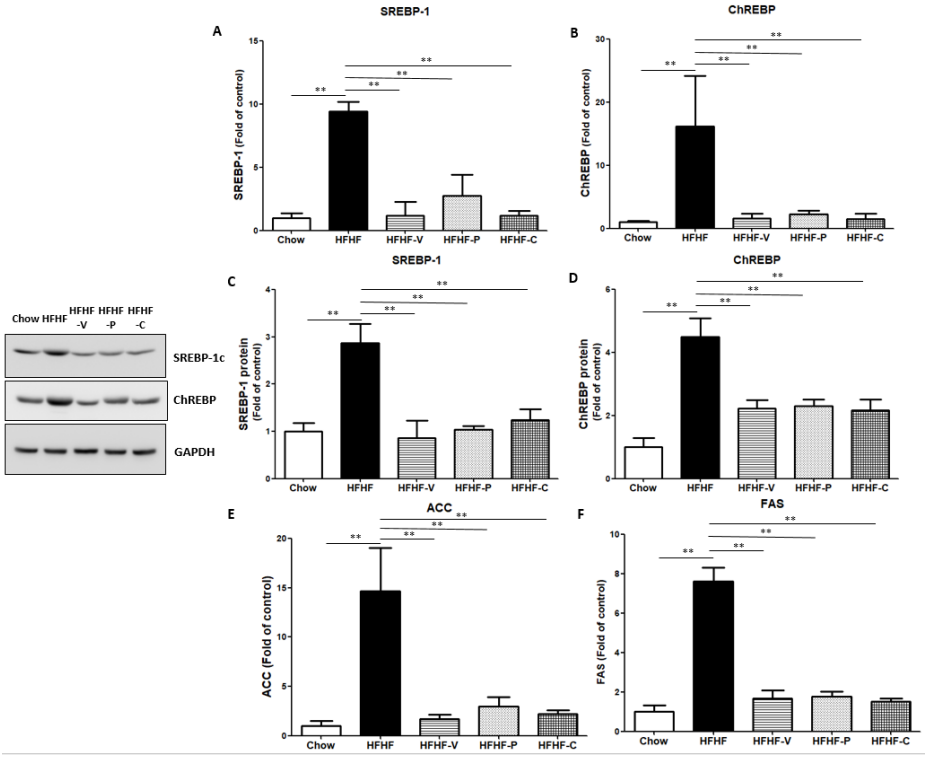


Figure 4. Hepatic mRNA and protein expression of SREBP-1 and ChREBP, mRNA

expression of ACC and FAS. Voglibose (HFHF-V) reduced mRNA expression of hepatic *de novo* lipogenesis markers such as SREBP-1 (A) and ChREBP (B). Protein levels of SREBP-1 (C) and ChREBP (D) were also suppressed in HFHF-V group. HFHF-V also reduced mRNA expression of hepatic ACC (E) and FAS (F), which were the downstream genes of SREBP-1 and ChREBP. These effects were comparable to pioglitazone (HFHF-P). Combination therapy (HFHF-C) showed significant improvements, but there was no additive effect compared to the monotherapy groups.

Abbreviation. SREBP-1, sterol regulatory element-binding transcription factor-1; ChREBP, carbohydrate response element binding protein; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; HFHF, high-fat, high-fructose diet group; HFHF-V, HFHF diet with voglibose group; HFHF-P, HFHF diet with pioglitazone group; HFHF-C, HFHF diet with combination (voglibose plus pioglitazone) group.

$^{*}P<0.05$, $^{**}P<0.01$

4. Reduced glucose inflow to hepatocytes significantly decreases hepatic lipogenesis even in a high-fat, high-fructose circumstance

As mentioned above, the diet used in this study has high quantity of fructose and lipids, which induced NAFLD aggressively. Voglibose is known to reduce or delay the absorption of glucose, whereas it does not have a direct effect on fructose and lipid. Therefore, we hypothesized that even in the high concentration of lipid and fructose, reducing the glucose inflow to hepatocytes will significantly reduce hepatic lipogenesis. To prove the hypothesis, we designed an additional *in vitro* study using HepG2 cell line. HepG2 cells were treated with sufficient OA (1.0mM) and fructose (20mM), which reflected HFHF-diet condition for 48 h with different concentrations of glucose; 5mM, 20mM, or none. Hepatocytes treated with OA, fructose and low concentration of glucose (5mM) showed significantly lower level of hepatic TG than those treated with OA, fructose and high concentration of glucose (20mM) (Figure 5). Lower glucose (5mM) group also showed reduced mRNA expression of SREBP-1 and ChREBP, showing the

importance of reducing glucose inflow to hepatocytes on hepatic lipogenesis even in the high concentration of lipids and fructose.

