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Liraglutide alters gut microbiota and improves endothelium-dependent relaxation in db/db mice

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ABSTRACT

Endothelial dysfunction is a hallmark of type 2 diabetes mellitus (T2DM) and a major contributor to cardiovascular complications. Although glucagon-like peptide-1 receptor agonists (GLP-1RAs) improve glycemic control and cardiovascular outcomes, the mechanisms linking GLP-1RA therapy, gut microbiome modulation, and endothelial function remain incompletely understood. In this study, we investigated whether the GLP-1RA liraglutide improves endothelial dysfunction in T2DM through microbiome-associated mechanisms that support vascular homeostasis. Male db/db mice and non-diabetic controls were treated with liraglutide (300 μg/kg/day, intraperitoneally) or saline for two weeks. Vascular function was assessed in mesenteric resistance arteries using wire myography. Human umbilical vein endothelial cells (HUVECs) were exposed to high glucose with or without liraglutide or the short chain fatty acid (SCFA), butyrate. Endothelial nitric oxide (NO) signaling was evaluated by eNOS (at Ser1177) phosphorylation and nitrite production. Gut microbiota composition was analyzed by 16S rRNA gene sequencing. Liraglutide significantly improved endothelium-dependent relaxation in db/db mice and restored high glucose-induced impairment of eNOS phosphorylation and NO production in HUVECs. *In vivo*, diabetes was associated with marked gut dysbiosis characterized by reduced alpha diversity and depletion of SCFA-producing taxa. Liraglutide treatment substantially restored microbial diversity and enriched beneficial genera, including Lachnospiraceae and *Lactobacillus*. Consistently, low-dose butyrate modestly enhanced NO production in endothelial cells. These findings support the concept of a GLP-1RA–microbiome–vascular axis, in which liraglutide-associated remodeling of the gut microbiota may contribute to improved endothelial NO signaling and vascular function in diabetes.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by insulin resistance and impaired insulin secretion, leading to hyperglycemia and various vascular complications [1]. These complications arise due to persistent endothelial dysfunction, which represents one of the earliest and most critical changes in the vascular system of diabetic patients [2]. Endothelial dysfunction is characterized by a reduction in the bioavailability of vasodilators, such as nitric oxide (NO), and an increase in endothelium-derived contracting factors, such as endothelin-1 (ET-1) and cyclooxygenase (COX)-derived prostanoids (e.g., thromboxane A2) [3]. These alterations disrupt the delicate balance between vasodilation and vasoconstriction, contributing to impaired endothelium-dependent relaxation, increased vascular tone, inflammation, and atherosclerosis. Consequently, these processes significantly elevate the risk of cardiovascular diseases (CVD), which remain the leading cause of morbidity and mortality in T2DM patients [4].

In addition to hyperglycemia, other factors such as increased oxidative stress, altered lipid metabolism, and the inflammatory state of T2DM exacerbate endothelial dysfunction, further impairing vascular health [5]. Recent research has also highlighted the role of the gut microbiome in modulating metabolic and cardiovascular health [6]. Disruptions in the microbiome, known as dysbiosis, are increasingly

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recognized as a contributing factor to the pathogenesis of T2DM and its associated vascular complications [7]. Dysbiosis can lead to systemic inflammation, insulin resistance, and endothelial dysfunction, thereby aggravating the risk of CVD in diabetic patients [8].

Liraglutide, a glucagon-like peptide-1 receptor agonist (GLP-1RA), has emerged as a promising therapeutic agent in the management of type 2 diabetes mellitus (T2DM) [9]. Beyond its well-established glucose-lowering effects, liraglutide has been shown to exert beneficial actions on cardiovascular risk factors, including improvements in endothelial function and reduction in inflammation [10]. Recent studies suggest that liraglutide may also directly ameliorate endothelial dysfunction through several mechanisms. It has been shown that liraglutide increases endothelial nitric oxide synthase (eNOS) activity via the downregulation of the nuclear factor-κB pathway in diabetic rats [11].

Additionally, liraglutide's effect to improve insulin sensitivity and regulate blood lipid levels further supports its potential to protect against the development of atherosclerosis and related cardiovascular complications [12,13]. Furthermore, emerging evidence suggests that liraglutide may influence the gut microbiome [14], potentially restoring microbial balance and improving systemic inflammation, both of which could contribute to its beneficial effects on vascular function [15].

Given the critical role of endothelial dysfunction in the pathogenesis of diabetic vascular complications and the emerging role of the microbiome in metabolic diseases, understanding the mechanisms by which liraglutide influences both endothelial health and microbial balance is essential. Therefore, we investigated whether liraglutide improves endothelial function in db/db mice and whether these effects are associated with changes in gut microbiota composition and SCFA-related signaling.

2. Materials and methods

All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85–23, 2011) and were approved by the Animal Experimental Committee of Yonsei University College of Medicine (Approval No. 2024–0203, Seoul, Korea).

