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Comparative effects of sodium glucose co-transporter inhibitors and glucagon like peptide 1 receptor agonists on metabolic parameters in diabetic animal model

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ABSTRACT

Comparative effects of sodium glucose co-transporter inhibitors and glucagon like peptide 1 receptor agonists on metabolic parameters in diabetic animal model

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Introduction: Cardiovascular disease (CVD) is a major cause of death in type 2 diabetes. Sodium-glucose cotransporter 2 (SGLT2) inhibitors are novel class of drugs that inhibit the renal absorption of glucose, and thus, lower blood glucose levels and share similarities with glucagon-like peptide-1 receptor agonists (GLP-1RA) as both reduce cardiovascular morbidity and mortality by lowering through blood glucose levels and causing body weight loss. We compared the effects of empagliflozin (SGLT2 inhibitor) and lixisenatide (GLP-1RA) in type 2 diabetic animal models.

Materials and Methods: Empagliflozin (3 mg/kg/day for 12 weeks, the OL-E group, p.o., n=4), lixisenatide (10 μg/kg/day for 12 weeks, the OL-G group, intraperitoneally, n=4), or vehicle (the OL-C group, n=3) were administered to Otsuka Long-Evans Tokushima Fatty (OLETF) rats (an animal model of T2DM). Long-Evans Tokushima Otsuka (LETO) rats (the LT group, n=3) were used as non-T2DM controls. All rats

underwent echocardiography using the Vivid Q ultrasound system to assess cardiac function at the end of the 12-week treatment period, when after euthanasia, hearts, aortic arches and adipose tissues were removed. Hearts and adipose tissues were subjected to histology.

Triglyceride (TG) accumulation and lipolysis were measured in cardiac and white adipose tissues, which were also analyzed by qPCR for the expressions of oxidative stress related genes (BAX, Casp3, SOD1, SOD2, NOX4, GPX1, GPX3, XBP1, and CHOP), mitochondria related genes (COX2, DRP1, MFN1, CytC, NRF2, TFAM, ATPsyn, and FIS1), ketolysis related genes (MCT1, BDH1 and SCOT), fatty acid oxidation related genes (PGC1α, CPT1α, CPT1β, and CPT2), inflammation related genes (ICAM, VCAM, IL-1β, NLRP3, IL-6, TNFα, MMP9, MCP1, p65, IL-8, IL-18, and HO-1), and lipase related genes (G0s2-1, G0s2-2, CGI58-1, CGI58-2, CGI58-3, ATGL1, ATGL2, ATGL3, HSL1, HSL2, and HSL3). Western blot analysis was conducted to determine levels of pro-inflammatory markers.

Results: During the 12-week treatment period, body weights reduced in the OL-E and OL-G groups as compared with the OL-C group. No significant body weight difference was observed between the OL-E and OL-G groups after treatment for 6 or 12 weeks. Non-fasting blood glucose levels in the OL-E group were significantly lower than in the OL-C, however the non-fasting blood glucose levels were not significantly different between OL-G and OL-C groups. Intraperitoneal glucose tolerance test results after 12 weeks of treatment showed that glucose values from 30 to 120 minutes after glucose loading were significantly lower in the OL-E and OL-G groups than in the OL-C group. TG and free fatty acid levels which were significantly higher in the OL-C group than in the LT group, were suppressed by the treatment of empagliflozin and lixisenatide. In the echocardiographic examination, E/A was significantly decreased in the OL-C group

than in the LT group. E/As in OL-E and OL-G group were not significantly different from that in OL-C group. Oil red O staining of thoracic aortas failed to detect any gross and microscopic atherosclerotic plaque in all group, and the histological examination did not detect any intergroup difference in terms of intracellular lipid droplet, interstitial fibrosis and macrophage infiltration in cardiac tissues. On the other hand, in adipose tissues, numbers of crown-like structures were significantly less in the OL-E and OL-G groups than in the OL-C group. Interestingly, adipocyte size was greater in the OL-E group and less in OL-G group compared to those of the OL-C group.

In OL-E group, no significant changes were observed in terms of triglyceride accumulation, lipolysis and expressions of fatty acid oxidation-, oxidative stress-, mitochondrial function-, ketolysis- and inflammation-related genes in cardiac and adipose tissue. In OL-G group, decreased inflammation in adipose tissue and adipocyte size were observed. Expressions of lipase-, fatty acid oxidation-, oxidative stress-, mitochondrial function-, ketolysis- and inflammation-related genes were decreased in adipose tissue. On the other hand, lipolysis and inflammatory pathway were up-regulated in cardiac tissue in OL-G group.

Conclusions: GLP-1RAs is considered to act mainly in adipose tissue via oxidative stress, mitochondrial function and inflammation related pathway. On the other hand, SGLT2 inhibitor had less effect on cardiac and adipose tissue than GLP-1RA. Empagliflozin may exhibit cardiovascular protective effect through different molecular mechanisms or effects on other tissues than those found in our study.

Keywords: Sodium-glucose cotransporter 2, Glucagon-like peptide-1 receptor agonists, Cardiovascular disease, Diabetes mellitus, Lipolysis, Triglyceride

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INTRODUCTION

Cardiovascular disease (CVD) is a major cause of death in type 2 diabetes (T2DM) [1, 2]. The International Diabetes Federation (IDF) estimated in 2019 that about 463 million people worldwide had T2DM [1]. In fact, T2DM affects 9.3% of the world's population, and the IDF predicts that the number of cases will rise to 700 million by 2045 [1]. Historically, adults with diabetes have a higher prevalence of CVD than those without [3]. The risk of CVD increases in-line with fasting plasma glucose levels, and is meaningful even before they reach levels warranting a diagnosis of diabetes [4]. However, no convincing evidence has been presented that glucose-lowering therapy significantly reduces the rates of cardiovascular (CV) events, death, or macrovascular complications [5-10].

Sodium-glucose cotransporter 2 (SGLT2) inhibitors [11, 12] are novel class of drugs that inhibit the renal absorption of glucose by inhibiting sodium-glucose transport protein 2 in kidneys, and thus, lower blood glucose levels. Three approved SGLT2 inhibitors (canagliflozin, empagliflozin, and dapagliflozin) have been surveyed for CV effects in large randomized clinical trials [13-15]. All three drugs significantly reduced the primary major adverse cardiac events (MACE) and adverse outcomes (CV death, nonfatal myocardial infarction, nonfatal stroke) and had metabolic benefits, that is, they decreased HbA1c levels, body weights, and blood pressures and increased HDL cholesterol levels [13-15]. In addition, it has been reported that ketones are produced by SGLT2 inhibitors, which will affect the improvement of CV outcomes [16].

SGLT2 inhibitors share some similarities with glucagon-like peptide-1 receptor agonists (GLP-1RAs). GLP-1RAs are agonists of GLP-1 receptor and are used to treat T2DM [17]. Their effect on glucose regulation is via glucose-dependent insulin

secretion, suppression of glucagon secretion, delaying gastric emptying and increasing satiety [18]. GLP-1RAs has been shown to reduce CV related mortality and body weight. In the LEADER trial, the effects of the GLP-1RA liraglutide were assessed in patients at high CV risk, and the results obtained confirmed a significant 13% reduction in MACEs and a 15% reduction in death from any cause when patients were treated once-daily with liraglutide as compared with placebo, when both were added to standard care [19]. SGLT2 inhibitors and GLP-1RAs also have similar outcomes result in terms of reducing CV related mortality by lowering blood glucose levels and causing body weight loss. In a meta-analysis, SGLT2 inhibitors (absolute risk reduction, −0.8%; HR, 0.79 [95%CrI, 0.69 to 0.91]) and GLP-1 agonists (absolute risk reduction, −0.5%; HR, 0.85 [95%CrI, 0.77 to 0.94]) were significantly associated with lower CV mortality than controls [20]. However, although SGLT2 inhibitors and GLP-1 agonists produce similar clinical results, no direct comparison of their mechanisms has been conducted.

Accordingly, in the present study, we compared the similarities and differences between an SGLT2 inhibitor (empagliflozin) and a GLP-1RA (lixisenatide) in an animal model of T2DM.

MATERIALS AND METHODS

Experimental animals

Otsuka Long-Evans Tokushima Fatty (OLETF) rats are a well-known animal model of T2DM and display characteristic features of obesity, hyperglycemia, hyperlipidemia, and diabetic complications such as diabetic nephropathy [21, 22]. Male 6-week-old OLETF and Long-Evans Tokushima Otsuka (LETO) rats purchased from Central Lab. Animal Inc. (Seoul). All rats were housed under controlled conditions in a 12-hour light-dark environment and supplied an 18% protein rodent diet (Teklad Diets, Madison, WI) and tap water *ad libitum* throughout. Empagliflozin (OL-E, 3 mg/kg/day dissolved in drinking water, n=4, Boehringer Ingelheim Pharma GmbH & Co KG, Germany) or lixisenatide (OL-G, 10 μg/kg i.p., n=4, Sanofi-Aventis Co., Ltd., France) were administered to OLETF rats. Empagliflozin or lixisenatide were administered daily to 14-week-old animals for 12 weeks. LETO (non-diabetic control group, LT, n=3) and OLETF (diabetic control group, OL-C, n=3) rats were administered saline.

After the 12-week treatment period, all animals were euthanized and hearts and adipose tissues were removed. All experiments, including immunoblots, were performed on heart and adipose tissues, which were rapidly dissected after sacrifice and stored in 4% paraformaldehyde or liquid nitrogen for immunohistochemical analyses. Blood was obtained from left ventricles and stored at –70°C for analyses. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Bundang Seoul National University Hospital.

Biochemical measurements

Body weights were measured weekly during the 12-week treatment period. Blood glucose levels were measured weekly using an Accu-Check glucometer (Roche, Basel, Switzerland). Intraperitoneal glucose tolerance testing (IPGTT) (2 g/kg of glucose) was performed prior to euthanasia after an overnight fast. Serum creatinine was measured using an autoanalyzer (Hitachi 917, Tokyo) and a commercial kit (Wako, Osaka, Japan).

Echocardiographic studies

Echocardiography was performed using the Vivid Q ultrasound system (GE Medical Systems, Horten, Norway) at age 23-24 weeks. Briefly, rats (n=3-4/group) were anesthetized with 1% isoflurane and secured to an imaging platform. Complete 2-dimensional (2-D), M-mode, and Doppler examinations were performed using a 12S-RS 12MHz transducer to quantify left ventricular dimensions and diastolic and systolic functions. Standard 2-D measurements (LV diastolic and systolic dimensions, ventricular septum and posterior wall thicknesses, and left atrial volume) were obtained as recommended by the American Society of Echocardiography. All dimensional measurements were indexed with respect to body weight.

Histologic examinations

Rats were sacrificed by cardiac puncture under anaesthesia. White adipose tissues (WATs), hearts, and aortas were collected and fixed in 4% paraformaldehyde (Biossesang, South Korea). Remaining tissues were immediately frozen in liquid

nitrogen. Paraffin blocks were prepared and stained with haematoxylin and eosin (H&E), oil red O (ORO), and CD68 (Abcam, USA). Slides were scanned under a photomicroscope (Axioskop 40, Carl Zeiss, Germany), and adipocyte size distributions were determined using the Adiposoft program.

Triglyceride and lipolysis measurements

Heart and white adipose tissue triglyceride (TG) and glycerol levels were determined (Thermo Fisher Scientific and Sigma). Data were normalized with respect to protein concentration and results are reported as μg per mg of protein.

Real-time polymerase chain reaction (PCR) quantification

Total RNA was isolated from hearts and white adipose tissues, and cDNA was synthesized using a reverse transcriptase kit (Thermo Fisher Scientific, USA). Primers used for qRT-PCR were obtained from Bioneer (South Korea) (Table 1).

To determine the molecular modes of action of empagliflozin and lixisenatide, we assessed the expressions of the following genes in cardiac and adipose tissues by qPCR:- oxidative stress related genes (BAX, CASP3, SOD1, SOD2, NOX4, GPX1, GPX3, XBP1, and CHOP), mitochondria related genes (COX2, DRP1, MFN1, CYTC, NRF2, TFAM, ATPsyn, and FIS1), ketolysis related genes (MCT1, BDH1, and SCOT), fatty acid oxidation related genes (PGC1α, CPT1α, CPT1β, and CPT2, inflammation related genes (ICAM, VCAM, IL-1β, NLRP3, IL-6, TNFα, MMP9, MCP1, p65, IL-8, IL-18, and HO-1), and lipase related genes - G0s2-1, G0s2-2, CGI58-1, CGI58-2, CGI58-3, ATGL(adipose triglyceride lipase)1, ATGL2, ATGL3, HSL (hormone

sensitive lipase)-1, HSL-2, and HSL-3.

Table 1. Primer sequences used for qPCR

Primers	Forward (5'--> 3')	Reverse (3' --> 5')
SOD2	CGGGGGCCATATCAATCACA	GCCTCCAGCAACTCTCCTTT
SOD1	TAACTGAAGGCGAGCATGGG	CCTCTCTTCATCCGCTGGAC
NOX4	GTCTGCTTGTTTGGCTGTCC	ACACAATCCTAGGCCCAACA
GPX1	CCTGGTATCTGGGCTTGGTG	TTAGGCGTAAAGGCATCGGG
GPX3	AACCCAGGGACAGACCATCT	GCTCATTTGGAACGGGAAGC
CASP3	GGAGCTTGGAACGCGAAGAA	ACACAAGCCCATTTCAGGGT
BAX	GGGCCTTTTTGCTACAGGGT	TTCTTGGTGGATGCGTCCTG
PGC1α	CATGTGCAGCCAAGACTCTG	GTGAGGACCGCTAGCAAGTT
CPT1α	TTATCGTGGTGGTGGGTGTG	CCCAGAGCCCTGTACCAAAG
CPT1β	TCGAGTTCAGAAACGAACGC	GGTCGGCGATATCCAACAGT
CPT2	GACTGTTTCTGCAGTCCGGT	CAGGGGATATGCGTTGACCA
MCT1	ACTGGTCGGTCGTGTAGGTG	TTCCAAGATGCGGCGAAAAC
BDH1	CCGGAGAGGGTCTTGAGAAAC	GACAGGGGTCTGGAAAGACG
SCOT	GTGCAAGAGGGAGGATCACC	AGCAAAGTCTCCCGTGATGG
ATPsyn	ATGGCACTGAAGGCTTGGTT	TCCGATTTTCCCACCCTTGG
TFAM	CCAAAAAGACCTCGGTCAGC	GTGACTCATCCTTAGCCCCC
NRF2	CAGCATGATGGACTTGGAATTG	GCAAGCGACTCATGGTCATC
CYTC	CATGCCTGATTGGGAGTGGT	AGTGCTTTCACTAGACCACGG
COX2	TGTGAAAGGGTGTCCCTTCG	AGTACAACACAGGAATCTTCACA
MFN1	TGACTTGGACTACTCGTGCG	GTGGCCATTTCTTGCTGGAC
DRP1	GCTCAGTGCTGGAAAGCCTA	ACTCCATTTTCTTCTCCTGTTGT
FIS1	ACTTCTTCTACCCGGAGGCT	CTCTACAGGCACTTTGGGGG

VCAM	CACTACGCCTTCACTCAGCA	TCGGGCGATCATTCGATCTG
HO-1	TTAAGCTGGTGATGGCCTCC	GTGGGGCATAGACTGGGTTC
IL-8	GAAGATAGATTGCACCGATG	CATAGCCTCTCACACACATTTC
β-actin	GAGATTACTGCCCTGGCTCC	AAACGCAGCTCAGTAACAGTC
ICAM	GCTCTCAGTAGTGCTGCTCC	GCCTGACCTCGGAGACATTC
MCP1	GGGCCTGTTGTTCACAGTTG	AGCTTCTTTGGGACACCTGC
TNFα	TGGGCTTTCGGAACTCACTG	CTGTGCCTCAGGGAACAGTC
IL-6	GTGGCTAAGGACCAAGACCA	TAGCACACTAGGTTTGCCGAG
IL-1β	GACTTCACCATGGAACCCGT	GGAGACTGCCCATTCTCGAC
MMP9	GCTATGGTTACACTCGGGCA	TGGCCTTTAGTGTCTCGCTG
p65	GAGACTGCGATGCAGCACTA	CCTCTTTTCGTCTCCCGCAT
IL-18	AACCGCAGTAATACGGAGCA	TCTGGGATTCGTTGGCTGTT
NLRP3	CTGCAAGATCCTGTGCAAGC	AGGCAGCAACTTCTTTCGGA
ATGL1	CTCACCTGTGCCTTACCGTT	AAAGTGTCTGCTGCCCAAGT
ATGL2	ACCCCGCACTAAAACACCTC	ATGTGGTAGACCCCGAGGAA
ATGL3	GAGCCCTGGGGTGGAATAAG	TGTGAGTGGCTGGTGAAAGG
HSL1	CATTCACGGTGGTGGCTTTG	TCCGGTTGAACCAAGCAGTT
HSL2	GACACGTCCCTGTTCCTCAG	ACACACTCCTGCGCATAGAC
HSL3	CTCTTTACGGGTGGCCGATT	CCATGTTGGCCAGAGACGAT
CGI58-1	AATTGCTATCCTCGGGGCAG	GCTCAGTCTACTGTGTGGCA
CGI58-2	GACGATTCCTTACGGGTGGG	CACGTATGACTTGGGTCGCA
CGI58-3	TGCAGGACCTTTTGGGTTGA	CCACCCGTAAGGAATCGTCA
G0s2-1	AGACACAGGAGGCGACTTTG	GCAGCAAGTCAGTCCCAGAT
G0s2-2	CTAGCGTTCTTCGGTGTGGT	AAGTCGCCTCCTGTGTCTTG

Western blot analysis

Rat hearts were lysed in protein lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Tween-20, 0.2% NP-40, 1 mM PMSF, 1 mM NaF, 1 mM Na3VO4 and protease inhibitor mixture) (Cell Signaling, USA). Proteins in lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore), which were blocked with 1% BSA and probed with anti-pERK1/2 (Thr202/Tyr204), anti-p38 MAPK (Thr180/Tyr182), anti-NFκB (Ser563) and anti-pJNK (Thr183/Tyr185) (all from Cell Signaling Technology) or anti-βActin (Sigma).