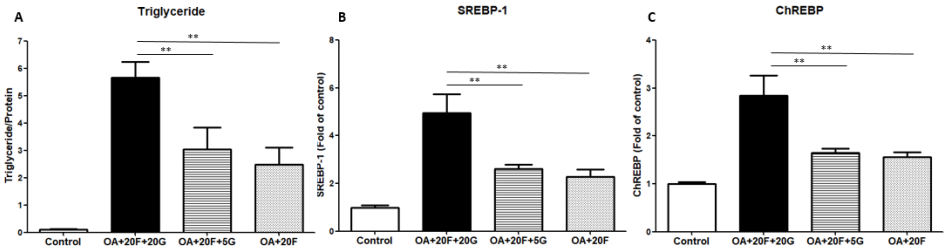


Figure 5. TG content, mRNA expression of SREBP-1 and ChREBP in HepG2 cells treated with OA, fructose, and different concentrations of glucose. High contents of OA, fructose and glucose (OA+20F+20G) increased hepatic TG content (A). Reduction of glucose inflow (OA+20F+5G) showed robust decrease in hepatic TG content. Decreased concentration of glucose (OA+20F+5G) also resulted in decreased expression of SREBP-1 (B) and ChREBP (C), even in the circumstance that high contents of fructose and fatty acid were still being provided.

Abbreviation. SREBP-1, sterol regulatory element-binding transcription factor-1; ChREBP, carbohydrate response element binding protein; OA, oleic acid; OA+20F+20G, HepG2 cell treated with 1.0mM OA, 20mM fructose and 20mM glucose; OA+20F+5G, HepG2 cell treated with 1.0mM OA, 20mM fructose and 5mM glucose; OA+20F, HepG2 cell treated with 1.0mM OA and 20mM fructose

$^{*}P<0.05$, $^{**}P<0.01$

5. Voglibose induces hepatic autophagy

Recently, there is accumulating evidence on the role of impaired autophagy on the pathogenesis of NAFLD[26-28]. Since voglibose is known to be hardly absorbed systemically, it is unlikely to directly affect the autophagy pathways. However, lowering hepatic glucose inflow or reduction of hepatic lipogenesis might influence on the autophagy. Therefore, we measured mRNA expression of autophagy markers, such as

ATG7 and BECN1 (Figure 6).

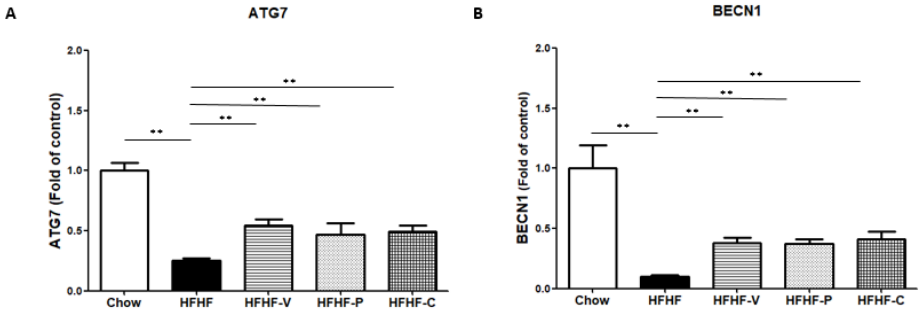


Figure 6. Hepatic mRNA expression of ATG7 and BECN1. Autophagy markers, such as ATG7 (A) and BECN1 (B), was significantly improved in the liver of voglibose-treated mice (HFHF-V). This trend was similar in mice with pioglitazone treatment (HFHF-P) or combination therapy (HFHF-C).

Abbreviation. ATG7, autophagy-related gene 7; BECN1, beclin; HFHF, high-fat, high-fructose diet group; HFHF-V, HFHF diet with voglibose group; HFHF-P, HFHF diet with pioglitazone group; HFHF-C, HFHF diet with combination (voglibose plus pioglitazone) group.