2.1. Animals and treatments

Ten- to 12-week-old male type 2 diabetic mice (db/db) and age-matched C57BL/6 mice (control) were obtained from Orient Bio Co. (Seongnam, Gyeonggi-do, Korea). Briefly, mice were housed in plastic cages with stainless steel grid tops at 23–24 ℃ with a 12-hour light/dark cycle and allowed access to commercial rodent chow and water *ad libitum*. Mice were divided into 4 groups: (1) control mice treated with vehicle (saline), Control + Vehicle; (2) control mice treated with liraglutide (300 μg/kg/day, by intraperitoneal injection, once daily), Control + Liraglutide; (3) diabetic mice treated with vehicle, db/db + Vehicle; (4) diabetic mice treated with liraglutide (300 μg/kg/everyday, by I.P. injection), db/db + Liraglutide. Liraglutide and vehicle were treated for 2 weeks. Mice were randomly assigned to each group, and investigators performing vascular function and microbiome analyses were blinded to treatment. Body weight and blood glucose levels were recorded once a week during the experimental period. Blood glucose level was measured in the tail blood samples using a blood glucose meter (Accu-Chek, Roche Diagnostic, Berlin, Germany) in all of the groups of mice after a 6-hour fast. To characterize changes in gut microbiota, fecal samples were obtained after treatment of liraglutide or vehicle.

At the end of the treatment period, mice were euthanized using 5 % isoflurane followed by CO_2 inhalation. Death was confirmed by the absence of chest movement, lack of response to a toe pinch, absence of a detectable heartbeat, and the appearance of ocular opacity. Once euthanasia was verified, the heart was rapidly excised and tissue samples were collected. To isolate mesenteric artery, the mesenteric small artery beds were removed and placed in ice-cold Krebs-Henseleit (K-H) solution (composition in mmol/L: NaCl, 119; $CaCl_2$, 2.5; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; KCl, 4.6; and glucose, 11.1). The second branch of mesenteric arteries were isolated and cut into 2- to 3-mm segments for subsequent analysis.

2.2. Preparation of isolated mesenteric artery

After the mice were sacrificed, the mesenteric resistance arteries were excised and placed into chilled Krebs-Henseleit (K-H) solution (composition in mM/L: NaCl 119, KCl 4.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11.1). The solution was continuously bubbled with a mixture of 95 % O_2 and 5 % CO_2. Adipose and connective tissues were removed under an optical microscope (model SZ-40, Olympus, Japan). The second branch of the mesenteric resistance arteries was dissected into 2–3 mm long segments for use in isometric tension experiments.

2.3. Measurement of isometric tension in mesenteric arteries

The mesenteric artery segments were mounted on an isometeric wire myograph system (model 620 M, Danish Myotechnology, Aarhus, Denmark) using 25 μm wires. The mesenteric arteial rings were stretched to their optimal resting tension approximately 2 mN. The segments were incubated in 37℃ K-H solution, aerated with 95 % O_2 and 5 % CO_2 for 20 min to allow equilibration. All mesenteric arteries were initially contracted with a high K^+ solution (70 mM) and then washed with normal K-H solution. The arteries were pre-contracted with thromboxane A_2 receptor agonist (U46619, 500 nM) and endothelium-dependent relaxation was measured in response to cumulative concentrations of acetylcholine (ACh, 10^{-9} to 10^{-5} M, Sigma-Aldrich). To evaluate endothelium-independent relaxation, sodium nitroprusside dehydrate (SNP, a NO donor, 10^{-9} to 10^{-4} M, Sigma-Aldrich) was used. Vasoconstriction was analyzed in response to various concentrations of U46619 (10^{-10} to 10^{-5} M).

2.4. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in vascular cell basal media (ATCC) supplemented with endothelial cell growth kit-BBE (ATCC). The cells were maintained at 37℃ in a humidified atmosphere with 5 % CO_2. In the experiments, HUVECs from passages 3–5 were used. The cells were plated and exposed to a 25 mM high glucose media for 12 h with liraglutide (1 μM). Controls were kept in a 5 mM normal glucose with 20 mM of mannitol (osmotic control) with or without liraglutide (1 μM). Sodium butyrate (Sigma Aldrich, St. Louis, MO, USA) was treated to the cells with various concentrations (0.1, 0.5, 1, 5 mM). Cells were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) for further analysis.

2.5. Western blotting analysis

Human umbilical vein endothelial cells treated with or without high glucose and liraglutide were homogenized with ice-cold RIPA buffer containing a complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). After protein quantification using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific), the samples were loaded onto 8–10 % sodium dodecyl sulfate-polyacrylamide (SDS) gels and transferred to a nitrocellulose (NC) membrane. The membranes were blocked for 2 h at room temperature with 5 % (w/v) skim milk (BD Biosciences, San Diego, CA) in tris-buffered saline with 0.1 % Tween 20 (TBST) and then incubated with primary antibodies against total endothelial nitric oxide synthase (t-

eNOS, 1:1000, Cell Signaling Technology, Boston, MA, USA) and phosphorylated eNOS (p-eNOS, Ser1177, 1:1000, Cell signaling Technology) overnight at 4℃, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Protein bands were visualized using the SuperSignal West Pico Plus chemiluminescent substrate (Thermo Fisher Scientific). Band intensities were normalized to β-actin and expressed as the ratio of p-eNOS to t-eNOS.

2.6. Assessment of nitrite levels

Nitrite levels in HUVECs were measured using the Griess reagent kit (Sigma-Aldrich) to quantify NO production. The cell culture medium was mixed with equal volume of Griess reagent and incubated at room temperature for 30 min in a light protected condition. Absorbance measurements were carried out at 548 nm using a microplate reader to quantified the nitrite concentration.