Statistical analysis

Results are expressed as the means ± SDs of at least two independent experiments. Kruskal-Wallis analysis was used to compare groups, and Mann-Whitney U test was used to compare differences between two groups. *P* values of < 0.05 were deemed significant. However, it was difficult to achieve statistical significance in most analyses due to small sample size. For this reason, *P* values of < 0.1 was expressed as marginally significant. The analysis was conducted using Graphpad Prism and SPSS ver. 23.0 (SPSS, Chicago, IL).

RESULTS

Effects of empagliflozin and lixisenatide on body weight and glucose tolerance in OLETF rats

At baseline, mean body weight of OL-E group was lower than those of OL-C and OL-G groups. After 12-week treatment, mean body weights of OL-E (empagliflozin, a SGLT2 inhibitor) and OL-G (lixisenatide, a GLP-1RA) groups were significantly lower than those of LT and OL-C group ($p < 0.05$). . OL-E and OL-G groups showed no significant difference in body weight at 6 or 12 weeks of treatment (Fig 1). Non-fasting blood glucose levels in the OL-E group were significantly lower than in the OL-C or OL-G groups ($p < 0.05$), and levels were not significantly different in OL-G and OL-C groups (Fig 2A). IPGTT results after 12 weeks of treatment showed that glucose values from 30 to 120 minutes after glucose loading were significantly lower in the OL-E and OL-G groups than in the OL-C group ($p < 0.05$, Fig 2B).

Figure 1. Body weight change

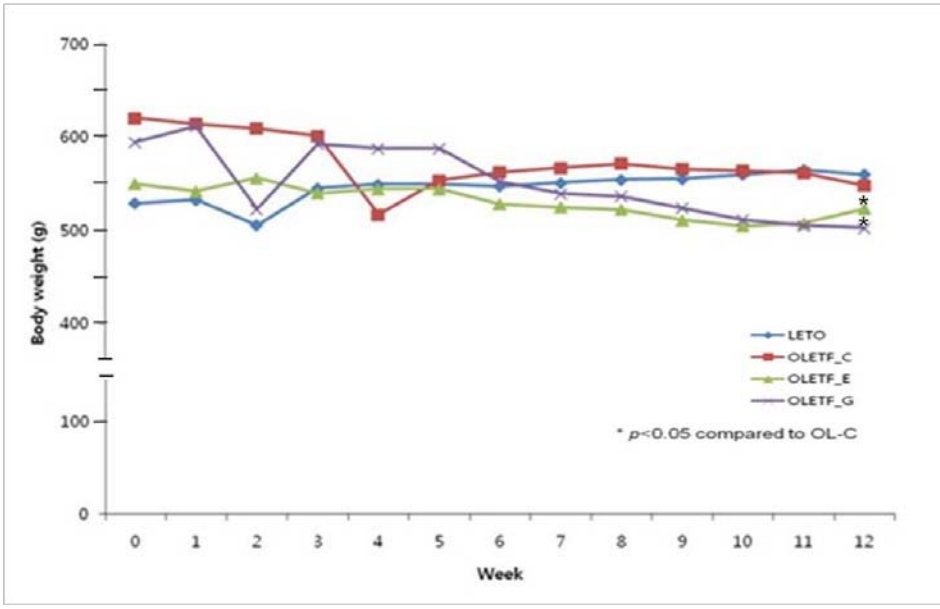
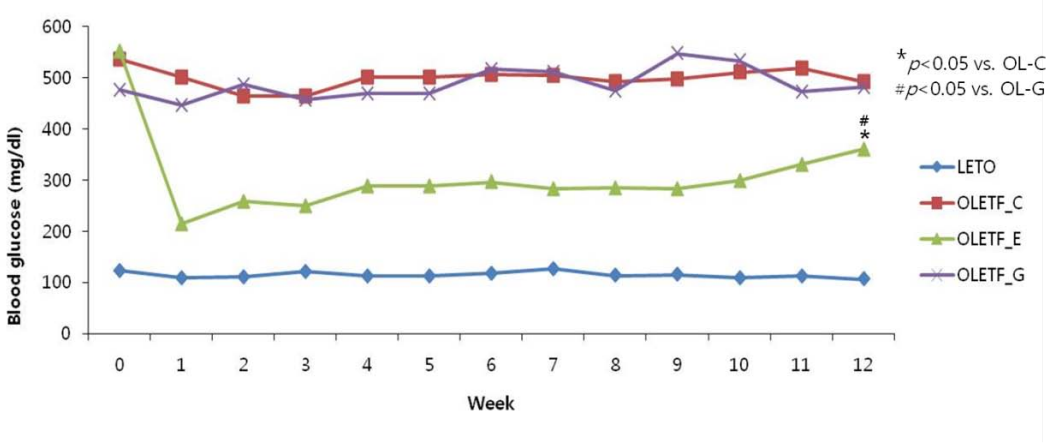
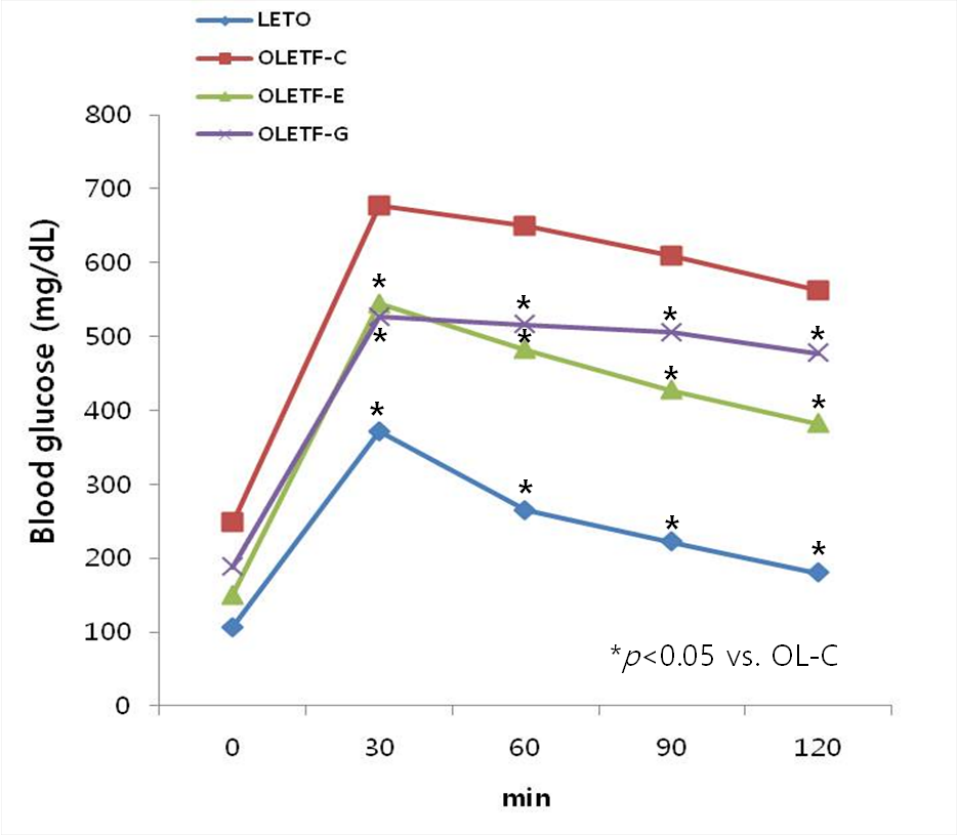


Figure 2. Blood glucose and intraperitoneal glucose tolerance test

A. Blood glucose level



B. Intraperitoneal glucose tolerance test



Effects of empagliflozin and lixisenatide on biochemical parameters

Blood AST, ALT, total cholesterol, TG, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and free fatty acid (FFA) levels were measured after 12 weeks of treatment. All lipid profiles were significantly higher in the OL-C group than in the LT group. TG and FFA levels were suppressed by empagliflozin and lixisenatide. Lipid profiles were no different in the OL-E and OL-G groups (Table 2).

Table 2. Lipid, liver function and renal function profiles

	LT (n=3)	OL-C (n=3)	OL-E (n=4)	OL-G (n=4)
AST (IU/L)	75.7±11.0	63.7±6.4	60.5±6.0	58.5±14.3
ALT (IU/L)	40.3±3.5	58.0±6.1	56.5±9.0	61.8±11.7
Creatinine (mg/dL)	0.42±0.07	0.32±0.04	0.32±0.03	0.31±0.01
Total Cholesterol (mg/dL)	111.7±7.0	177.3±11.0[a]	180.3±27.2	174±31.0
TG (mg/dL)	58.0±6.6	146.7±45.7[a]	124.8±30.1	110.3±9.6
HDL (mg/dL)	20.0±2.0	30.0±2.6[a]	32.5±7.6	36.0±11.5
LDL (mg/dL)	109.6±5.8	156.3±18.4[a]	166.4±22.3	161.5±30.3
FFA (mg/dL)	296.7±28.7	683.0±313.8[a]	560.5±34.0	625.8±129.5

a, $p < 0.05$ vs. LT

Cardiac Structure and Function

Group echocardiographic parameters are summarized in Table 3. E (p=0.13), A (p=0.18), E/A (p=0.01), and DT (p=0.015) were significantly between all groups by Kruskal-Wallis analysis. Unfortunately, *post hoc* analysis was not possible because of small sample sizes. However, Mann-Whitney analysis showed the LT and OL-C groups were significantly different with respect to E (p=0.019) and E/A ($p < 0.0001$), that the LT and OL-E groups were significantly different with respect to A (p=0.011) and E/A (p=0.001), and that the LT and OL-G groups were significantly different with respect to E (p=0.007) and E/A (p=0.008). However, E/A of OL-E and OL-G were not significantly different from OL-C. Furthermore, A was significantly higher (p=0.022) and DT was significantly lower (p=0.026) in the LT group than in the control group. And also there were significant differences in LVIDd index (p=0.042) and LVIDs index (p=0.022), and A (p=0.020) was significantly higher lower in the OL-E group than in the OL-G group (Table 3 and Fig 3).

Table 3. Echocardiographic measurements

	LT (n=3)	OL-C (n=3)	OL-E (n=4)	OL-G (n=4)
Structural				
LVPWId, mm/kg	3.37±0.7	3.05±0.4	3.18±0.6	3.18±0.5
LVPWIs, mm/kg	4.26±0.5	4.29±0.7	4.15±0.9	4.06±0.2
IVSId, mm/kg	3.01±0.4	3.31±0.5	2.93±0.6	3.04±0.3
IVSIs, mm/kg	4.57±0.6	4.65±0.7	4.12±0.5	4.41±0.3
LVIDId, mm/kg	13.4±1.4	14.65±1.7	13.89±2.2	15.6±2.9[c]
LVIDIs, mm/kg	7.15±0.9	7.49±1.4	7.48±1.4	8.53±2.1[c]
LVMI, mg/g	2.46±0.2	2.67±0.3	2.44±0.4	2.63±0.4
Functional				
FS, %	46.5±5.9	49.18±4.4	47.41±1.7	46.96±4.8
EF(Teich), %	82.3±5.5	84.74±3.9	83.53±1.6	82.78±4.1
SV(Teich), ml	0.8±0.2	1.02±0.3	0.9±0.2	0.96±0.2
EDV(Teich), ml	0.96±0.3	1.22±0.4	1.08±0.2	1.13±0.3
ESV(Teich), ml	0.17±0.1	0.2±0.1	0.18±0.1	0.22±0.1
E[a], m/s	0.76±0.2	0.6±0.1[b]	0.66±0.1	0.58±0.1[b]
A[a], m/s	0.38±0.1	0.5±0.1[b]	0.55±0.1[b]	0.43±0.1[c]
E/A[a]	2.14±0.8	1.22±0.1[b]	1.21±0.2[b]	1.36±0.2[b]
DT[a], ms	63.0±15.4	60.59±6.3[b]	69.21±12.7	57.98±5.5

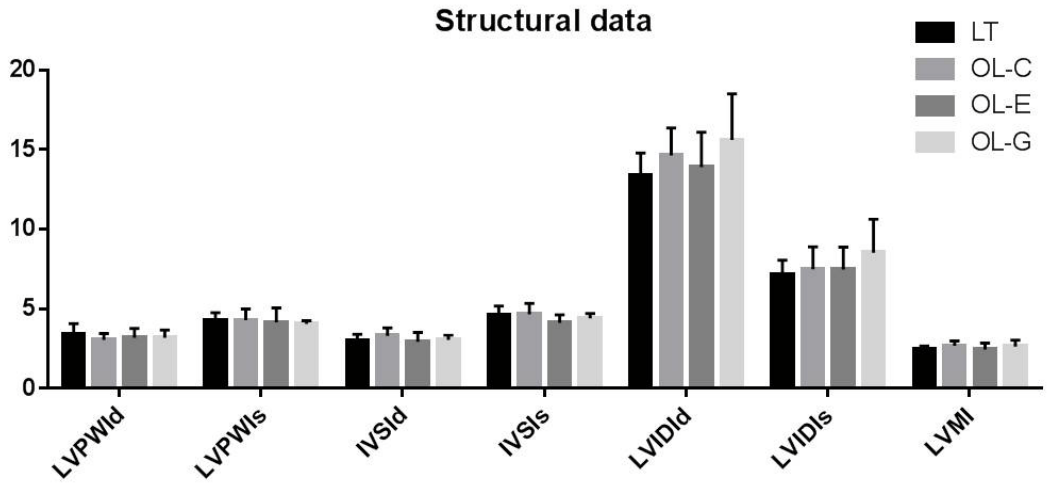
LVPWId, left ventricular posterior wall thickness during diastole;LVPWIs, left ventricular posterior wall thickness during systole;IVSId, interventricular septal wall thickness during diastole;IVSIs, interventricular septal wall thickness during systole;LVIDId, left ventricular internal dimension during diastole;LVIDIs, left ventricular internal dimension during systole;LVMI, left ventricular mass index;FS, fractional shortening;EF, ejection fraction;EDV,

left ventricular end-diastolic volume;ESV, left ventricular end-systolic volume;E, mitral inflow E wave velocity;A, mitral inflow A wave velocity;DT, mitral inflow E wave deceleration time.

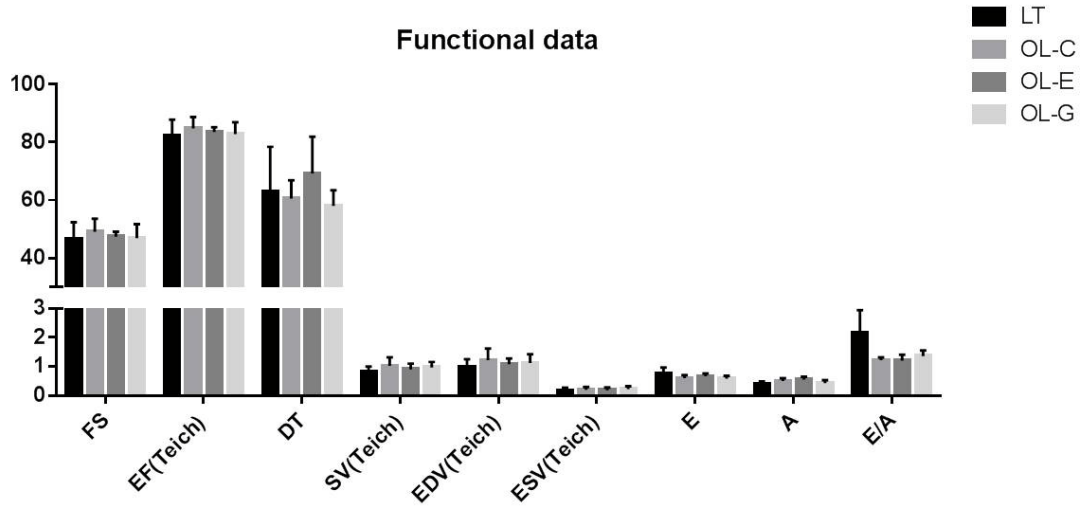
a. $p < 0.05$ by ANOVA; b. $p < 0.05$ vs. LT by Mann-Whitney analysis; c. $p < 0.05$ vs. OL-E by Mann-Whitney analysis

Figure 3. Echocardiographic measurements

A. Structural Parameters



B. Functional Parameters



LVPWId, left ventricular posterior wall thickness during diastole;LVPWIs, left ventricular posterior wall thickness during systole;IVSId, interventricular septal wall thickness during diastole;IVSIs, interventricular septal wall thickness during systole;LVIDId, left ventricular internal dimension during diastole;LVIDIs, left ventricular internal dimension during systole;LVMI, left ventricular mass index;FS, fractional shortening;EF, ejection fraction;EDV, left ventricular end-diastolic volume;ESV, left ventricular end-systolic volume;E, mitral inflow E wave velocity;A, mitral inflow A wave velocity;DT, mitral inflow E wave deceleration time.

Oil-Red O staining of Aortas

Oil red O staining of thoracic aortas was used to identify atherosclerosis. The de-staining was not good and the vessels were stained as a whole. However, no gross and microscopic atherosclerotic plaque was observed in any of the four groups (Fig 4 and 5).