$^{*}P<0.05$, $^{**}P<0.01$

As expected, the autophagy markers were significantly reduced in the mice with HFHF diet. In mice treated with voglibose, hepatic expression of autophagy markers was robustly improved. We speculated that reducing excess hepatic glucose influx could have a beneficial effect on restoring autophagy process in the liver. Mice with pioglitazone or with combination therapy showed similar trends.

6. Voglibose does not alter the expression of intestinal glucose transporters

Voglibose is poorly absorbed after oral administration and the majority of the drug remains in the intestinal lumen. Although there were no previous studies reporting that α-GI resulted in the change of glucose transporters, it might influence on the levels of

glucose or fructose transporters in the enterocytes. When we assessed the mRNA expression of SGLT-1, which takes glucose from the intestinal lumen to the intestinal mucosa, voglibose did not alter the expression level of SGLT-1 (Figure 7). This trend was also observed in the case of GLUT5, the fructose transporter in brush border, and GLUT2 which exports glucose and fructose from the enterocyte across the basolateral membrane.

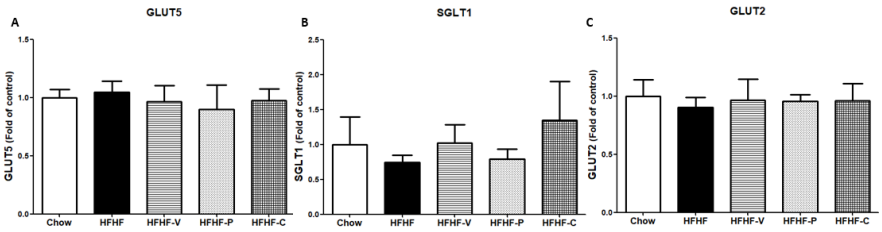


Figure 7. mRNA expression of hexose transporters in the jejunum. Voglibose did not alter the mRNA expression of GLUT5, SGLT-1, and GLUT2 in the small intestine.

Abbreviation. GLUT5, glucose transporter 5; SGLT-1, sodium glucose cotransporter 1; GLUT2, glucose transporter 2; HFHF, high-fat, high-fructose diet group; HFHF-V, HFHF diet with voglibose group; HFHF-P, HFHF diet with pioglitazone group; HFHF-C, HFHF diet with combination (voglibose plus pioglitazone) group.

IV. DISCUSSION

In this study, we investigated the therapeutic effects of voglibose, an AGI, on HFHF-induced nondiabetic NAFLD mice. Although the HFHF diet used in this experiment might be unfavorable to show its therapeutic effects on NAFLD, 10-week treatment of voglibose robustly improved NAFLD in all aspects of hepatic steatosis, inflammation, and fibrosis. The therapeutic effects of voglibose were comparable to those of pioglitazone, one of the drugs recommended for the treatment of NAFLD in various guidelines and reviews[2,3,8]. The effects of combination therapy with voglibose plus pioglitazone did not show any additive or synergistic effects compared to those of the monotherapy. It is considered that the effect of each monotherapy was so strong that it was difficult to show additive or synergistic effects.

Voglibose is poorly absorbed after oral administration and the majority of the drug remains in the intestinal lumen, is excreted rapidly in stools thereafter[29,30]. There are no metabolites identified to date. Therefore, we focused on the action of voglibose in the intestinal lumen, inhibition of intestinal glucose absorption. By inhibiting α-glucosidase, voglibose delay and reduce the amount of glucose uptake through the enterocytes, which decreases glucose inflow to liver as well as postprandial blood glucose level. Since glucose is one of the major material for *de novo* lipogenesis in the liver[31], which is considered as the prominent pathophysiologic abnormality of NAFLD[32], we hypothesized that the therapeutic effects of voglibose on NAFLD was due to the reduction of hepatic *de novo* lipogenesis. As expected, administration of voglibose showed marked suppression of two major transcriptional regulator of *de novo* lipogenesis, SREBP1 and ChREBP. The expression of downstream lipogenic enzymes, ACC and FAS was also decreased. These results reflect the decrease in hepatic *de novo* lipogenesis, which is considered to be the main therapeutic mechanism of voglibose on NAFLD. The results of *in vitro* experiment showed how the reduction of glucose uptake into hepatocytes had a significant effect on *de novo* lipogenesis even in the HFHF diet, supporting the therapeutic effect of voglibose on the HFHF-induced NAFLD.