2.7. 16S rRNA sequencing

The gut microbiome was analyzed using 16S rRNA gene sequencing of stool samples collected from each group of mice. Genomic DNA was extracted using a Qiagen DNeasy PowerSoil Pro extraction kits (Qiagen, MD, USA) according to the manufacturer's instructions. The extracted DNA was quantified and quality controlled by 1 % agarose gel electrophoresis. Then, we amplified the V3-V4 variable region of the 16S rRNA gene for sequencing using forward and reverse primers 16S-F (5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWG CAG) and 16S-R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGACTACHVGGGTATCTAATCC). 16S Metagenomic Sequencing Library Preparation protocol by Illumina was followed. Amplicons were sequenced on Nextseq2000 (Illumina, CA, US) at Sanigen Co., Ltd. (Gyeonggi-do, Republic of Korea) to generate 2×150 bp paired-end reads aiming for a sequencing depth of $> 100{,}000$ reads per sample.

2.8. Microbiota analysis

Raw reads were trimmed, quality filtered, pair-merged and denoised using DADA2 plugin (dada2 denoise-paired option) [16] in Qiime2 software 2023.2 distribution [17]. With end product of DADA2, amplicon sequence variants (ASVs) were defined and used in downstream analyses. Taxonomic classification analysis was performed for the ASVs using Qiime2 Naïve Bayes classifier against SILVA database v138.

The ASVs were aligned using multiple sequence alignment tool MAFFT [18] and phylogenetic tree was constructed with FastTree [19] plugin. Based on the constructed data, alpha and beta diversity was calculated using q2-diversity plugin in Qiime2. Alpha diversity was assessed using observed operational taxonomic units (OTUs) and Shannon index, while beta diversity was evaluated using Bray-Curtis dissimilarity matrices and visualized via Principal Coordinate Analysis (PCoA).

For differential abundance analysis, LEfSe (Linear discriminant analysis Effect Size) [20] analysis was performed to identify significantly enriched microbial taxa between treatment groups. Kruskal-Wallis H-test was applied to detect taxa with significantly different abundances across groups ($p < 0.05$), followed by pairwise Wilcoxon rank-sum tests. Taxa with an LDA (Linear Discriminant Analysis) score threshold greater than 2.0 were identified as significantly differential features.

2.9. Drugs

The following drugs were used: liraglutide (Angene, Nanjing, China); U46619 (Cayman Chemicals, Ann Arbor, MI, USA); acetylcholine and the general laboratory reagents (Sigma Aldrich)

2.10. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism Version 10.0.0 software (GraphPad Software Inc., San Diego, CA, USA). Comparison between two groups was performed using an unpaired Student's *t*-test. One-way or two-way ANOVA with the Bonferroni post hoc test was used to analyze the differences among multiple groups. A value of $^*P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of liraglutide on body weight and blood glucose

To evaluate the effects of liraglutide on body weight and blood glucose levels, mice were administered liraglutide (300 μg/kg/day, intraperitoneally) or vehicle (200 μL saline) for 2 weeks. Body weight was significantly, although modestly, reduced in liraglutide-treated db/db mice compared with vehicle-treated db/db mice, whereas liraglutide had no effect on body weight in control mice (Fig. 1A). Similarly, liraglutide treatment markedly lowered blood glucose levels in db/db mice but did not alter glucose levels in control mice (Fig. 1B).

3.2. Effect of liraglutide on endothelium-dependent relaxation

To determine whether administration of liraglutide affects vascular function, we measured vascular responses in mesenteric resistance arteries of liraglutide-treated and vehicle-treated mice using a multi-wire myograph system (Fig. 2). Endothelium-dependent relaxation in mesenteric arteries was significantly reduced in db/db mice compared to control mice. Liraglutide treatment significantly improved this endothelial dysfunction in db/db mice (Fig. 2E). Neither SNP-induced, endothelium-independent relaxation (Fig. 2 F) nor U46619-induced vasoconstriction (Fig. 2G) differed among the experimental groups.

3.3. Effect of liraglutide on eNOS phosphorylation and NO level in HUVECs

To further explore the mechanism by which liraglutide administration affects endothelium-dependent relaxation, we measured phosphorylation level of eNOS (Ser1177) in HUVECs. To mimic diabetic condition, cells were cultured in high glucose medium (25 mM) for 12 h. As an osmotic control, mannitol (20 mM) was treated to the cells cultured in normal glucose medium (5 mM).

The phosphorylated eNOS level was significantly reduced in the high glucose-treated HUVECs compared to normal glucose- and mannitol-treated cells. Administration of liraglutide significantly increased phosphorylated eNOS level (Fig. 3A) in high glucose-treated HUVECs.

Due to the rapid oxidation of nitric oxide (NO) into nitrite and nitrate, nitrite serves as a reliable, stable indicator for NO production. Therefore, NO levels were determined by quantifying the nitrite concentrations in the cell culture medium using the Griess reagent kit. Consistent with the western blot data, exposure to high glucose markedly reduced nitrite levels in HUVECs. Interestingly, liraglutide treatment substantially restored nitrite production, elevating the levels to nearly those observed under control conditions (Fig. 3A and B).

3.4. Liraglutide treatment restores alpha diversity in db/db mice

To evaluate the effect of liraglutide treatment on the diversity of the gut microbiota, 16S rRNA sequencing was performed on fecal samples from each treatment group (Control + Vehicle; Control + Liraglutide; db/db + Vehicle; db/db + Liraglutide; n = 3 per group).