Figure 4. Aorta en face Oil-Red O staining

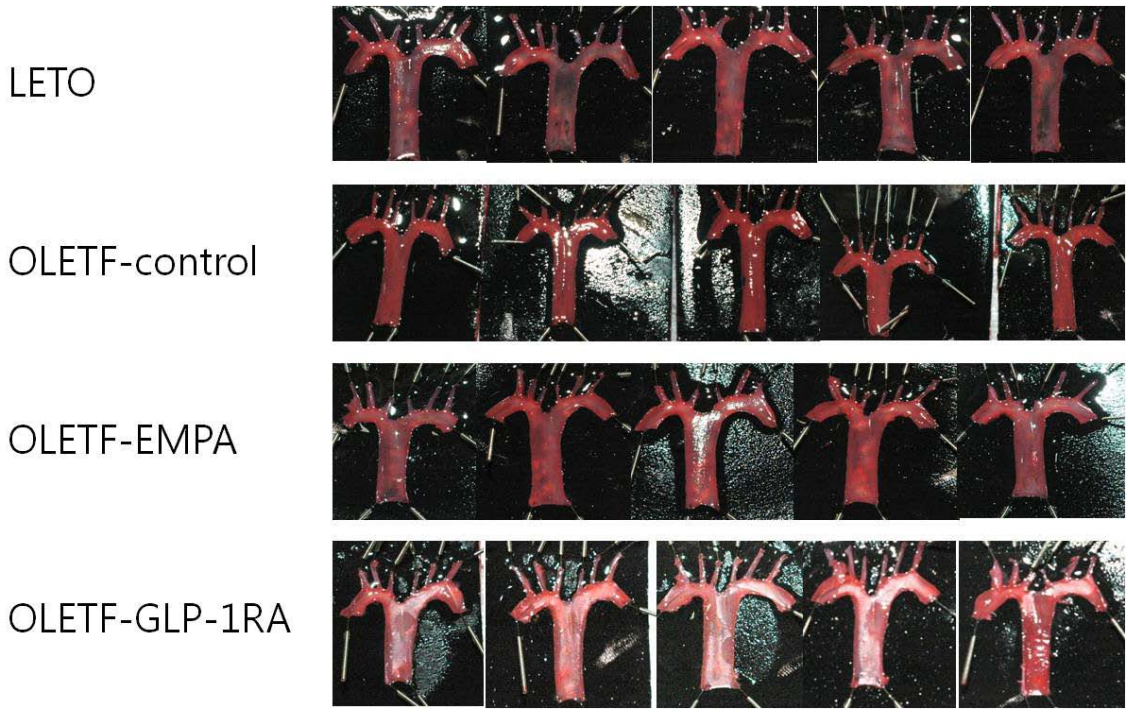


Figure 5. Microscopic atherosclerotic change of aorta

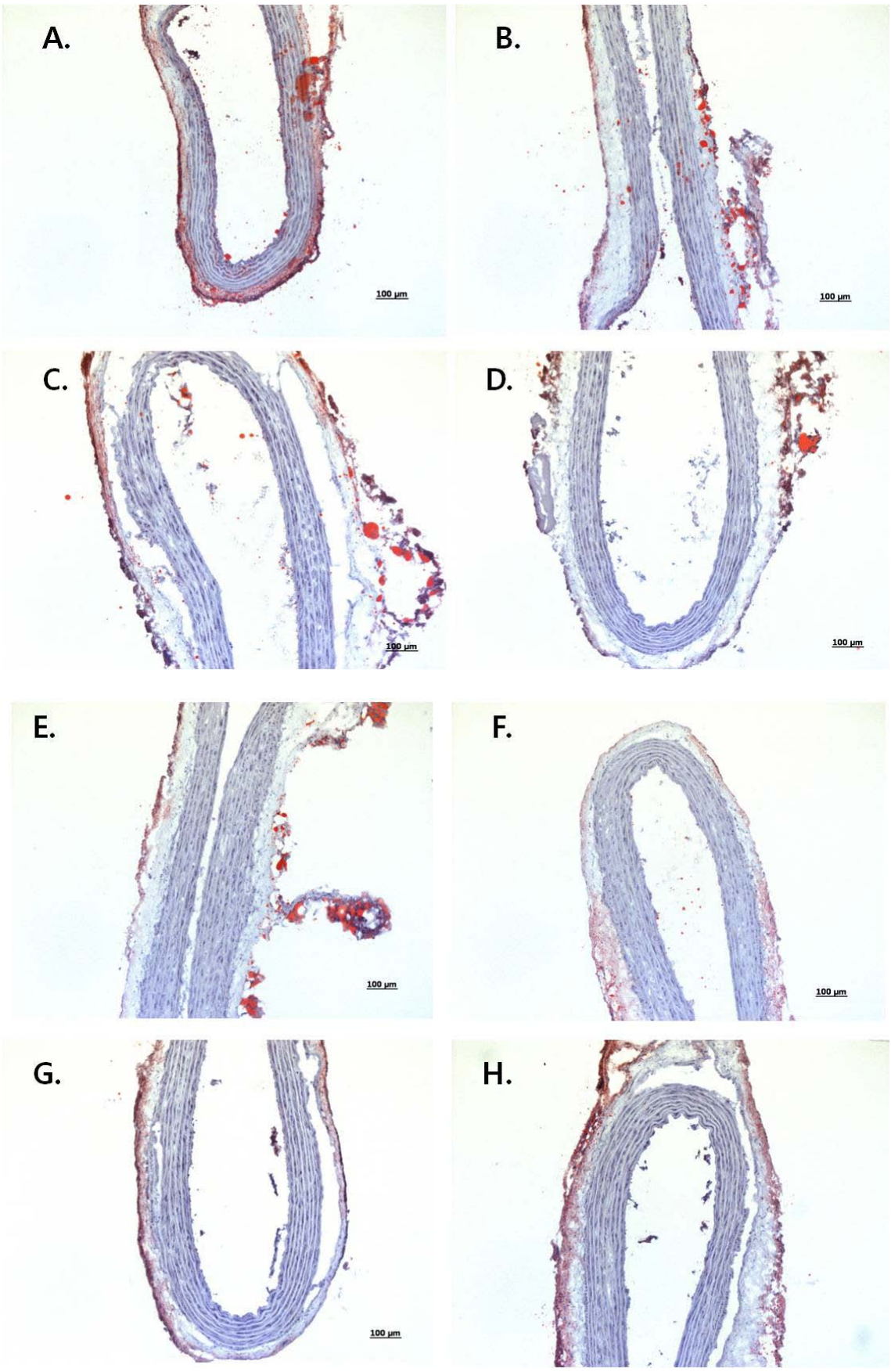
Oil-Red-O staining was done to determine microscopic atherosclerotic change in aorta.

A and B. LETO (x100)

C and D. OLETF – control (x100)

E and F. OLETF – empagliflozin (x100)

G and H. OLETF – lixisenatide (x100)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B.
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H.

Histologic analysis

H&E staining was performed to identify histological differences in heart and adipose tissues. Cardiac tissues did not show significant differences among the four groups. No lipid droplets were observed in cardiac tissues and no other histological changes such as interstitial fibrosis and macrophage infiltration were observed (Fig 6A-6H). CD68 staining also failed to reveal any intergroup difference (Fig 6I-6P).

Figure 6. Histology of cardiac tissues

H&E staining was performed to identify histological differences in heart. Cardiac tissues did not show significant differences among the four groups. There were no lipid droplets in cardiac tissues and no other histological changes such as interstitial fibrosis and macrophage infiltration were observed in all groups. In addition, CD68 stain showed no differences, either.

A and B. LETO, H&E (x200)

C and D. OLETF – control, H&E (x200)

E and F. OLETF – empagliflozin, H&E (x200)

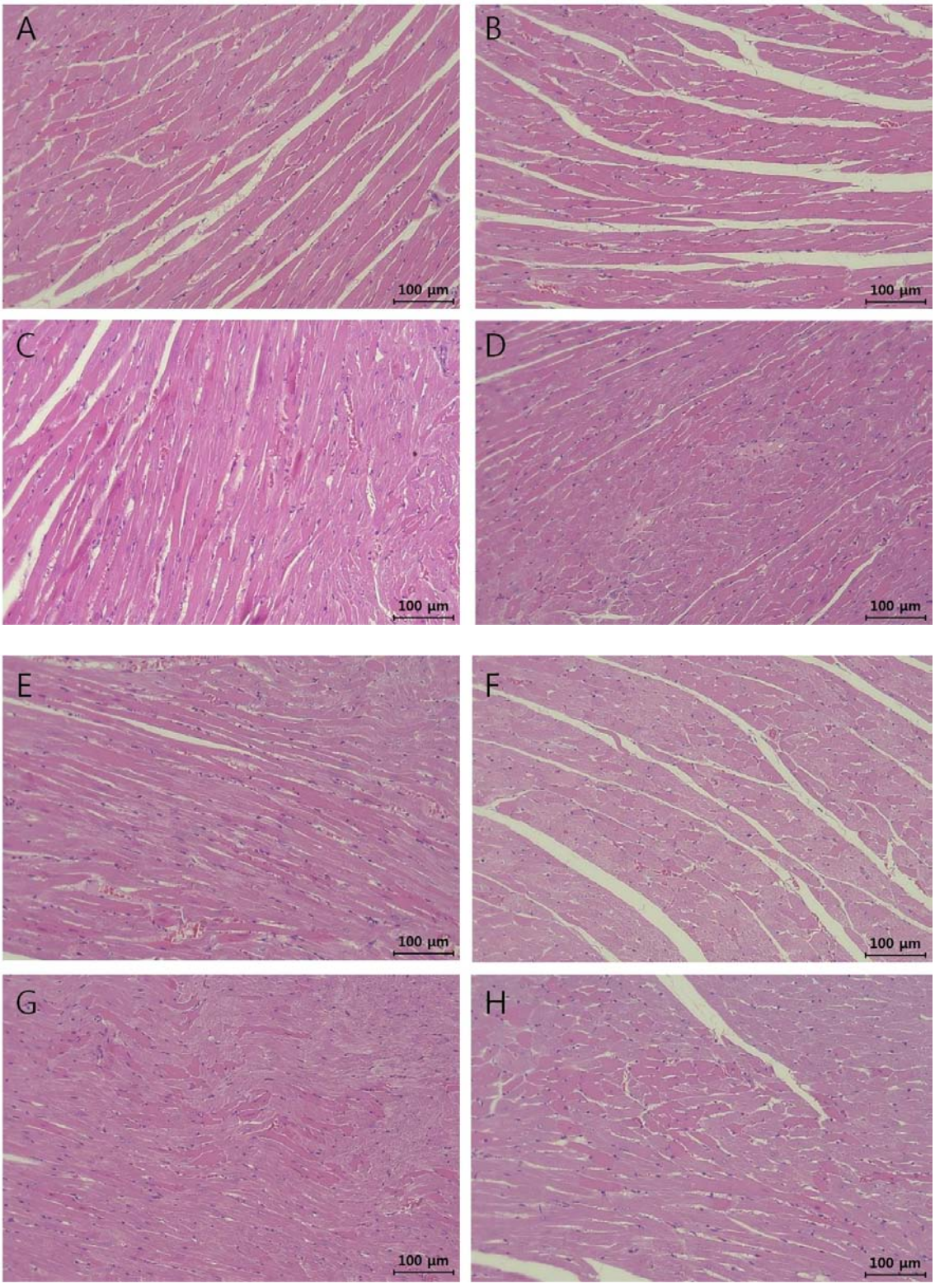
G and H. OLETF – lixisenatide, H&E (x200)

I and J. LETO, CD68 (x100)

K and L. OLETF – control, CD68 (x100)

M and N. OLETF – empagliflozin, CD68 (x100)

O and P. OLETF – lixisenatide, CD68 (x100)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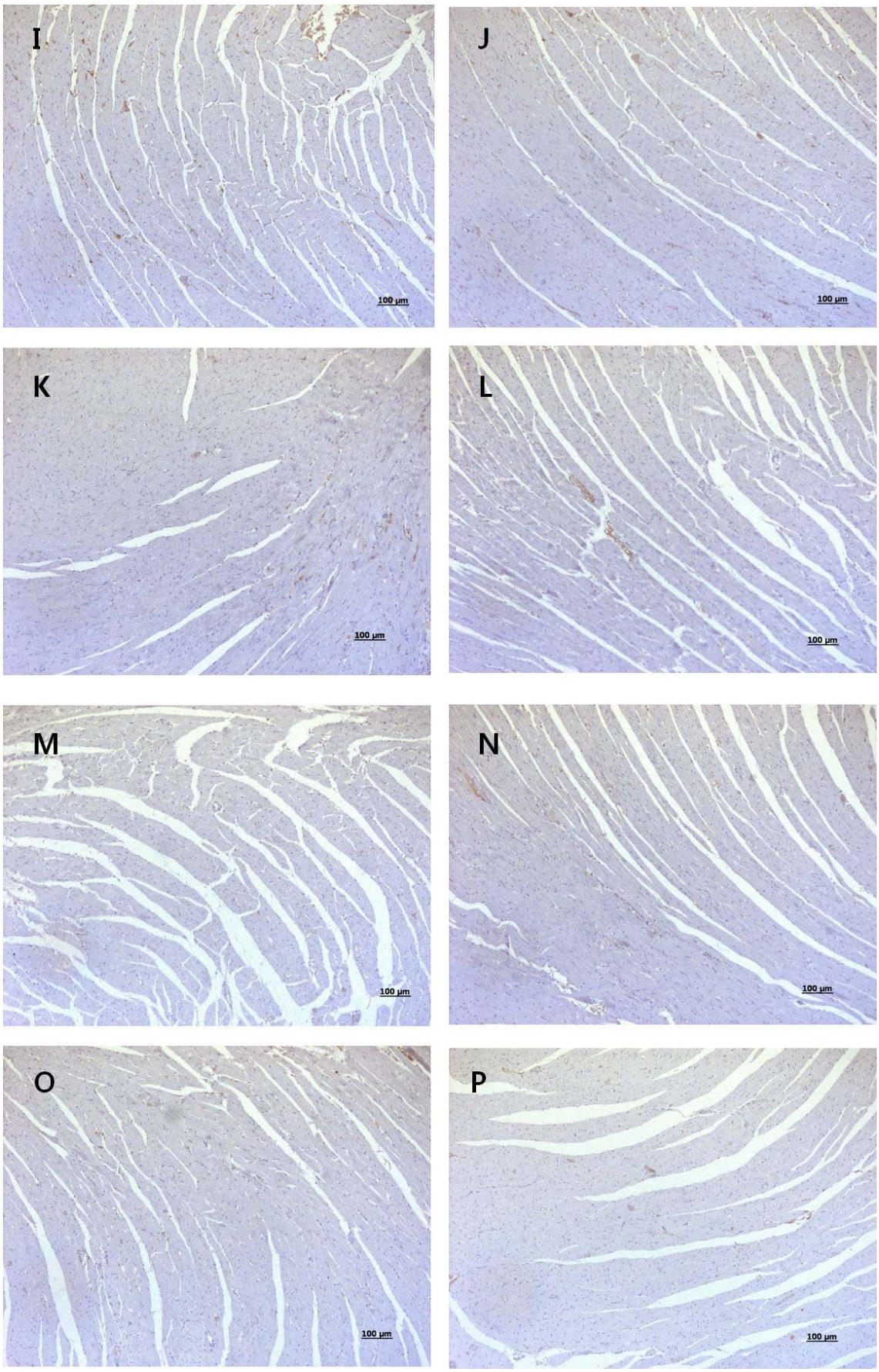
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K
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By contrast, histologic examination revealed some differences between adipose tissues, in which the presence of dead adipocytes is a hallmark of obesity. Macrophages surrounding dying or dead adipocytes form crown-like structures (CLSs) and these were observed by H&E staining in the OL-C group. CLS numbers were lower in the OL-E group than in the OL-C group. In the OL-G group, CLS numbers were slightly lower and lipid droplet sizes were smaller than in OL-C group (Fig 7A-7H). CD68 and H&E staining showed macrophage infiltration was reduced in the OL-E group as well as OL-G group compared to the OL-C group (Fig 7I-7P).

Figure 7. Histology of adipose tissues

There were some differences in adipose tissues histologic exam. Crown like structures (CLSs, index) were identified in OL-C, which was significantly reduced in OL-E group. In OL-G group, CLSs decreased slightly and lipid droplet size decreased. In addition, CD68 stating revealed the similar results.