The effects of voglibose administration ranged to the relative recovery of autophagy. Autophagy is a cellular homeostatic pathway involved in protein and organelle degradation, associated with various pathophysiology[33]. Previous studies have reported that impaired hepatic autophagy is closely related to the pathogenesis of NAFLD[27,34]. In addition, evidence is accumulating that the improvement of autophagy is effective in treating NAFLD[26,28]. In this study, administration of voglibose resulted in the improvement of autophagy. As described above, since voglibose is hardly absorbed systemically, this effect is presumed to be due to a decrease in glucose inflow to liver and thus a decrease in hepatic *de novo* lipogenesis, rather than its direct effect. This assumption is supported by previous studies which reported that lipotoxicity suppresses hepatic autophagy[35,36].

Pioglitazone is a class of antidiabetic agents, which improves insulin sensitivity by activating peroxisome proliferator-activated receptor γ (PPARγ)[37]. In addition to the improvement of insulin sensitivity, pioglitazone is known to have various, systemic effects such as anti-inflammation, vasoprotection, and inducing autophagy[38-40]. As mentioned above, pioglitazone is the only antidiabetic agent generally recommended by guidelines for NAFLD management. Interestingly, it has been reported that pioglitazone improves NAFLD even in the patients without diabetes[41,42]. Although there is a controversy whether PPARγ activation is more prominent in lipid distribution to adipose tissue compared to lipid accumulation to the liver[43-45], most clinical studies have reported that pioglitazone reduced hepatic steatosis[41,42,46]. In addition, those studies also reported that pioglitazone improved hepatic inflammation. In this experiment, pioglitazone showed improvement in all aspects of steatosis, inflammation and fibrosis in nondiabetic NAFLD mice.

Relatively novel antidiabetic agents such as GLP-1 agonists or SGLT-2 inhibitors have gained attention as a novel treatment option for NAFLD[2,7]. Because these agents have advantages such as weight loss and cardioprotective effects, they have attracted the interest of many researchers, and favorable results for NAFLD treatment have been reported, especially in patients with diabetes[9-12,47,48]. In our experiment, voglibose, a class of AGI, is a relatively old-fashioned antidiabetic agent, but effectiveness of it on NAFLD treatment was comparable to pioglitazone. Although our experiment was an animal study, our results are noteworthy in that voglibose showed a marked therapeutic effect in the nondiabetic NAFLD model induced by the HFHF diet, which resembles a problematic unhealthy diet these days.

Our study has some limitations. First, it would be ideal to administer voglibose before every meal, considering its mechanism of action. Therefore, in mice, it may be more physiological to provide voglibose as a feed mixture. However, in the case of mixing the drug in the feed, the properties of the diet would be changed and it was expected that considerable amount of the diet with the drug would be lost by mice. This made it hard to

keep the appropriate drug dosage during the experiment, so we decided to administer the drug via oral gavage, once daily. Second, the amount of food intake in HFHF-C group was relatively lower than other treatment groups in our experiment. However, since there was no difference in the food intake between HFHF-V, HFHF-P, and HFHF group, the influence of the amount of food on the therapeutic effect of voglibose can be excluded. Finally, we did not investigate the therapeutic mechanisms of voglibose other than the reduction of glucose inflow to liver, such as gut microbiota-related mechanisms. In fact, previous studies have reported that AGIs may affect the composition of the gut microbiota[49,50]. In addition, evidence is accumulating for a role of gut microbiota in NAFLD[51,52]. In the future, studies investigating the influence of AGIs on the gut microbiota, and its relationship with NAFLD treatment would be needed. Furthermore, clinical studies which evaluate the therapeutic effects of AGIs on NAFLD patients with or without diabetes is also considered necessary.

V. CONCLUSION

Administration of voglibose improved NAFLD in all aspects of hepatic steatosis, inflammation and fibrosis in HFHF-fed, nondiabetic mice. Reduction of glucose inflow to liver by voglibose administration is sufficient to suppress hepatic *de novo* lipogenesis even in the situation of HFHF diet. This finding suggests that AGIs would be effective for NAFLD treatment in the case of human under the unhealthy diet which is high in fructose and fat.