The rarefaction curves based on Shannon diversity indices reached a plateau across all samples, indicating that sequencing depth was adequate to capture the microbial community structure in each sample

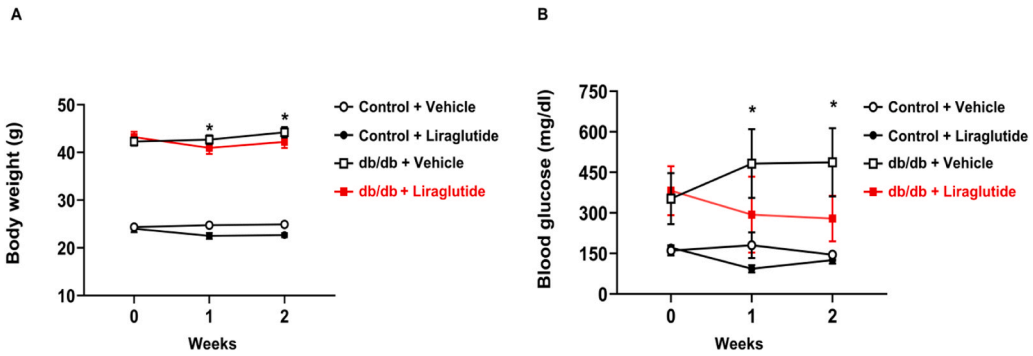

Fig. 1. Effect of liraglutide administration in control and db/db mice. (A) Body weight, (B) Blood glucose in vehicle (saline)- or liraglutide (300 μg/kg/everyday)-treated mice (n = 5). Data are shown as mean ± SD. The n-values represent the number of animals. *p < 0.05.

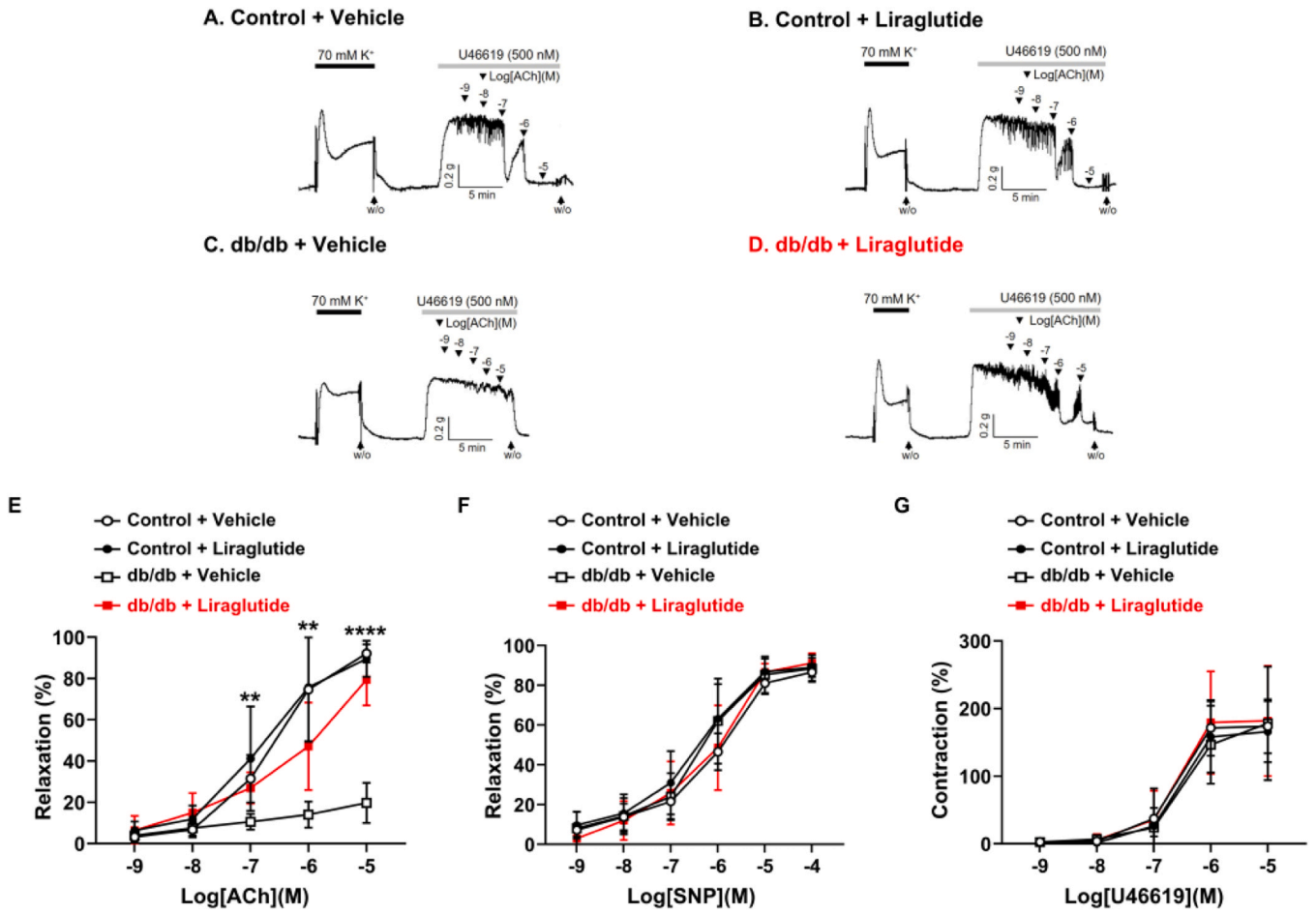

Fig. 2. Effect of liraglutide administration in mesenteric resistance arteries from control and db/db mice. (A-D) Representative traces showing the relaxation response to various concentrations (10^{-9} to 10^{-5} mol/L) of ACh on vasocontraction induced by U46619 (500 nM) in all groups. (E) Analysis of endothelium-dependent relaxation in response to ACh. (F) Analysis of endothelium-independent relaxation in response to SNP. (G) Analysis of contraction responses to various concentration of U46619 (10^{-9} to 10^{-5} M). Results are the means ± SD (n = 5). ** p < 0.01 and **** p < 0.0001 as compared between db/db and liraglutide-treated db/db mice.