A and B. LETO (x100)

C and D. OLETF – control (x100)

E and F. OLETF – empagliflozin (x100)

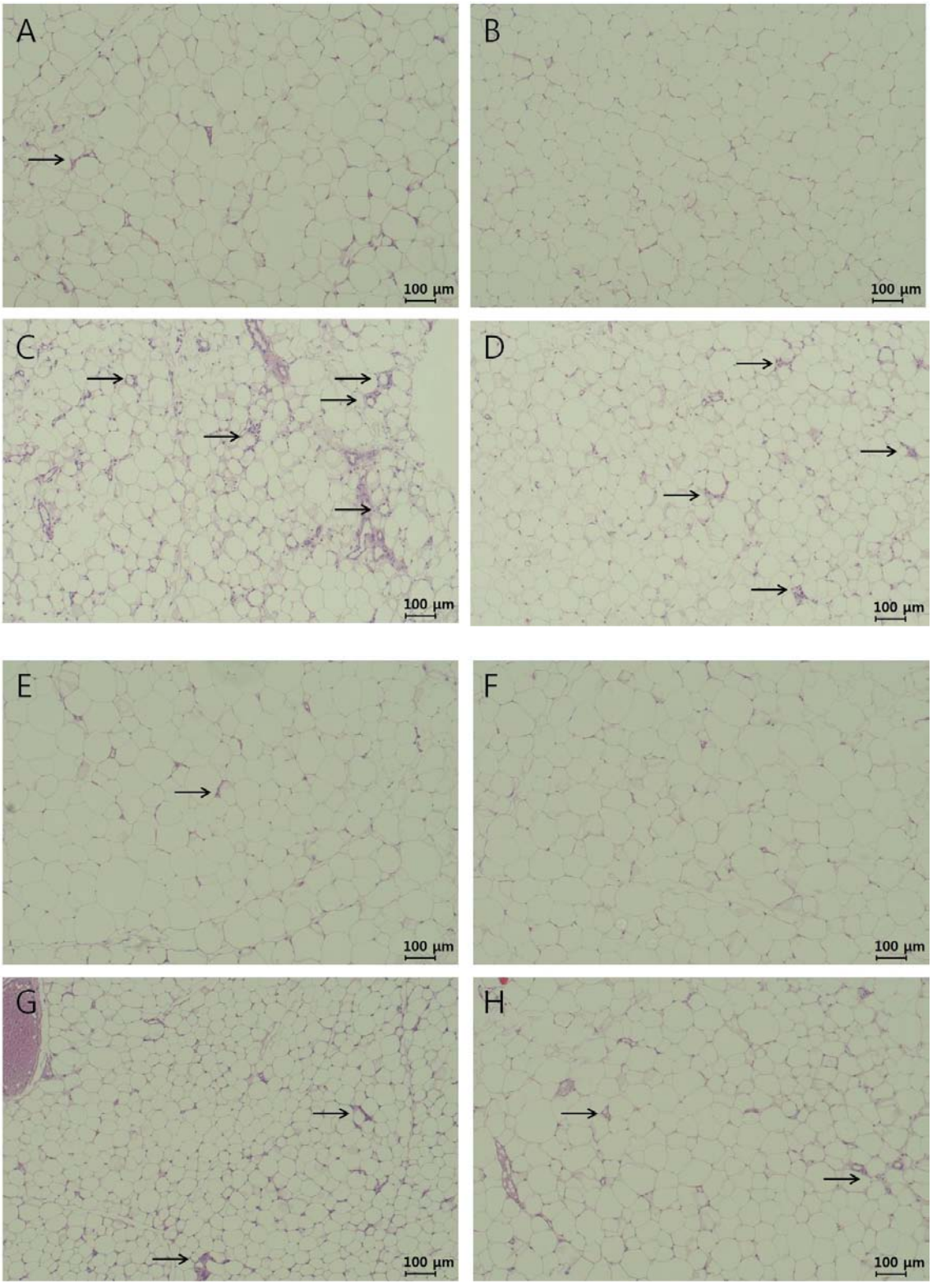
G and H. OLETF – lixisenatide (x100)

I and J. LETO, CD68 (x100)

K and L. OLETF – control, CD68 (x100)

M and N. OLETF – empagliflozin, CD68 (x100)

O and P. OLETF – lixisenatide, CD68 (x100)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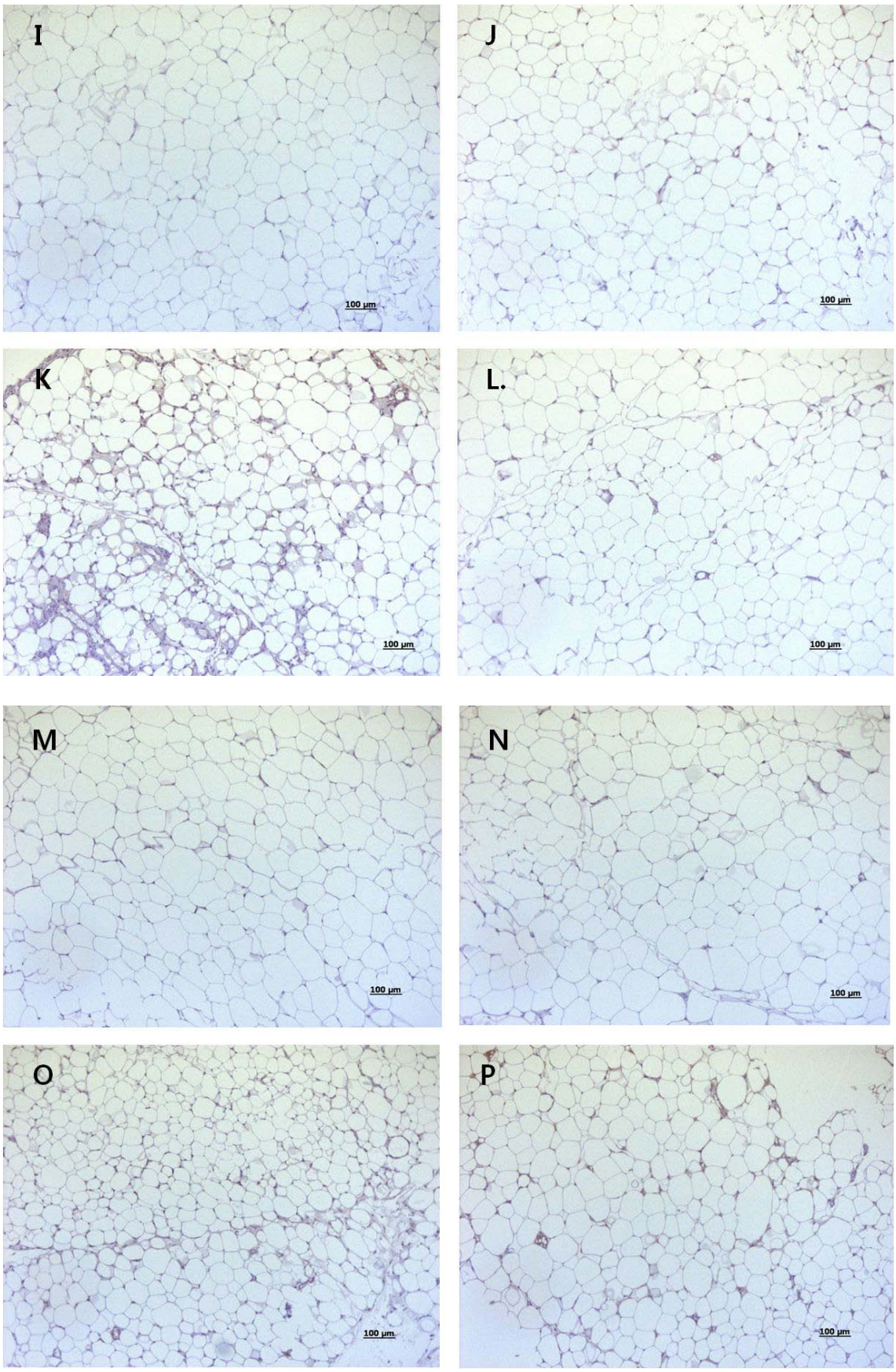
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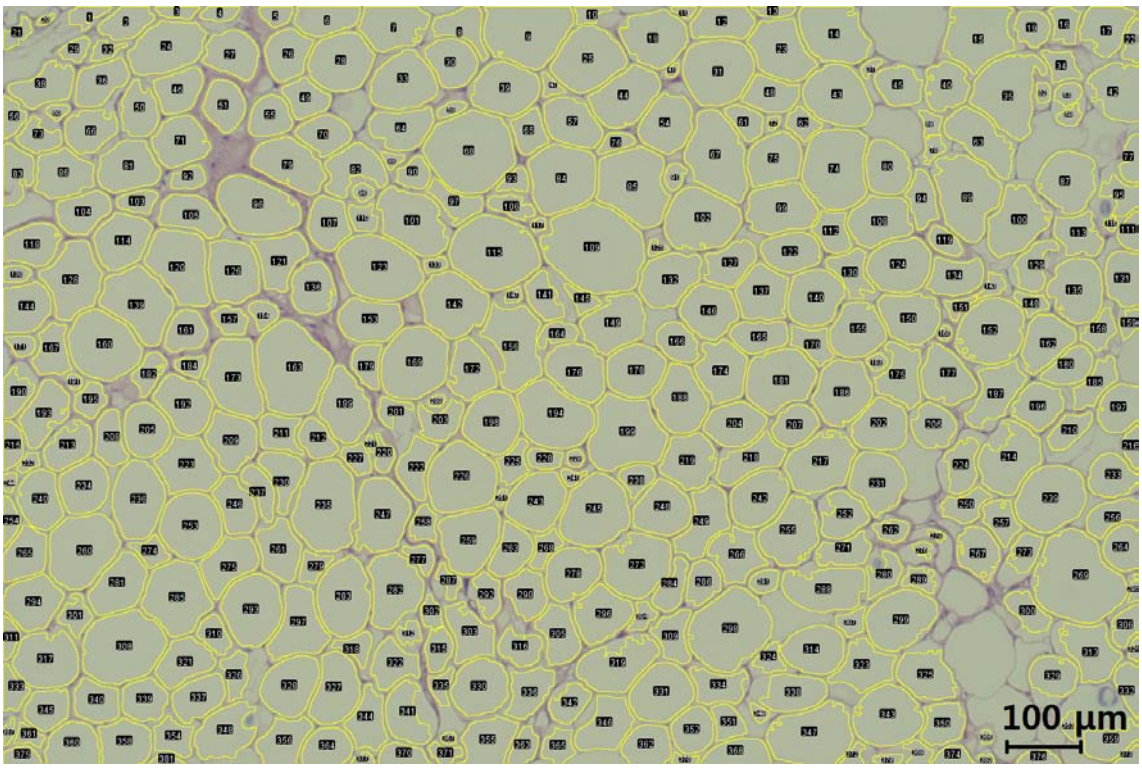
Adipocyte sizes were measured in six photographs per group using the Adiposoft program. In the LT group, ~35% of adipocytes had a diameter of < 15 µm, ~ 44% a diameter between 15 and 25 µm, and ~21% had a diameter > 25 µm. In the OL-C group, ~46% had a diameter of < 15 µm, ~40% a diameter of 15-25 µm, and ~14% a diameter > 25 µm. In the OL-E group, ~39% had a diameter < 15 µm, ~33% a diameter of 15-25 µm, and ~28% a diameter of > 25 µm. In the OL-G group, ~63% had a diameter of < 15 µm, ~ 33% a diameter of 15-25 µm, and ~4% a diameter > 25 µm (Table 4). Summarizing, adipocytes were smaller in the OL-C group than in the LT group, empagliflozin increased adipocyte sizes, but lixisenatide reduced adipocyte sizes (Fig 8A-8E).

Table 4. Adipocyte size distribution

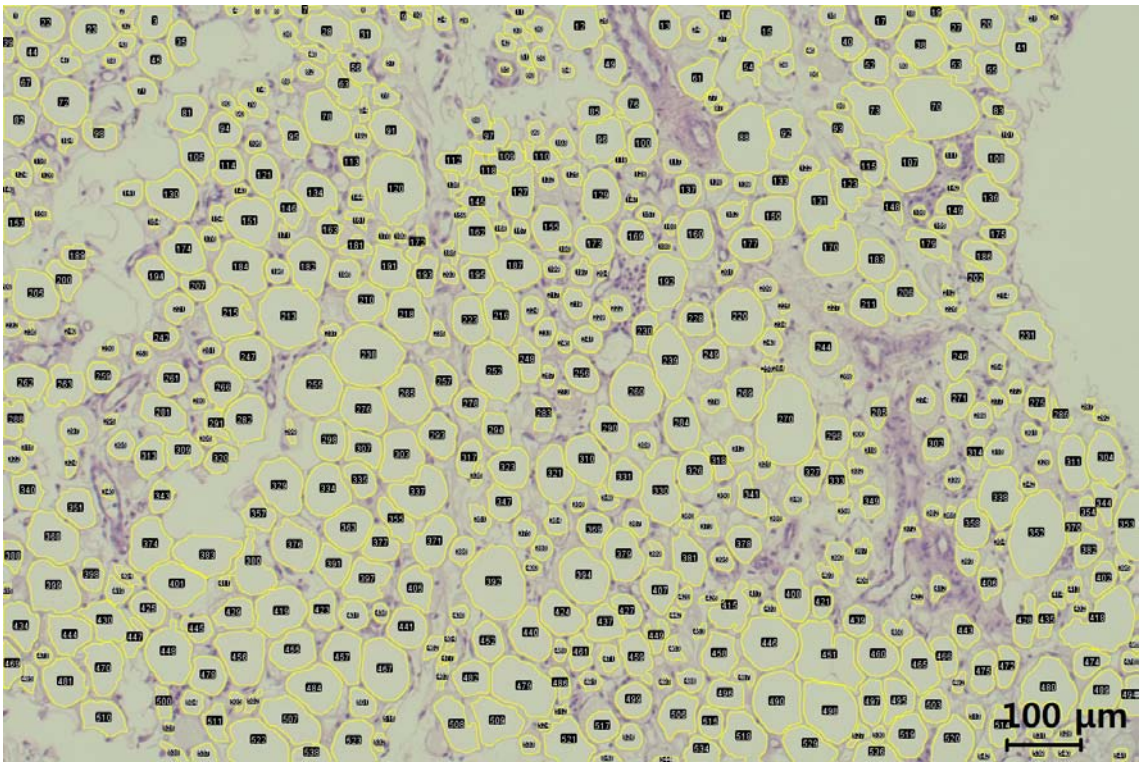
	radius (µm)									total
	r<10	10≤r<15	15≤r<20	20≤r<25	25≤r<30	30≤r<35	35≤r<40	40≤r<45	45≤r	
LETO	17.94	17.27	20.52	23.18	14.32	5.49	1.04	0.17	0.08	100
OL-C	23.64	22.93	22.57	17.43	8.36	3.36	1.25	0.43	0.04	100
OL-E	19.92	19.46	21.39	18.88	12.01	5.27	1.42	1.05	0.59	100
OL-G	28.63	33.87	23.86	9.62	2.71	0.93	0.27	0.07	0.02	100