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ABSTRACT (IN KOREAN)

식이로 유발된 마우스 지방간 모델에서 알파글루코시데이즈 억제제의 치료 효과

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배 재 현

비알콜성지방간은 만성 대사성 질환으로, 건강하지 않은 생활습관, 특히 건강하지 않은 형태의 식이와 밀접하게 연관되어 있다. 포도당과 과당의 섭취증가는 비알콜성지방간의 중요한 발병기전인 간에서의 지방신생합성을 증가시키는 것으로 알려져있다. 알파글루코시데이즈 억제제 (α-glucosidase inhibitor, 이하 AGI)는 소장에서의 포도당 합성을 지연시키는 기전으로 작동하는 당뇨약제이다. 포도당 섭취 증가와 비알콜성지방간의 연관 관계를 고려해보았을 때, 이러한 AGI의 기전은 비알콜성지방간의 예방 또는 치료에 효과적일 것으로 예상해볼 수 있다. 실제로 이전의 몇몇 동물실험에서 AGI는 비알콜성지방간을 효과적으로 예방하는 것으로 보고된 바 있으나, 비당뇨모델에서의 효과, 특히 지방간이 발생된 이후의 치료효과에 대해서는 연구가 부족한 상황이다. 더욱이, 요즘 중요한 문제로 부각되는 고지방, 고과당식이로 인해 유발된 비알콜성지방간에 대하여 AGI가 치료 효과를 보이는지에 대해서는 보고된 연구가 현재 없는 상황이다. 이에, 저자 등은 고지방, 고과당식이로 유발된 비당뇨, 비알콜성지방간 동물 모델에서 AGI 약제인 보글리보즈의 치료효과를 조사해보고자 하였다. 이미 입증된 비알콜성지방간 치료약제인 피오글리타존을 양성 비교군으로 삼았으며, 보글리보즈와 피오글리타존 병합요법의 효과에 대해서도 함께 살펴보았다.

실험 동물로는 7주령 C57BL/6J 마우스를 구매하였으며, 이들을 1주간 교화시킨 후, 처음에는 식이 종류에 따라 정상식이군 (chow)과 고지방, 고과당식이군으로 나누어, 각각의 해당 식이로 10주간 사육하며, 고지방, 고과당식이군에서 지방간이 발병하도록 유도하였다. 11주차부터 고지방, 고과당식이군을 다시 치료약제에 따라 보글리보즈군, 피오글리타존군,

병합요법군, 그리고 위약군으로 균등하게 나누었으며, 10주동안 치료를 진행하였다. 모든 치료는 경구로 제공되었으며, 정상식이군 및 치료를 하지 않는 고지방, 고과당식이군의 경우 동일 성상의 위약을 제공하였다.

총 20주의 실험이 종료되었을 때, 고지방, 고과당식이를 제공받되, 치료 없이 위약만 제공받은 군은 전반적으로 비만해졌으며, 간조직 소견상 지방간 및 지방간염, 그리고 간섬유화까지 진행된 소견을 보였다. 보글리보즈는 이러한 고지방, 고과당식이로 유발된 지방간에 대하여 간내 중성지방 및 염증, 그리고 섬유화까지 효과적으로 개선시켰다. 이러한 치료 효과는 입증된 치료 약제인 피오글리타존과 비슷한 수준이었다. 병합요법군도 우수한 치료효과를 보여 주었으나, 각각의 단독요법보다 더 우월하지는 못하였다. 보글리보즈는 또한 간에서의 지방신생합성을 반영하는 지표들을 유의미하게 감소시켰는데, 이는 보글리보즈를 통한 포도당 흡수 억제가 효과적으로 간에서의 지방신생합성을 감소시켰음을 보여준다. 간세포를 이용한 세포실험을 통해 저자 등은 고농도의 지방산과 과당에 노출된 간세포에 있어서, 포도당 농도만을 감소시켰을 때의 간세포의 지방 함량 및 지방신생합성 지표가 유의미하게 감소하였음을 확인하였고, 이는 보글리보즈를 통한 간으로의 포도당 유입 감소가 지방간에 충분히 효과적일 수 있음을 기전적으로 뒷받침해주는 결과이다. 또한 보글리보즈 치료는 지방간에서 억제된 자가포식을 유의미하게 개선시켰는데, 이는 지방간 치료에 있어서 보글리보즈의 부가적인 치료 기전을 시사하는 결과로 볼 수 있다.

결론적으로, 보글리보즈는 고지방, 고과당 식이를 통해 유발된 비당뇨, 비알콜성지방간에 있어서 유의미한 치료효과를 보여주었고, 그 주된 치료 기전은 간에서의 지방신생합성 억제로 여겨진다.

핵심되는 말 : 비알콜성지방간, 알파글루코시데이즈 억제제