(Supplementary Figure 1). Alpha diversity analysis revealed significant differences among the four groups (Fig. 4A). The observed number of operational taxonomic units (OTUs) was substantially reduced in vehicle-treated db/db mice compared to vehicle-treated control mice, reflecting the dysbiotic state associated with diabetes. Notably, liraglutide treatment significantly restored alpha diversity in db/db mice, with the observed OTU count in db/db mice treated with liraglutide approaching levels comparable to vehicle-treated control mice (Fig. 4A). These findings indicate that liraglutide intervention ameliorated the microbial diversity loss induced by the diabetic condition.

3.5. Distinct changes in beta diversity following liraglutide treatment

Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity revealed clear separation among the four treatment groups (Fig. 4B). The samples from control mice treated with vehicle and

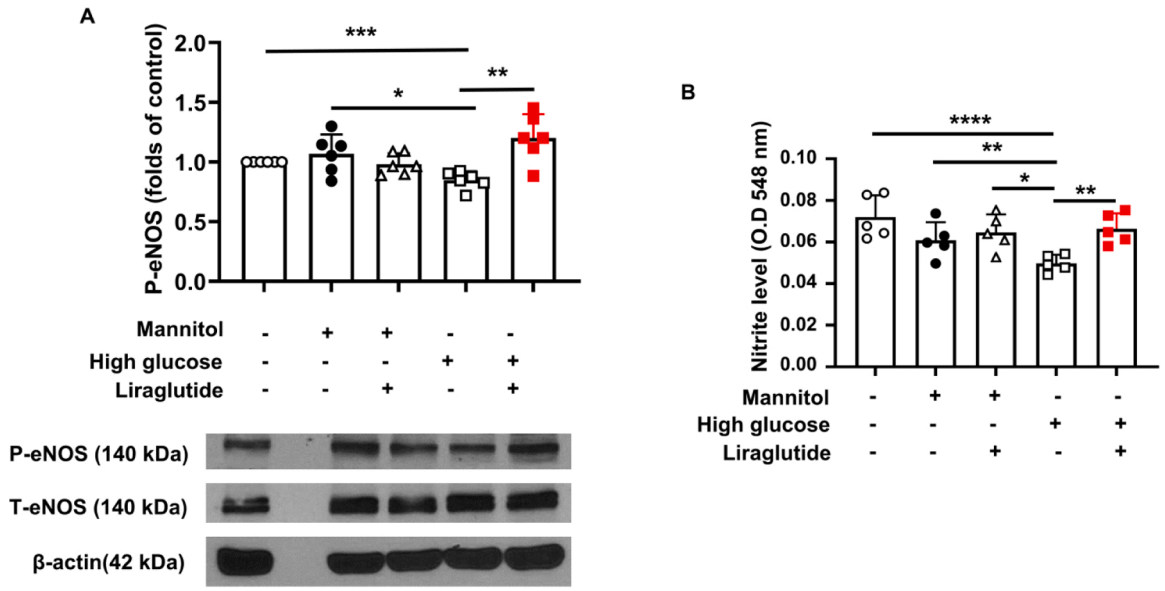

Fig. 3. Effect of liraglutide on eNOS phosphorylation and nitrite levels in HUVECs. (A) Representative Western blots and densitometric analysis of total and phosphorylated eNOS (Ser1177) in HUVECs cultured under normal or high glucose conditions, in the presence or absence of liraglutide. (B) Nitrite levels were measured in the corresponding samples as an index of NO production. Data are presented as mean ± SD (n = 6). *p < 0.05, **p < 0.01, *** p < 0.001, and **** p < 0.0001.