Figure 8. Adipocyte size measurement

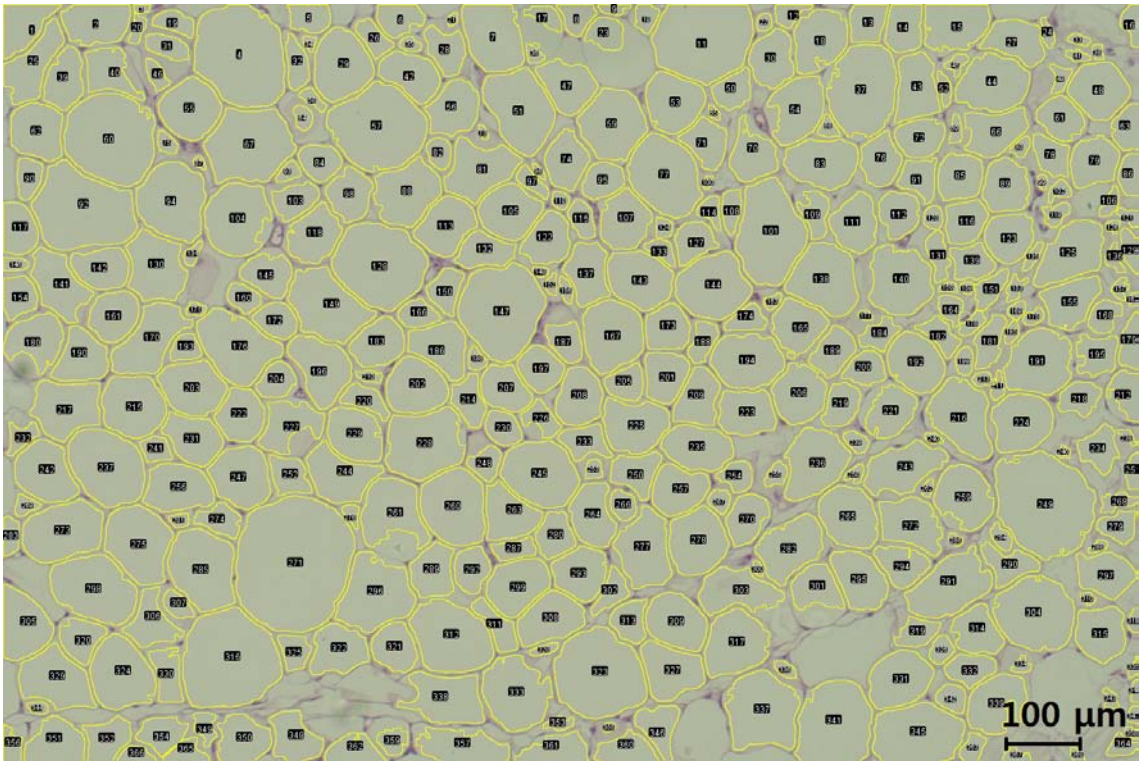
A. LETO



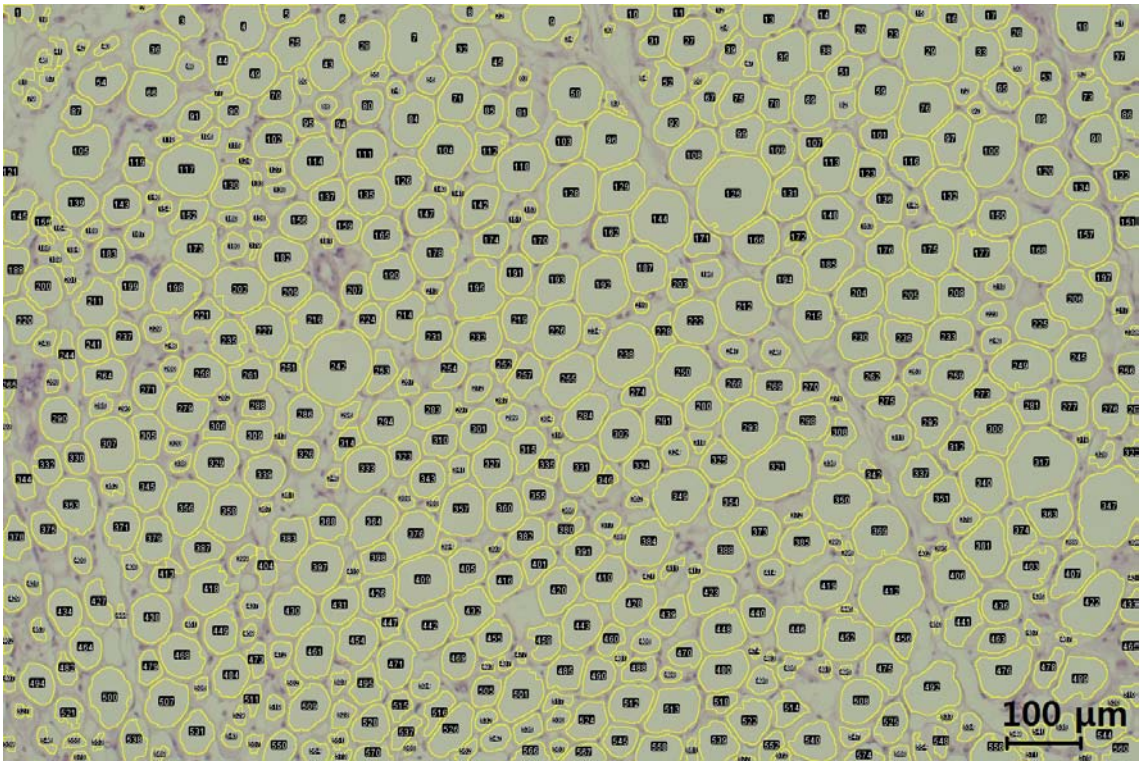
B. OL-C



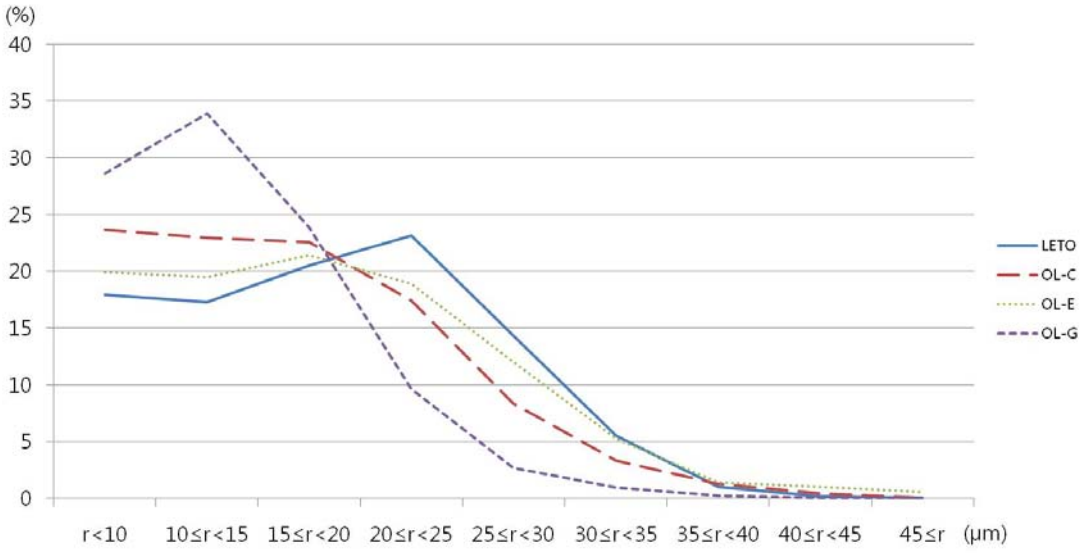
C. OL-E



D.OL-G



E. Comparison of adipocyte size distribution among 4 groups



Tissue triglyceride accumulation, lipolysis, and related gene expressions

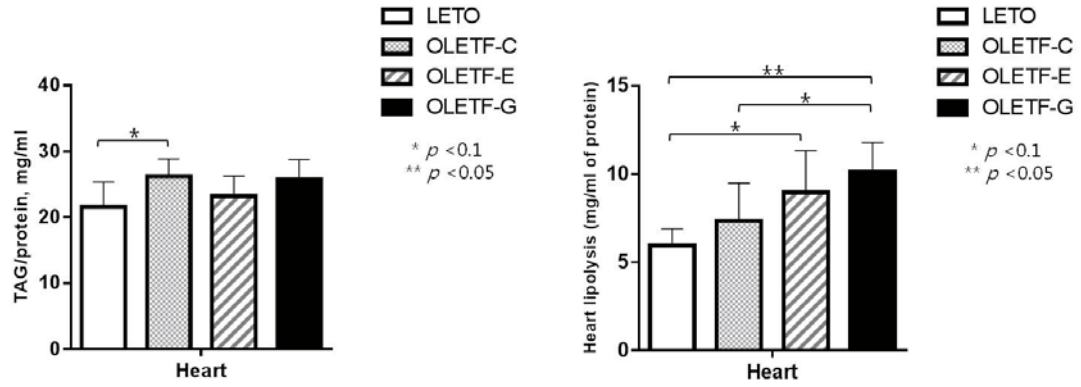
More TG accumulated in cardiac tissue in OL-C group than in the LT group ($p < 0.1$). TG accumulation was not significantly decreased in OL-E and OL-G groups compared to OL-C group, however, lipolysis was increased in OL-G group compared to OL-C group ($p < 0.1$, Fig 9A-a). Among lipase related genes, ATGL3 expression was significantly increased in OL-G group ($p < 0.05$), while it was decreased with marginal significance in OL-E group ($p < 0.1$) compared to OL-C group. Increase in lipolysis by lixisenatide in cardiac tissues seemed to be associated with increased lipase gene expression, especially ATGL3. On the other hand, lipase co-factor related genes were not significantly affected (Fig 9A-b). The expressions of fatty acid oxidation related genes were not significantly different between OL-E and OL-G groups (Fig 9A-c).

No significant intergroup differences in TG accumulation or lipolysis was observed in adipose tissues (Fig 9B-a). Lipase related gene expressions, which are increased in OL-C group compared to LT group, tended to be decreased by lixisenatide treatment, but without statistical significance (Fig 9B-b). Furthermore, fatty acid oxidation related gene expressions were significantly lower in the OL-G group than in the OL-E group (Fig 9B-c).

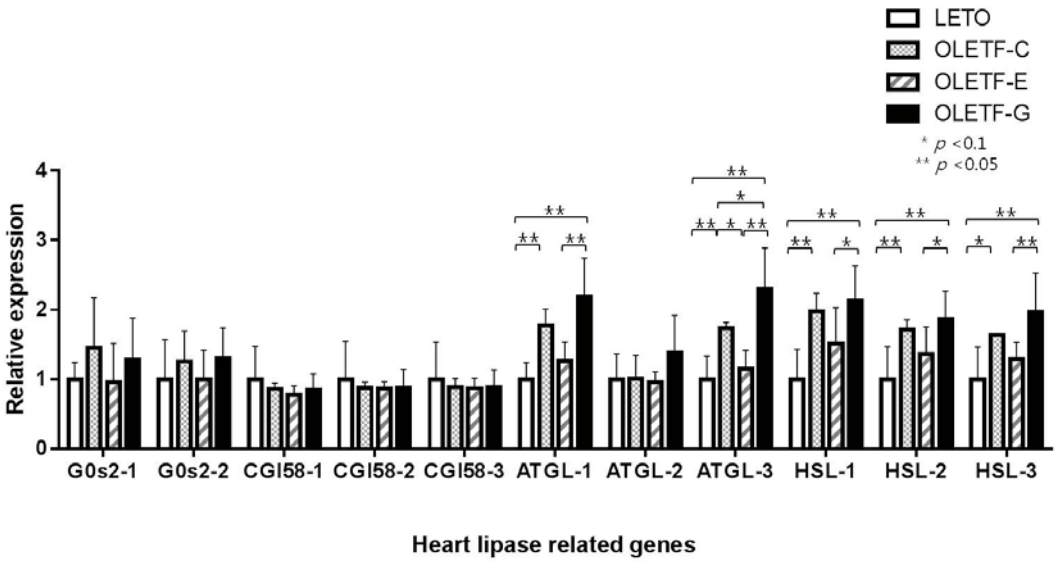
Figure 9. Triglyceride and lipolysis in cardiac and adipose tissues

A. Heart

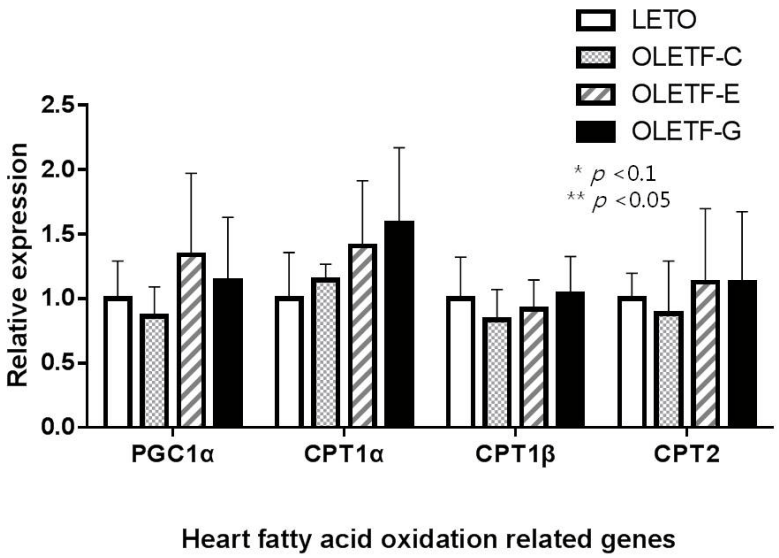
a. Triglyceride and lipolysis in cardiac tissues



b. Lipase related genes in cardiac tissues

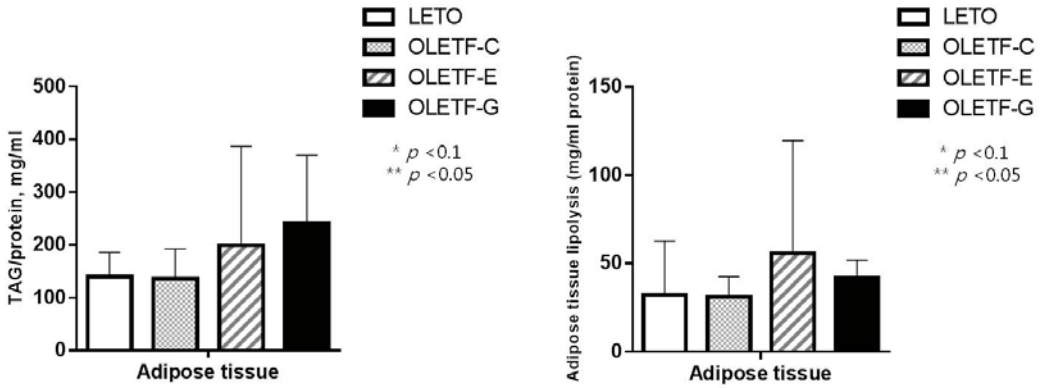


c. Fatty acid oxidation related genes in cardiac tissues

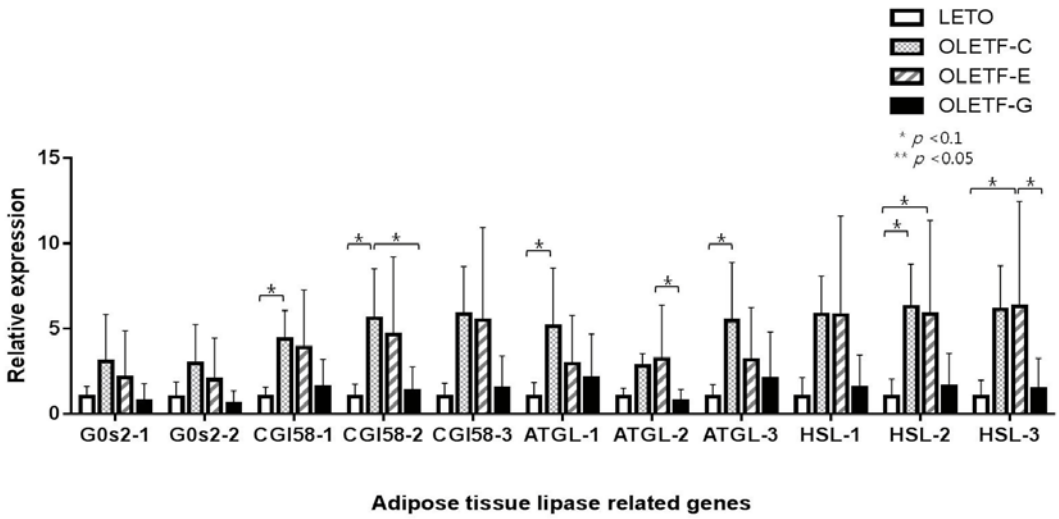


B. White adipose tissue

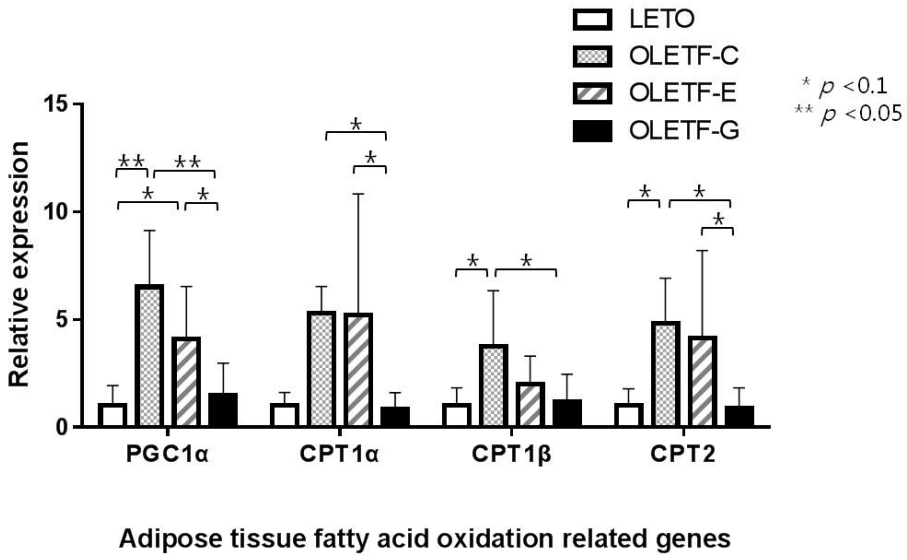
a. Triglyceride and lipolysis in white adipose tissues



b. Lipase related genes in white adipose tissues



c. Fatty acid oxidation related genes in white adipose tissues



Oxidative stress and mitochondria related gene expressions

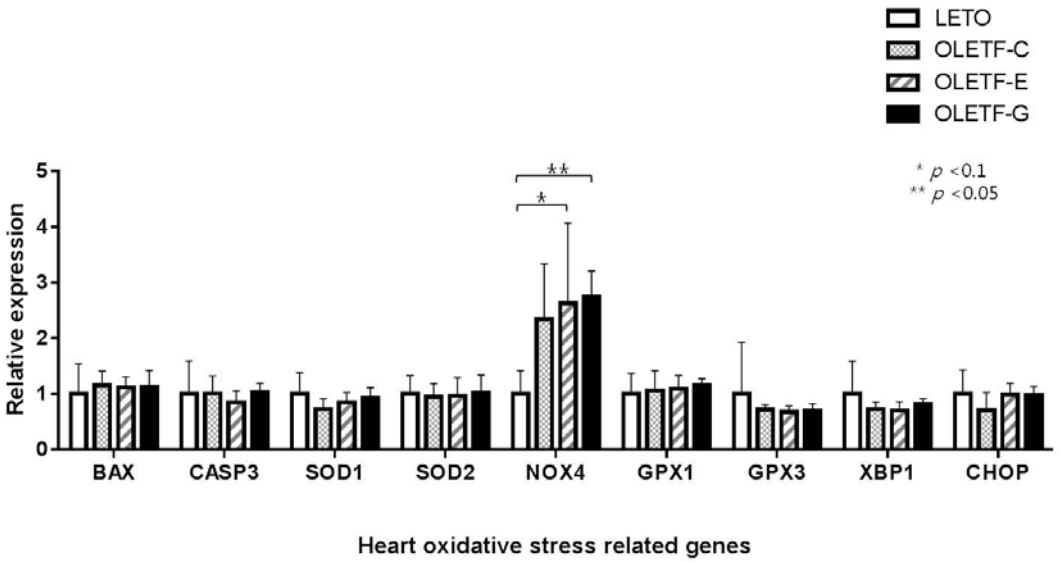
In cardiac tissues, the expressions of oxidative stress related genes were not significantly different between LT and OL-C group. Expression of NOX4, one of the oxidative stress related genes, tended to be higher in OL-C group than in LT group without statistical significance, but was not significantly decreased by empagliflozin or lixisenatide. Mitochondrial function related genes were also not significantly different among groups (Fig 10A-a and b).

In WATs, GPX1 and TFAM expressions were marginally increased in OL-C group compared to LT group ($p < 0.1$). Expressions of other oxidative stress and mitochondrial related genes also tended to be increased in OL-C group than in LT group without statistical significance. GPX1 ($p < 0.1$) and Cytc ($p < 0.05$) expressions were decreased in OL-G group compared to OL-C group. Other oxidative stress related genes such as CASP3 and NOX4, and mitochondrial related genes such as DRP1, Cytc and NRF2 tended to be lower in OL-G group than in the OL-C group (p = ns) or OL-E group ($p < 0.1$, Fig 10B-a and b).