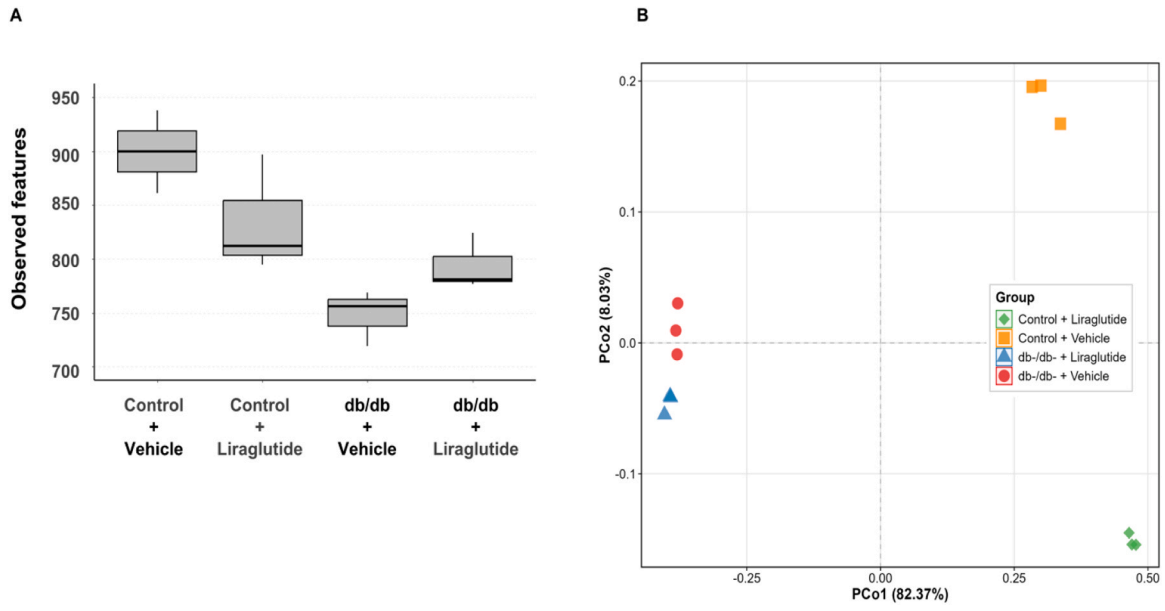

Fig. 4. Effect of liraglutide treatment on gut microbiota diversity. Gut microbiota diversity was analyzed across four experimental groups: Control + Vehicle, Control + Liraglutide, db/db + Vehicle, and db/db + Liraglutide (n = 3 per group). (A) Alpha diversity measured by observed ASVs (or OTUs). (B) Beta diversity visualized by principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity. Each point represents an individual sample, with variance explained indicated on each axis.

liraglutide clustered together and were distinctly separated from the db/db mice and db/db mice treated with liraglutide groups along the first principal coordinate (PC1). Interestingly, the liraglutide-treated db/db mice group demonstrated a partial shift in clustering position, suggesting that liraglutide treatment induced compositional changes in the gut microbiota of diabetic mice, moving toward a profile more similar to non-diabetic controls.

3.6. Liraglutide-induced changes in genus-level microbial composition

Genus-level analysis revealed differential responses to liraglutide between non-diabetic control and db/db mice. In control mice, comparison between vehicle-treated control and liraglutide treated mice demonstrated selective increases in specific taxa, including Muribaculaceae, *Ruminococcus*, *Akkermansia*, Rhodospirillales and *Clostridium*_UCG_014 (Fig. 5A), while Bacteroides remained relatively stable. Conversely, Lachnospiraceae and Lachnospiraceae_NK4A136_group showed modest decreases, indicating subtle compositional shifts in the microbiota.

In contrast, liraglutide treatment to db/db mice induced substantial restoration of the dysbiotic microbiota profile (Fig. 5B). Liraglutide-treated db/db mice demonstrated marked increases in beneficial taxa,

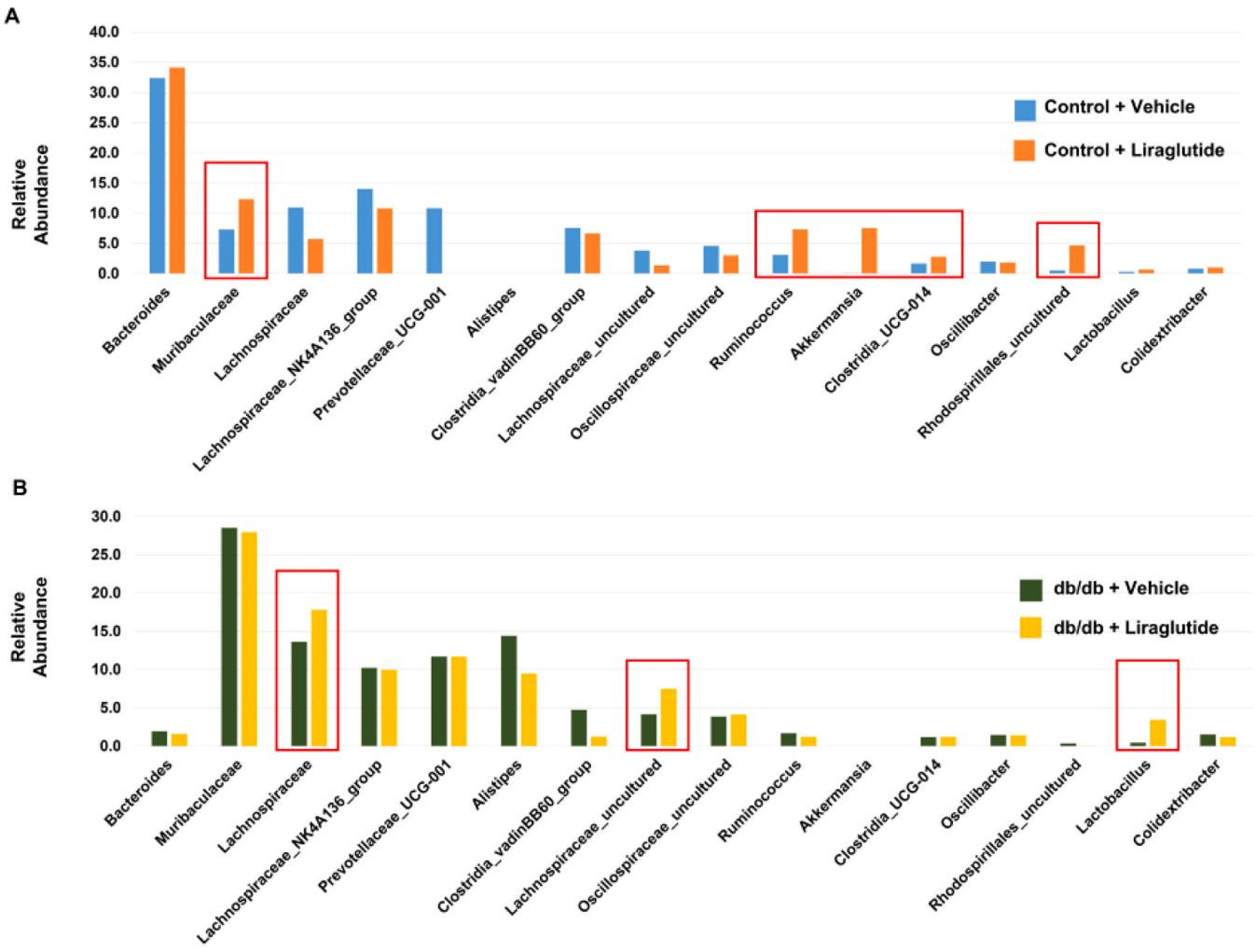

Fig. 5. Relative abundance of gut microbiota at the genus level. Comparison of microbial community composition between (A) Control + Vehicle and Control + Liraglutide groups, and (B) db/db + Vehicle and db/db + Liraglutide groups. Only taxa with a relative abundance > 1 % are shown. Bar plots represent the average normalized abundance from three biological replicates (n = 3). Red boxes indicate taxa showing notable differences between treatment groups.

including Lachnospiraceae, Oscillospiraceae (with a slight increase), and *Lactobacillus*, while simultaneously exhibiting pronounced decreases in *Clostridium*-related species and other potentially pathogenic genera. Heatmap analysis confirmed these compositional differences, with hierarchical clustering demonstrating distinct patterns between diabetic and non-diabetic groups (Supplementary Figure 2). Notably, the db/db mice treated with liraglutide showed partial clustering separation from the vehicle-treated db/db mice, indicating substantial liraglutide-induced restructuring of the diabetic microbiota toward a more eubiotic state.

3.7. Differential microbial taxa associated with liraglutide treatment

To identify taxa with significantly different abundances between groups, LEfSe (Linear discriminant analysis Effect Size) analysis was performed with an LDA score threshold of 2.0. Comparison between db/db mice and liraglutide treated db/db mice revealed numerous taxa with significantly different relative abundances (Fig. 6). Taxa enriched in the db/db mice (shown in red) included multiple *Clostridium*-related species and various This microbial restructuring. In contrast, liraglutide treatment promoted enrichment of beneficial taxa in the db/db mice (shown in green), including several Lachnospiraceae species, *Lactobacillus*, and other short-chain fatty acid (SCFA)-producing bacteria. The LEfSe results demonstrated that liraglutide treatment induced a substantial restructuring of the diabetic microbiota, with discriminative features predominantly represented by taxa known to support metabolic health and intestinal barrier function.

3.8. Effect of butyrate on NO level in HUVECs

To determine whether the microbiome-derived short chain fatty acid (SCFA), butyrate, modulates endothelial NO production, HUVECs were treated with increasing concentrations of sodium butyrate (0, 0.1, 0.5, 1, 5 mM), and nitrite levels in the culture medium were quantified as an index of NO production.

Sodium butyrate treatment induced a non-linear response in NO production in HUVECs (Fig. 7). Nitrite levels were slightly increased at 0.1 mM sodium butyrate-treated cells compared to the untreated control, whereas treatment with 0.5 mM sodium butyrate and 1 mM resulted in a reduction of nitrite levels. At 5 mM sodium butyrate, nitrite concentration returned to a level comparable to the control. These findings indicate that only low-dose butyrate modestly enhances NO production, while intermediate doses suppress NO levels and high-dose butyrate does not exert a measurable effect.

4. Discussion

In the present study, we demonstrate that liraglutide treatment markedly improves vascular endothelial function in db/db mice, accompanied by substantial restoration of gut microbial diversity and