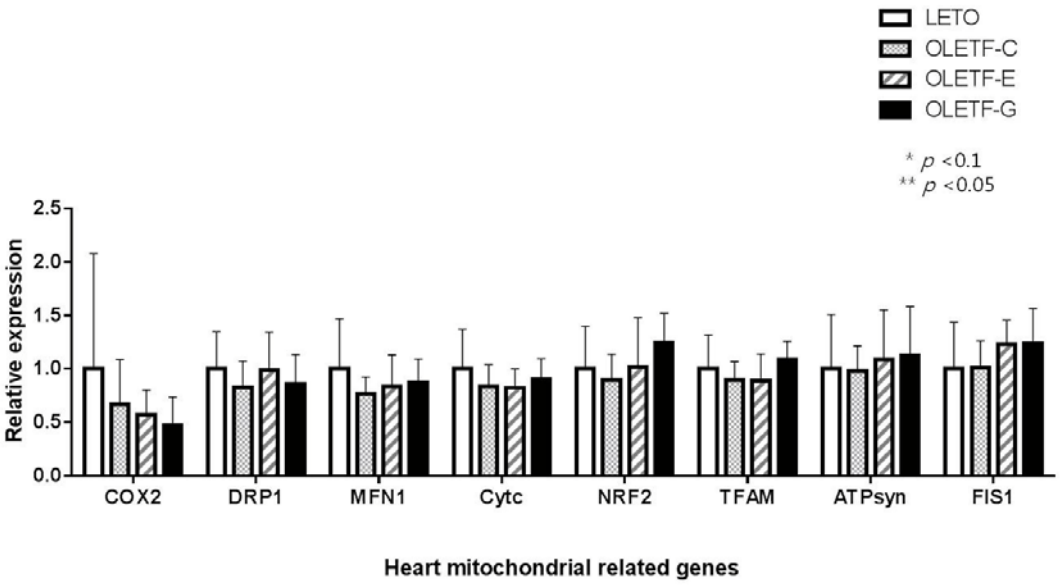
Figure 10. Oxidative stress and mitochondria related genes

A. Heart

a. Oxidative stress related genes in cardiac tissues

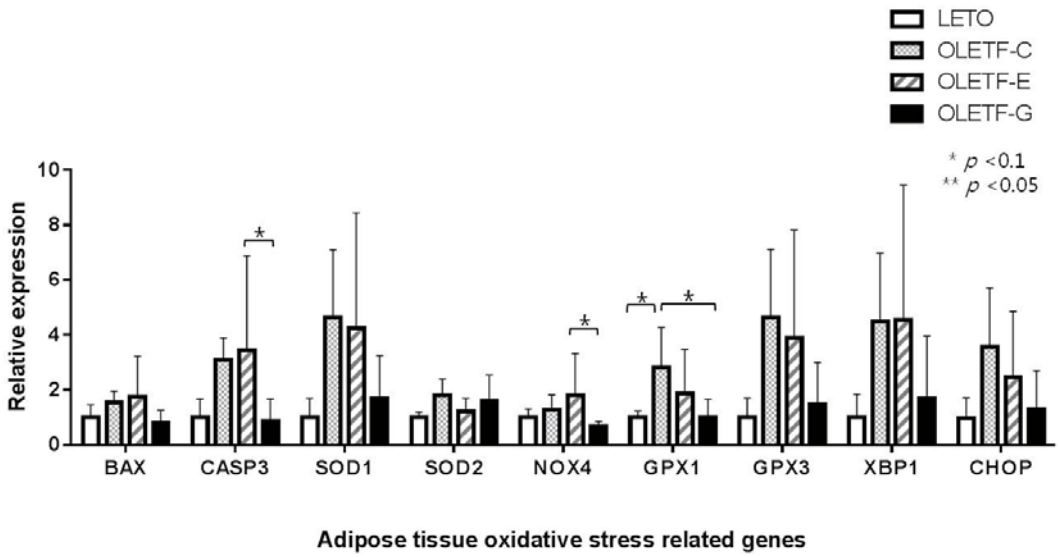


b. Mitochondrial related genes in cardiac tissues

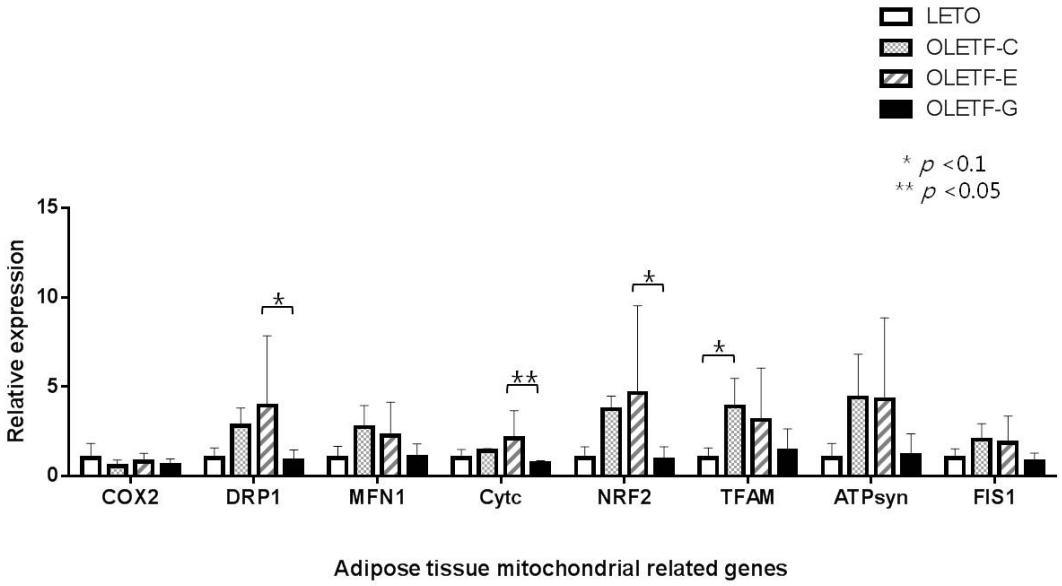


B. White adipose tissue

a. Oxidative stress related genes in adipose tissues



b. Mitochondrial related genes in adipose tissues



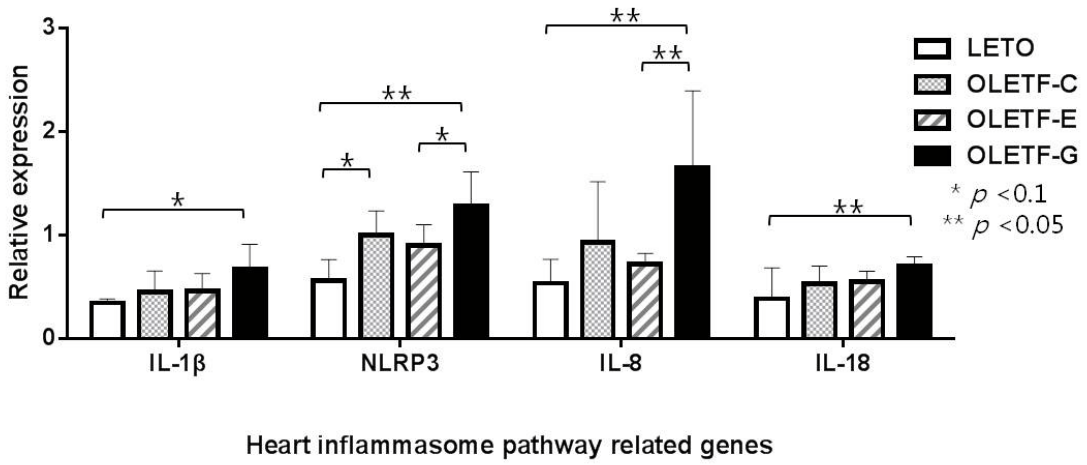
Inflammasome pathway related gene expressions

Inflammasome pathway related gene expressions, that is, of IL-1β, NLRP3, IL-8, and IL-18, were also evaluated. In cardiac tissues, NLRP3 expression in OL-C group was marginally higher than in LT group. Empagliflozin or lixisenatide treatment did not significantly decrease the NLRP3 expression. IL-8 ($p < 0.05$) and NLRP3 ($p < 0.1$) expressions were higher in OL-G group compared to OL-E group.

The effect of lixisenatide treatment in adipose tissues was to reduce the expression of inflammasome pathway related genes, unlike in heart tissues. NLRP3 expression is decreased in OL-G group compared to OL-E group with marginal significance. Empagliflozin treatment did not show significant difference in expression of inflammasome pathway related genes compared to control in WATs (Fig 11A and 11B).

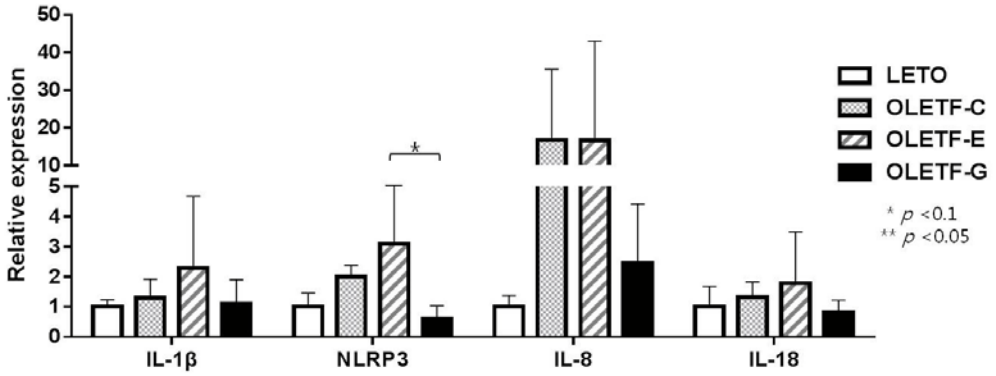
Figure 11. Inflammasome pathway related genes

A. Heart



Heart inflammasome pathway related genes

B. White adipose tissue



Adipose tissue inflammasome pathway related genes

Ketolysis and inflammation related gene expressions

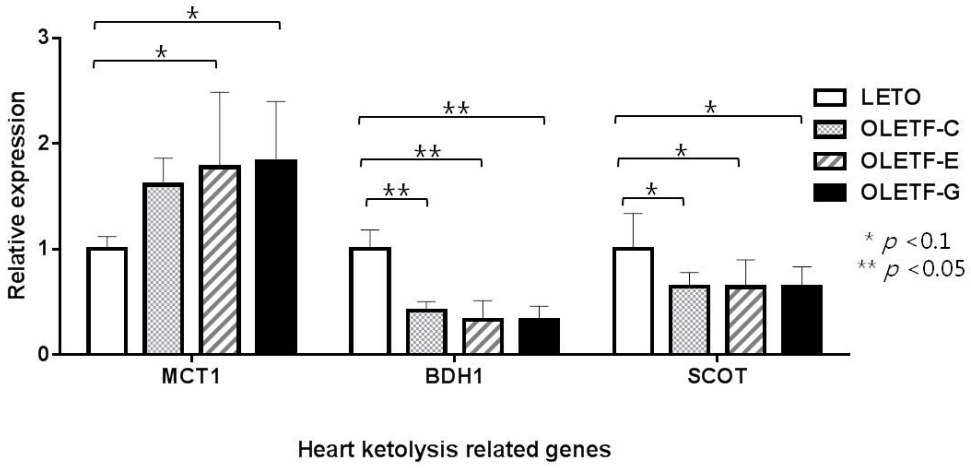
In cardiac tissues, expressions of BDH1 and SCOT, which are ketolysis related genes, was significantly and marginally lower in OL-C group compared to LT group. Ketolysis related gene expressions were unaffected by empagliflozin or lixisenatide. However, in adipose tissues, their expression levels were lower in the OL-G group than in the OL-E group with marginal significance (Fig 12A-a and 12B-a).

We also evaluated the expressions of the pro-inflammatory marker related genes, ICAM, VCAM, IL-6, TNFα, MMP9, MCP-1, p65, and HO-1. In cardiac tissue, VCAM and MCP-1 expressions were significantly increased on OL-C group compared to LT group ($p < 0.05$). No significant expressional decrease was observed in the OL-E and OL-G compared to OL-C group. Rather, in the OL-G group, expressions such as ICAM, TNFα, and p65 were higher than in the OL-C or OL-E group ($p < 0.05$ vs. OL-C and OL-E; $p < 0.1$ vs. OL-E; $p < 0.05$ vs. OL-C, respectively). In WATs, MMP9 expression was higher in the OL-C group than in the LT group with marginal significance ($p < 0.1$). Lixisenatide treatment decreased MMP9, VCAM and HO-1 compared to OL-C or OL-E group ($p < 0.05$ vs. OL-C; $p < 0.1$ vs. OL-C; $p < 0.1$ vs. OL-E, respectively). In the OL-E group, there was no significant change in the expression of inflammation related genes compared to the OL-C group (Fig 12A-b and 12B-b).

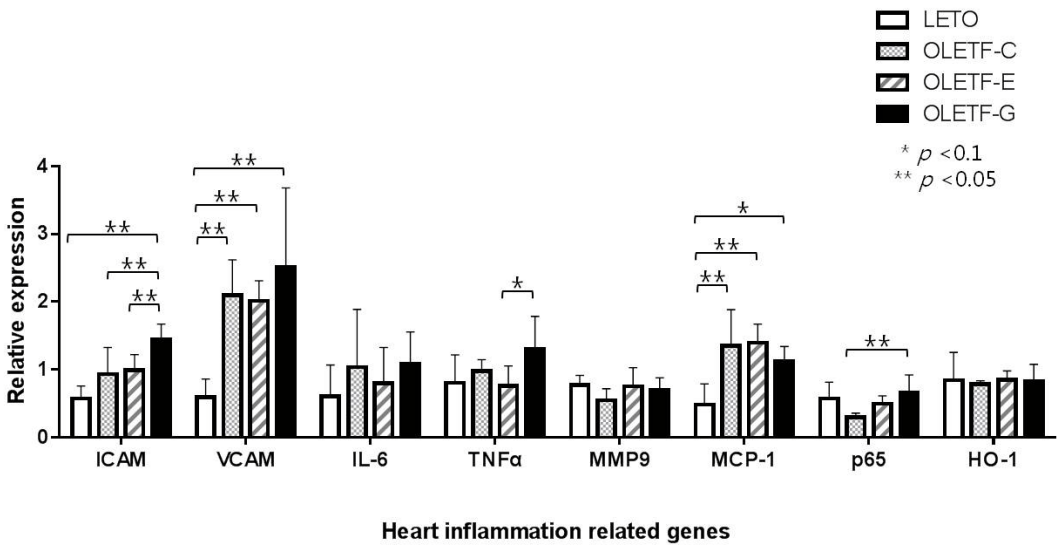
Figure 12. Ketolysis and inflammation related genes

A. Heart

a. Ketolysis related genes in cardiac tissues

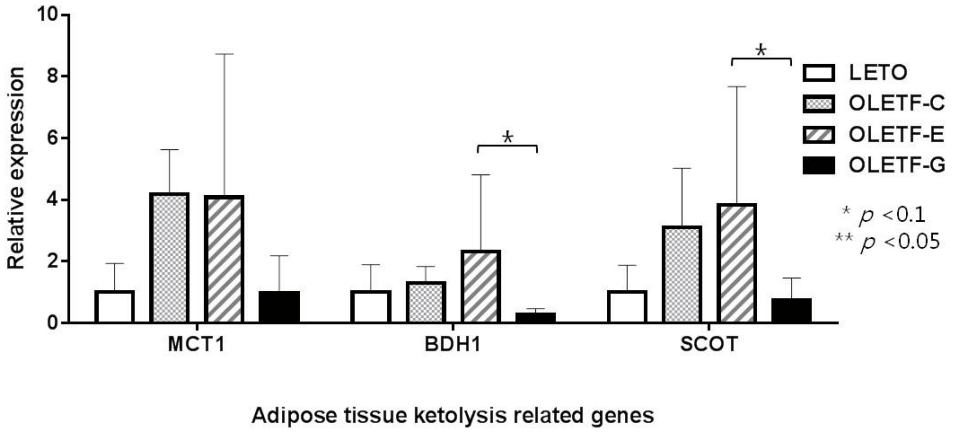


b. Inflammation related genes in cardiac tissues

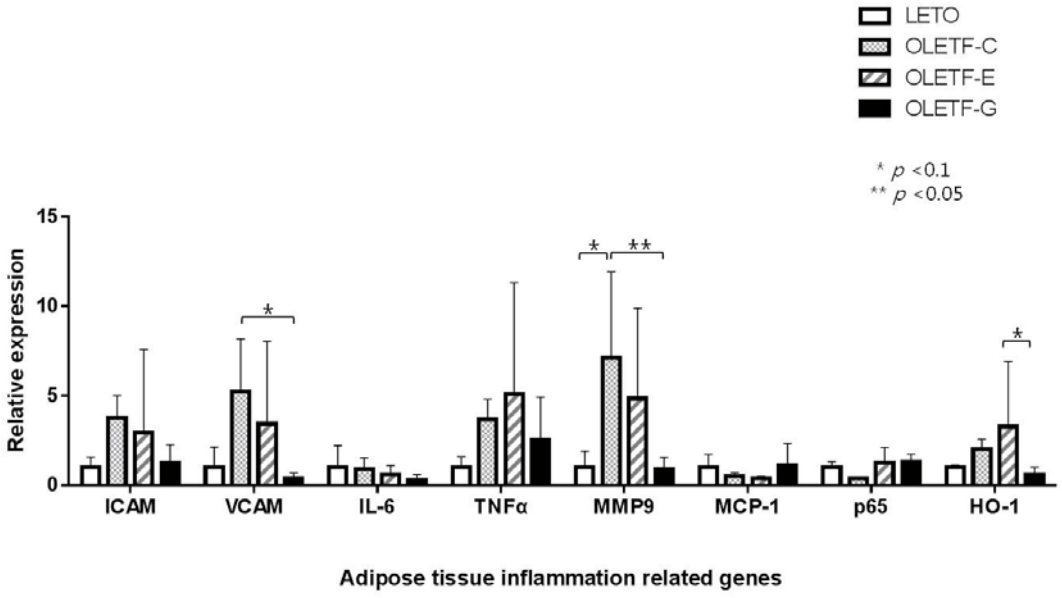


B. White adipose tissue

a. Ketolysis related genes in adipose tissues



b. Inflammation related genes in adipose tissues

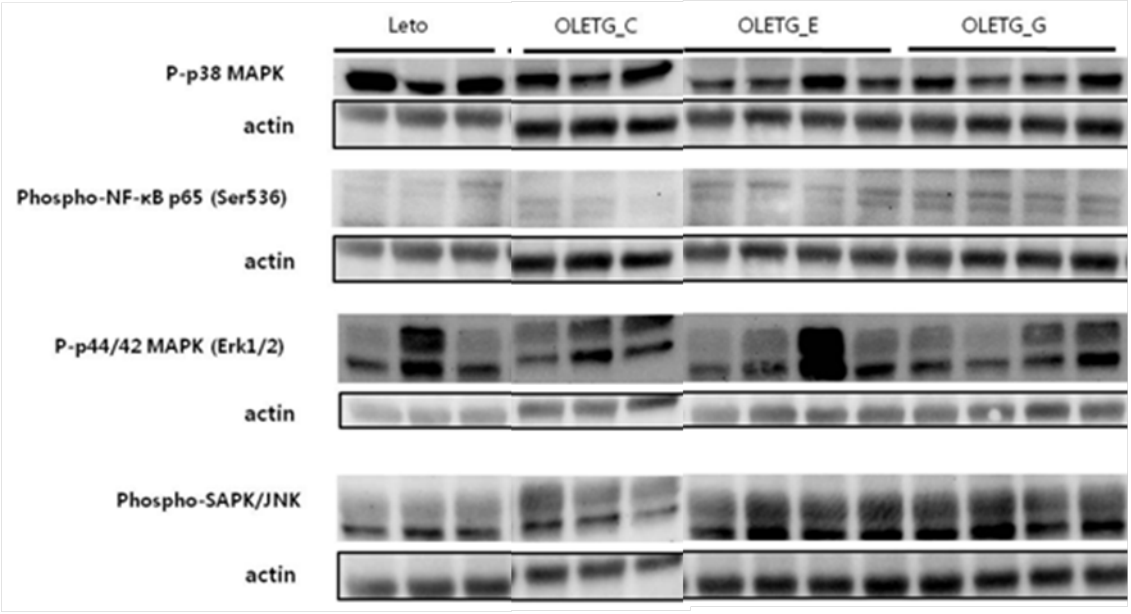


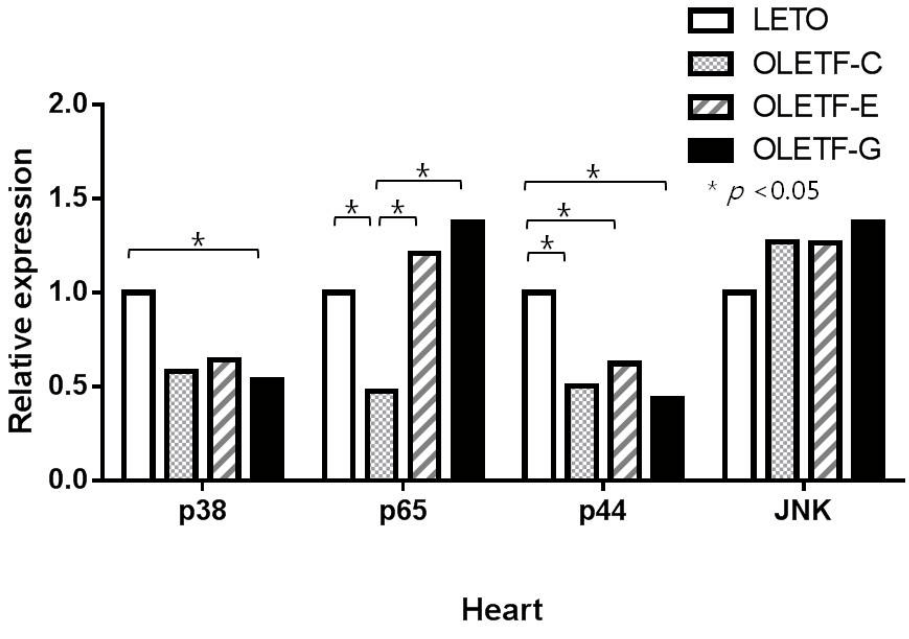
Western blotting of pro-inflammatory markers

Western blotting was performed for pERK1/2, anti-p38 MAPK, anti-NFκB and anti-pJNK. In cardiac tissues, anti-NFκB and pERK1/2 expression were decreased in OL-C group compared to LT group with statistical significance ($p < 0.05$). Anti-NFκB expression was significantly increased by empagliflozin and lixisenatide treatment ($p < 0.05$, respectively). pERK1/2 expression was not significantly changed by both drug treatment compared to control (Fig 13A). In WATs, pERK1/2 expression was significantly lower in the OL-G group than in the OL-C group, but the other pro-inflammatory markers were unaffected by treatments (Fig 13B).

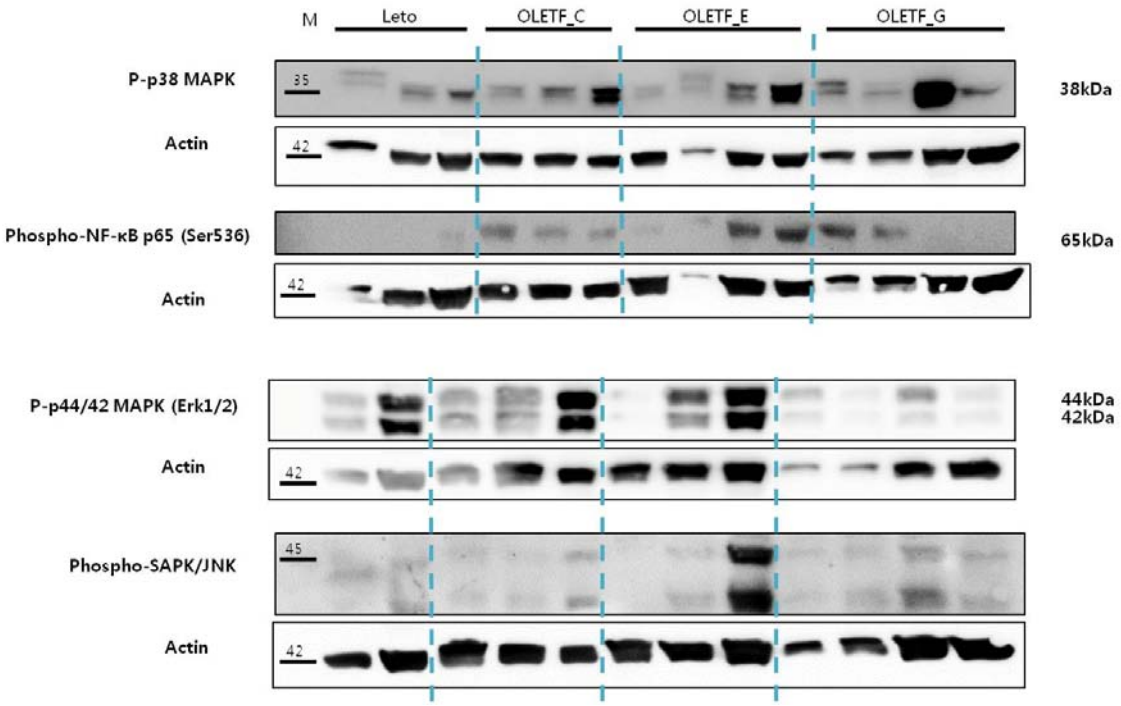
Figure 13. Western blotting

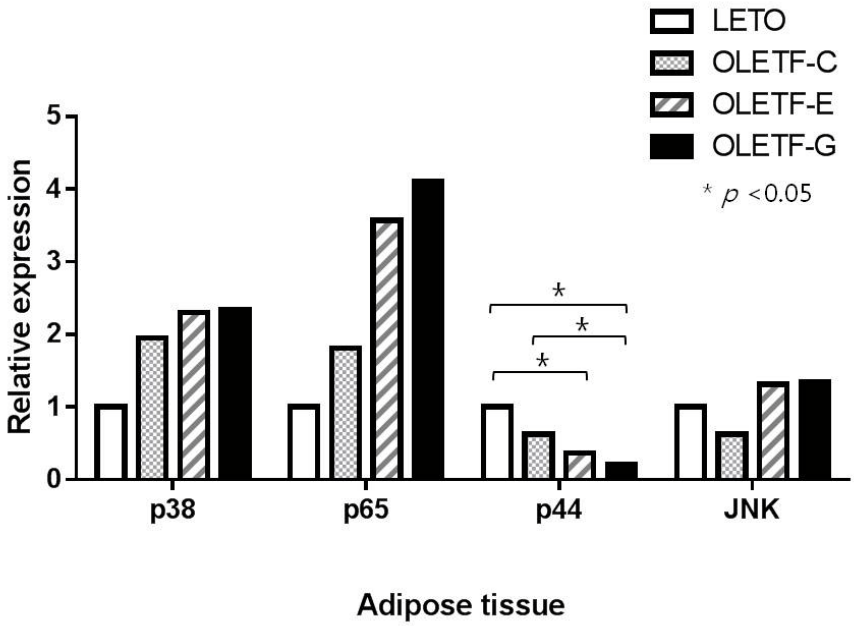
A. Heart





B. White adipose tissue





DISCUSSION

T2DM is a chronic metabolic disorder associated with obesity, insulin resistance, and non-alcoholic fatty liver disease. Accordingly, body weight control is the main therapeutic strategy for chronic metabolic diseases, and the treatment of T2DM should be initiated promptly with lifestyle intervention to maintain ideal body weight [23]. However, most T2DM patients fail to achieve the target glycemic goals by life style modification alone, and thus, antihyperglycemic agents are actively used [23], and thus, novel antidiabetic agents with antiobesity and lipid-lowering effects in addition to glucose-lowering efficacy are an attractive proposition for patients with T2DM.

SGLT2 inhibitors like empagliflozin inhibit the reabsorption of filtered glucose and subsequent urinary glucose excretion by blocking SGLT2, which is located on the apical side of kidney proximal tubular cells [24-27]. These inhibitors have glucose-lowering effects and offer additional beneficial effects on metabolic profiles, which include blood pressure and body weight reductions [25-27].

GLP-1RA regulates glucose levels by increasing insulin secretion from β-cells in a glucose-dependent manner, and by suppressing glucagon secretion, delaying gastric emptying and promoting satiety (28133970, 21975753). GLP-1RA has been known to have not only glucoregulatory, appetite-suppressant and weight-lowering effect, but also cardioprotective effect [28].

The Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes (EMPA-REG OUTCOME) randomized double blind controlled trial was the first SGLT2 inhibitor trial to focus on CV and renal outcomes. In this trial, 7020 T2DM patients with CVD were assigned to 10 or 25mg of empagliflozin daily or placebo over a 3.1 year median follow-up period [15]. The primary endpoint (a composite of CV

related death, nonfatal MI, and nonfatal stroke) occurred in 10.5% of empagliflozin patients and 12.1% of placebo patients, which corresponded to rates per 1000 patient-years of 37.4 vs. 43.9, respectively (HR = 0.86, 95% CI = 0.74-0.99, p = 0.04). Heart failure hospitalization (HHF) occurred in 2.7% of empagliflozin patients as compared with 4.1% of placebo patients, corresponding to a rates per 1000 patient-years of 9.4 vs. 14.5 (HR = 0.65, 95% CI = 0.50-0.85, $p = 0.002$). HHF or CV-related death (excluding fatal stroke) occurred in 5.7% of empagliflozin patients and in 8.5% of placebo patients (rate per 1000 patient-years of 19.7 vs. 30.1, HR = 0.66, 95% CI = 0.55-0.79, $p < 0.001$). The Canagliflozin Cardiovascular Assessment Study (CANVAS) Program, which includes T2DM patients aged ≥ 50 years with at least 2 CVD risk factors (but no established CVD), also showed significant risk reduction for HHF rates as well as the primary endpoint (a composite of CV death, nonfatal MI, or nonfatal stroke) [13]. The Dapagliflozin Effect on Cardiovascular Events (DECLARE-TIMI 58) trial did not show statistically significant risk reduction for primary safety outcome (a composite of CV death, nonfatal MI, or nonfatal stroke) probably due to the low number of events in both study arms. However, HHF occurrence was significantly lower in dapagliflozin group (rate per 1000 patient-years = 6.2 vs. 8.5, HR = 0.73, 95% CI = 0.61-0.88, $p = 0.0008$) [14].

In the LEADER trial, 9340 patients underwent randomization and median follow-up was 3.8 years. The primary outcome was death from a CV related cause or first occurrence of nonfatal myocardial infarction or nonfatal stroke. The primary outcome occurred in significantly fewer patients in the liraglutide (a GLP-1RA) group (608 of 4668 patients [13.0%]) than in the placebo group (694 of 4672 patients [14.9%]) (hazard ratio, 0.87; 95% confidence interval [CI], 0.78 to 0.97; $P < 0.001$ for noninferiority; $P = 0.01$ for superiority). Fewer patients succumbed to a CV related

cause in the liraglutide group (219 patients [4.7%]) than in the placebo group (278 [6.0%]) (hazard ratio, 0.78; 95% CI, 0.66 to 0.93; *P* = 0.007), and the all-cause mortality rate was lower in the liraglutide group (381 [8.2%]) than in the placebo group (447 [9.6%]) (hazard ratio, 0.85; 95% CI, 0.74 to 0.97; *P* = 0.02) [19].

Recently, empagliflozin was reported to improve cardiac diastolic function in a rodent diabetic model [29] and dapagliflozin was found to prolong ventricular-repolarization by augmenting mitochondrial function in a rat model of insulin-resistant metabolic syndrome [30].

Potential cardioprotective effects of GLP-1RAs are thought to be derived from their ability to improve established CVD risk factors, such as obesity, high blood pressure, hyperglycemia, and dyslipidemia. However, GLP-1RAs may also have direct effect on the myocardium, and this represents an area of growing interest. GLP-1 receptors are abundant in cardiac tissue and its stimulation has several effects [31].

Based on these findings, we hypothesized that SGLT2 inhibitors and GLP-1RAs have direct effect on cardiac tissue and affect cardiac structure, mechanical function, histology and gene expression profiles. Thus, we first compared the effects of empagliflozin and lixisenatide on cardiac tissue.

Echocardiography was performed on animals in all four groups after several weeks of dosing to confirm change in cardiac structure and function. E/A ratio, which is a surrogate marker of diastolic dysfunction was significantly decreased in diabetic control group than in LT group. We did not obtain firm evidence that empagliflozin or lixisenatide changed myocardial contractility. In addition, unlike that reported in a previous study [29], we found no difference between diastolic functions in the empagliflozin group and lixisenatide group. As a result, it is difficult to decide whether the protective effects of empagliflozin and lixisenatide on CVD were caused by their

beneficial effect on cardiac structure or function.

Previous studies have reported that OLETF rats showed no significant changes in echocardiographic parameters or in myocardial or vascular histology at 22 weeks of age as compared with LETO rats [32]. However, in this previous study, at 62 weeks of age, wall thickening of intramyocardial coronary arteries was evident. Intimal and medial walls of thoracic aortas were found to be thickened due to the infiltration of monocytes under endothelial cells or plaque formation in older (62-week-old) OLETF rats. Furthermore, functional and pathological changes of LV myocardium were not observed, except for a relaxation abnormality in older OLETF rats [32]. In the present study, cardiac and vascular tissues were obtained from 26-week-old animals, because we wanted to observe cardiovascular complications, and thus, were unable to confirm these previous results by examining differences between markers of cardiac complications.

It has been reported that lipid droplets were observed in the hearts of T2DM and metabolic syndrome patients [33-35] and in those of high fat diet fed rodents and genetically altered mice [36]. However, we did not observe lipid droplets or any treatment related histological changes in cardiac tissues. We suggest that this difference was possibly caused by the use of different animal models and treatment durations.

Considering that empagliflozin and lixisenatide proved to have cardiovascular protective effect, we examined the effects of these drugs on heart tissue in a comprehensive way.

First, we looked into the changes of lipid metabolism and fuel utilization in cardiac tissue. In the present study, TG accumulated more in cardiac tissues in the OL-C group than in the LT group as expected. Even though TG content was not significantly decreased by lixisenatide treatment, it seemed to increase lipolysis in cardiac tissue.

Lipases reduce TG accumulation in tissues [37], and ATGL, which is predominantly expressed in adipose tissue, and to lesser extents, in cardiac and skeletal muscles and other tissues, hydrolyses long-chain fatty acid TGs [38, 39]. In cardiac muscle, ATGL deficiency has been reported to cause severe TG accumulation, especially in cardiac tissues [37]. In the present study, ATGL3 expression was increased by lixisenatide in cardiac tissues, but not by empagliflozin, which suggests lixisenatide increased lipolysis by increasing lipase expression. It is known that cardiac fatty acid oxidation rate is increased in various forms of heart disease such as heart failure, ischemic heart disease and diabetic cardiomyopathies [40]. However, no significant changes in fatty acid oxidation related genes were observed by empagliflozin or lixisenatide treatment in our study. With this result, changes in fatty acid oxidation rate would not be an important mechanism which these drugs have protective effects on cardiovascular diseases. Oxidative stress and mitochondrial function related genes were not significantly increased in diabetic control. Empagliflozin or lixisenatide treatments also did not significantly change the expression level of these genes compared to diabetic control group. However, NOX 4 expression was higher in empagliflozin-treated and lixisenatide-treated group than in the LT group.

Reactive oxygen species (ROS) are highly reactive *in vivo* and include peroxides, superoxide, the hydroxyl radical, singlet oxygen [41], and alpha-oxygen and are produced during normal cellular metabolism. ROS have a range of effects in the vascular system [42]. ROS are produced intracellularly by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, NOX) complexes in cell membranes, mitochondria, peroxisomes, and endoplasmic reticulum [43, 44]. NADPH oxidase is a membrane-bound enzyme complex that faces the extracellular space and catalyzes the production of superoxide radicals by transferring one electron to oxygen from NADPH.

Human isoforms of the catalytic component of this complex include NOX 1, NOX 2, NOX 3, NOX 4, NOX 5, DUOX 1, and DUOX 2 [45]. NOX 1, NOX 2, NOX 4, and NOX 5 are expressed in the vascular system, and NOX 1 and NOX 2 have been identified as mediators of endothelial dysfunction [46, 47].

Some controversy exits regarding the metabolic effects of NOX 4. Its overexpression in mouse hearts has been reported to exacerbate cardiac dysfunction, fibrosis, and apoptosis in response to pressure overload [48]. NOX4 in cardiomyocytes is a major source of mitochondrial oxidative stress and mediates mitochondrial and cardiac dysfunction during pressure overload [48]. Also, NOX 4 in cardiac fibroblasts has been suggested to play an important role in mediating cardiac fibrosis by stimulating myofibroblast differentiation and to affect the development of pathological hypertrophy [49]. However, recently, NOX 4 attracted attention because it differs from NOX 1 and NOX 2 in several respects. NOX 4 mRNA expression is higher than those of other NOX homologues**,** whereas NOX 1 and NOX 2 are induced by angiotensin II in vascular smooth muscle cells [50]. In particular, a transient massive upregulation of NOX 4 was reported during the resolution phase of vascular injury [51]. In addition, NOX 4 contributes to basal vascular H2O2 formation, which in turn promotes angiogenic responses [42]. NOX 4 also acts to maintain the activities of protective endothelial mediators [42]. Further research is needed on the role of NOX 4.

Inflammation responses play important roles in diabetic cardiomyopathy [52]. In the present study, expression of NLRP3, which is one of the inflammasome pathway-related genes, was increased in diabetic control group. Empagliflozin did not significantly reduce the expression of inflammasome pathway related genes. In lixisenatide treated group, NLRP3 and IL-8 expressions were higher than the empagliflozin treated group but not than the diabetic control group. Lixisenatide group

also showed higher expression of ICAM and TNFα, which are inflammation related genes, compared to empagliflozin group but not to diabetic control group. NF-kB (p65) protein expression was also increased in empagliflozin and lixisenatide compared to diabetic control group. The high expression of inflammation related genes and proteins in the lixisenatide group was somewhat unexpected. There are several studies reporting that GLP-1 agonists reduce endothelial inflammation [53] and decrease markers of systemic inflammation [54, 55]. However, there is limited evidence that GLP-1RA reduces inflammatory responses in heart tissue. BDH1 and SCOT are reduced in the diabetic control group, but do not appear to be altered by drug administration, and this also may not be the main mechanism of the cardioprotective effects of empagliflozin and lixisenatide.

Since empagliflozin and lixisenatide are associated with weight loss and adipose tissue reduction, we considered that changes in adipose tissue may have roles in CVD risk reduction. Therefore, changes in histology and gene expressions were compared.

In the present study, decrease in CLS numbers in adipose tissue was observed in empagliflozin-treated group and, to a lesser degree, lixisenatide-treated group. Macrophage infiltration was reduced in empagliflozin- and lixisenatide-treated group. Change in adipocyte sizes by drug administration was an interesting finding in this experiment. The adipocyte size increased in the empagliflozin group, whereas it decreased in the lixisenatide group.

Adipocyte size and inflammation in adipose tissue are increased in diabetic patients and this is related to insulin resistance [56, 57]. Previous study showed GLP-1 reduced adipocyte size and inhibited adipose tissue macrophage infiltration and inflammation in diabetic animal model [57]. Decrease in the expression of IL-6, TNFα,

MCP-1, phosphorylation of ERK1/2 and JNK was also observed [57]. In our study, lixisenatide-treated group showed decreased expressions of inflammasome pathway-related and inflammation-related genes, which are consistent with previous study, and this probably explains the histologic change in this group.

Even though lixisenatide-treated and empagliflozin-treated group had similar body weight after treatment, only lixisenatide-treated group had a significant reduction in the adipocyte size and inflammation in adipose tissue, suggesting that these changes in adipose tissue in lixisenatide group was caused by drug treatment, and not by weight reduction. It has been shown that the expression of lipogenic gene transcripts such as Srebp1c, Acc1 and Fas mRNA was significantly decreased in adipose tissue of GLP-1-treated mice [57]. Since exendin-4 treatment on 3T3-L1 adipocytes increased the expression of lipogenic genes, authors presumed that the reduction of lipogenic gene expression and decrease in adipocyte sizes observed earlier were indirect effects by reduced inflammatory response, combined with improved blood glucose and insulin resistance [57]. However, in our experiment, empagliflozin-treated group also showed improved blood glucose and inflammation in the adipose tissue, suggesting that there are additional mechanisms that influence lipogenic gene expression.

Expression of fatty acid oxidation related genes, oxidative stress relate genes, mitochondrial related genes and ketolysis related genes were decreased only in lixisenatide-treated group.

SGLT2 receptors are expressed in the kidney and their expressions are negligible in other tissues [58]. Even though reduction of CLS number and inflammatory cell infiltration was observed in the empagliflozin-treated group, no significant change in gene expression was observed in adipose tissue by drug treatment. It is likely that empagliflozin acted through systemic effects rather than directly on

adipose tissue, possibly a systemic effect involving glucagon and butyrate elevations.

SGLT2 inhibitors promote the urinary excretion of glucose [59], and this is balanced in part by endogenous glucose production [60], which has been linked to increases in glucagon levels and glucagon-to-insulin ratio [60-62]. However, the main cause and impact of altered glucagon regulation after the inhibition of SGLT2 has not been established [63]. GLP-1RAs have been reported to reduce levels of circulating glucagon in T2DM [64], but possibly to increase levels when administered long-term [65, 66]. Although we did not measure serum glucagon concentrations directly, we inferred empagliflozin increased and lixisenatide decreased glucagon concentrations (lixisenatide was administered for only 12 weeks in the present study).

Glucagon is considered to a cardiostimulant that increases heart rate and contractility [67, 68] by stimulating glucagon receptors associated with Gs protein, leading to adenylyl cyclase activation and a consequent increase in 3',5'-cyclin adenosine monophosphate (cAMP) production in myocardium [68]. Glucagon also seems to inhibit the activity of cyclic nucleotide phosphodiesterase enzymes, which convert cAMP to 5'AMP [68]. Thus, the combination of enhanced cAMP production and suppressed cAMP hydrolysis increases levels of myocardial cAMP, which is responsible for the cardiac effects of glucagon [69]. However, as mentioned above, it was difficult to confirm the effect of glucagon in the present study because of its short term nature and the use of a relatively young animal model.

Low insulin and elevated glucagon levels in adipose tissue upregulate lipolysis [70, 71] and promote a shift in whole-body substrate utilization from carbohydrate to fat, which mechanistically explains high levels of ketone bodies in patients treated with SLGT2 inhibitors [72]. Ketone bodies are a vital alternative source of energy during periods of starvation [73, 74], and together with β-hydroxybutyrate (BHB), acetoacetate,

and acetone accumulate during starvation. BHB is the most stable form of ketone body [75]. During periods of starvation, fatty acid β-oxidation up-regulation promotes the conversion of acetoacetate into BHB [76, 77]. Ketone bodies have broadly favorable effects, for example, it acts as a fuel source to produce adenosine triphosphate (ATP) more efficiently than glucose or FFAs in a failing heart [78, 79]. Furthermore, it was recently reported a ketogenic diet extended longevity and health span in animal models [80]. Ketone bodies have an anti-inflammatory property resulting from the inhibition of the Nod-like receptor pyrin domain of protein 3 (NLRP3) inflammasome [36, 81, 82]. However, in our study, no significant reduction in the expressions inflammasome pathway related markers, including NLRP3, was observed in empagliflozin-treated group.

The present study has a number of limitations that warrant consideration. First, the number of experimental animals in each group was too small, non-fasting glucose levels were not diminished by lixisenatide. Second, the short duration of treatment made it difficult to identify histological changes in myocardium or heart function. Third, because of the characteristics of the two drugs, they were administered via different routes, that is, empagliflozin was administered orally, whereas lixisenatide was administered intraperitoneally. Thus, it is possible animals in the lixisenatide group experienced stress, which may have affected blood glucose.

In summary, empagliflozin (a SGLT2 inhibitor) treatment did not show significant changes in the expression related to fatty acid oxidation, oxidative stress, mitochondrial function and ketolysis in cardiac and adipose tissue of diabetic rat model in our study. Lixisenatide (a GLP-1RA) seems to decrease inflammation in adipose tissue and adipocyte size via reduction of oxidative stress and of inflammatory cytokine production. On the other hand, lipolysis and inflammatory pathway was enhanced in

cardiac tissue by lixisenatide treatment. Lixisenatide appeared to act directly on adipose tissue as well as cardiac tissue, presumably because GLP-1RA receptors are present in almost all organs including the heart. On the other hand, empagliflozin had less effect on cardiac and adipose tissue than lixisenatide, and may be affected by systemic mediators such as glucagon or butyrate.

	SGLT-2 inhibitor	GLP-1 RA
Body weight	↓	↓
Serum glucose	↓	↓
Adipose tissue		
Adipocyte size	↑	↓
CLS	↓	↓
TG content	→	→
lipolysis	→	→
Lipase related genes (CGI58-1, ATGL2, HSL3)	→	↓
Fatty acid oxidation related genes (PGC1α, CPT1α, CPT1β, CPT2)	→	↓
Oxidative stress related genes (CAP3, NOX4, GPX1)	→	↓
Mitochondrial related genes (DRP1, Cytc, NRF2)	→	↓
Inflammasome pathway related gene (NLRP3)	→	↓
Ketolysis related genes (BDH1, SCOT)	→	↓
Inflammation related genes (VCAM, MMP9, HO-1)	→	↓
Cardiac tissue		
TG content	→	→
lipolysis	→	↑
Lipase related genes (ATGL1, ATGL3, HSL1, HSL2, HSL3)	→↓	→↑
Fatty acid oxidation related genes	→	→
Oxidative stress related genes	→	→
Mitochondrial related genes	→	→
Inflammasome pathway related genes (NLRP3, IL-8)	→	↑
Ketolysis related genes	→	→
Inflammation related genes (ICAM, TNFα, p65)	→	↑

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요약(국문초록)

나트륨/포도당 공동수송체2 억제제와 글루카곤양펩타이드1 수용체 효현제가 당뇨병 동물 모델에서 대사 지표에 미치는 영향

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서론: 심혈관 질환은 제 2형 당뇨병 환자의 주요 사망원인이다. 나트륨-포도당 공동수송체 2 억제제는 신장에서 포도당의 재흡수를 억제하여 혈당을 낮추는 기전으로 작용하는 새로운 계열의 항당뇨병 약제이다. 이는 글루카곤양펩타이드1 수용체 효현제와 몇몇 유사점을 가지고 있다. 두 약제는 공통적으로 혈당 강하, 체중감량을 통해 심혈관 질환 사망률 및 이환률을 감소시킨다. 본 연구에서는 나트륨-포도당 공동수송체 2 억제제인 엠파글리플로진과 글루카곤양펩타이드1 수용체 효현제인 릭시세나타이드가 제 2형 당뇨병 동물 모델에서 미치는 영향을 비교해보고자 한다.

방법: 제 2형 당뇨병 모델인 OLETF rat에서 엠파글리플로진 (3 mg/kg/day, 12주간 경구 투여, n=4) 및 릭시세나타이드 (10 μg/kg/day, 12주간 복강내

투여, n=4)를 처리한 군과 OLETF control (n=3) 및 LETO control (n=3)을 대상으로 실험을 진행하였다. 모든 군은 12주간의 약물 처치가 끝나가는 시점에 심장초음파검사를 통해 심기능을 측정하였다. 안락사 후, 심장 근육, 대동맥, 지방조직을 채취하여, 심장 조직 및 지방 조직의 조직학적 분석을 시행하였다.

심장 조직 및 지방 조직에서 중성지방 축적 및 지질분해 정도를 측정하였다. 또한 qPCR 을 이용하여 산화 스트레스 관련 유전자 – BAX, Casp3, SOD1, SOD2, NOX4, GPX1, GPX3, XBP1, and CHOP, 미토콘드리아 기능과 관련된 유전자 – COX2, DRP1, MFN1, Cytc, NRF2, TFAM, ATPsyn, and FIS1, 케톤 분해와 관련된 유전자 – MCT1, BDH1, SCOT, 지방산 산화와 관련된 유전자– PGC1α, CPT1α, CPT1β, and CPT2, 염증반응과 관련된 유전자 - ICAM, VCAM, IL-1β, NLRP3, IL-6, TNFα, MMP9, MCP1, p65, IL-8, IL-18, and HO-1, 지방분해 효소와 관련된 유전자 - G0s2-1, G-S2-2, CGI58-1, CGI58-2, CGI58-3, ATGL(Adipose triglyceride lipase)1, ATGL2, ATGL3, HSL(hormone sensitive lipase)1, HSL2, and HSL3들의 발현 양상을 확인하였다. 염증반응의 지표를 확인하기 위해 웨스턴 블롯을 시행하였다.

결과: 12주간의 약제처리 기간 동안 OL-E 및 OL-G군의 체중은 OL-C 군에 비해 감소하였다. 약제처리 6주 및 12주 시점에서 OL-E 군과 OL-G 군 사이에 체중의 유의한 차이는 없었다. 비공복상태 혈당은 OL-E 군에서 OL-C 군에 비해 유의하게 낮았으나 OL-G와 OL-C 군 사이에는 의미 있는 차이가 없었다. 복강내당부하검사 결과 당부하 후 30분~120분 사이의 혈당은 OL-E 및 OL-G 군에서 OL-C 군에 비해 유의하게 낮았다. 중성지

방과 유리지방산 농도는 LT 군에 비해 OL-C 군에서 유의하게 높았으며, 이는 empagliflozin 및 lixisenatide 투여에 의해 감소되는 경향을 보였다. 심장초음파검사에서 E/A는 LT군에 비해 OL-C 군에서 유의하게 낮았다. 그러나, OL-E, OL-G군의 E/A는 OL-C군에 비해 유의한 차이를 나타내지 않았다. 대동맥 혈관에서 Oil red O staining 을 하였으나, 육안적 검사 및 현미경적 검사 모두에서 모든 군에서 동맥경화 플라크는 관찰되지 않았다. 조직학적 검사에서 심장 조직에서는 네 군간에 세포내 지방 축적, 간질 섬유화, 대식세포 침윤 등의 차이를 관찰할 수 없었다. 반면에 지방조직에서는 OL-C군에 비해 OL-E 및 OL-G 군에서 crown like structure 이 감소한 것을 확인할 수 있었다. 지방세포의 크기를 측정하였을 때, OL-C 군에 비해 나트륨-포도당 공동수송체 2 억제제 처리군에서 지방세포의 크기가 크고, 글루카곤양펩타이드1 수용체 효현제 처리군에서 지방세포의 크기가 작은 것을 확인할 수 있었다.

OL-E군에서는 심근 조직 및 지방 조직 모두에서 중성지방 축적이나, 지질분해, 지방산 산화, 산화스트레스, 미토콘드리아 기능, 케톤분해 및 염증관련 유전자들의 발현에 의미 있는 차이가 없었다. OL-G군에서는 지방조직에서 염증 감소와 지방세포 크기 감소가 관찰되었다. 지방분해효소, 지방산 산화, 산화스트레스, 미토콘드리아 기능, 케톤분해 및 염증관련 유전자들의 발현이 지방 조직에서 감소되어 있었다. 반면 심근 조직에서는 지방분해 및 염증관련 유전자들이 항진되어 있는 것이 관찰되었다.

결론: 글루카곤양펩타이드1 수용체 효현제는 산화스트레스, 미토콘드리아, 염증관련 경로를 통해 주로 지방조직에 영향을 주는 것으로 생각된다. 반면

나트륨-포도당 공동수송체 2 억제제는 글루카곤양펩타이드1 수용체 효현제에 비해 심장 및 지방조직에서의 효과는 적은 것으로 보인다. 나트륨-포도당 공동수송체 2 억제제의 경우 본 연구에 포함되지 않은 다른 분자적 경로를 통해 작용을 하거나, 심장 또는 지방 외의 다른 조직에 대한 효과를 통해 심혈관 질환 예방 효과를 보일 가능성이 있을 것으로 사료된다.

주요어: 나트륨-포도당 공동수송체 2 억제제, 글루카곤양펩타이드1 수용체 효현제, 심혈관 질환, 당뇨병, 지질분해, 중성지방

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