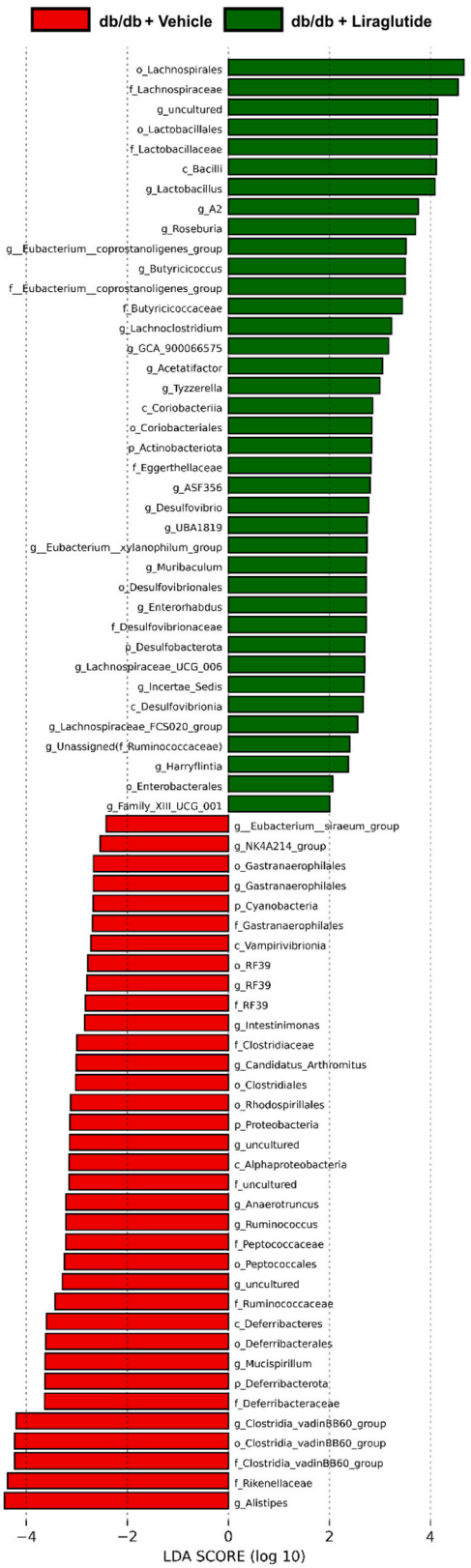

Fig. 6. Differentially abundant taxa identified by LEfSe analysis. Linear discriminant analysis effect size (LEfSe) comparing gut microbial composition between db/db + Vehicle and db/db + Liraglutide groups. Horizontal bars represent LDA scores ($\log_{10}$) for taxa meeting the significance threshold (LDA > 2.0, $p < 0.05$). Red bars indicate taxa enriched in db/db + Vehicle; green bars indicate taxa enriched in db/db + Liraglutide. Taxonomic levels are indicated by prefixes: p_, phylum; c_, class; o_, order; f_, family; g_, genus.

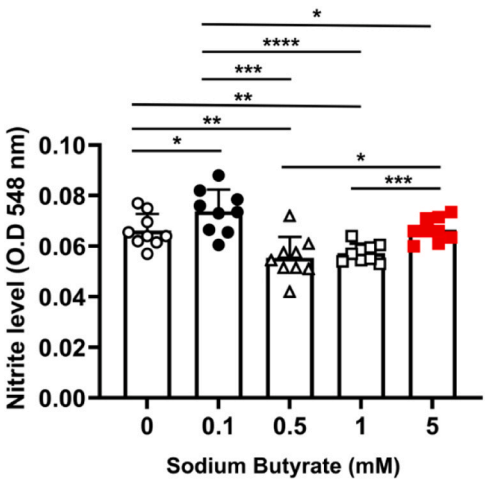

Fig. 7. Effects of sodium butyrate on endothelial NO production in HUVECs. HUVECs were treated with increasing concentrations of sodium butyrate (0, 0.1, 0.5, 1, 2, 5, and 10 mM), and nitrite levels in the culture medium were quantified as an index of NO production. Data are presented as mean ± SD (n = 8). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

community composition. By integrating *in vivo* vascular physiology, *in vitro* endothelial assays, and comprehensive microbiome analyses, our findings suggest that liraglutide-enhanced endothelial NO bioavailability is closely associated with liraglutide-induced remodeling of the gut microbiota. This microbial restructuring is accompanied by enrichment of SCFA-producing taxa, which may contribute to a metabolic environment that supports endothelial homeostasis, thereby providing a mechanistic link between liraglutide-induced microbiome changes and improved vascular function in diabetes.

Consistent with previous reports demonstrated that endothelium-dependent relaxation was impaired in diabetes [21–23], db/db mice exhibited a significant reduction in endothelium-dependent relaxation of mesenteric arteries. Liraglutide treatment restored this response without altering endothelium-independent relaxation or vasoconstrictor sensitivity, indicating a selective improvement in endothelial function (Fig. 2). These findings align with clinical and experimental evidence showing that GLP-1RA improve endothelial homeostasis, reduce oxidative stress, and promote eNOS activation [11,24].

We further observed that liraglutide increased eNOS phosphorylation at Ser1177 and restored NO production in HUVECs exposed to high-glucose conditions, indicating improved endothelial NO signaling under diabetic stress (Fig. 3A). Consistent with these cellular findings, liraglutide treatment significantly improved endothelium-dependent relaxation in vivo. However, endothelial function in the intact organism is influenced by multiple systemic factors beyond endothelial cells alone. To further characterize physiological changes associated with liraglutide treatment, we examined alterations in gut microbiota composition.

Previous studies have demonstrated that microbiome-derived metabolites, particularly short chain fatty acids (SCFAs), enhance eNOS phosphorylation, promote NO availability, and attenuate oxidative stress in vascular tissues [25–27]. Importantly, db/db mice exhibit marked gut dysbiosis with reduced abundance of SCFA-producing taxa [28–30], which are associated with impaired endothelial NO bioavailability and vascular dysfunction. In this context, the liraglutide-induced restoration of SCFA-producing bacterial populations observed in our study suggests a microbiome-associated contribution to the improved endothelial function. Together, these findings indicate that liraglutide-mediated microbiome remodeling is closely associated with enhanced endothelial NO signaling and vascular protection in diabetes.

Diabetes induced a significant reduction in α-diversity and generated a clearly dysbiotic microbial profile characterized by the loss of

beneficial SCFA-producing taxa and an expansion of *Clostridium*-related genera associated with metabolic and inflammatory dysfunction. Liraglutide substantially reversed these abnormalities. The treatment restored α-diversity and shifted β-diversity patterns toward those of non-diabetic controls (Fig. 4). Taxonomic analyses revealed increases in bacterial groups linked to metabolic and vascular health, including members of Lachnospiraceae, Oscillospiraceae, and *Lactobacillus* (Figs. 5 and 6). Many species within these groups are established producers of SCFAs, particularly butyrate [31–34], suggesting that liraglutide may help re-establish a microbial environment capable of generating metabolites relevant to endothelial recovery. These findings are consistent with emerging evidence that GLP-1RAs can modulate gut microbiota composition [35–38], although our results extend previous observations by linking these microbial changes specifically to improvements in endothelial function.

The functional relevance of these microbial shifts is supported by our *in vitro* experiments showing that physiologically relevant concentrations of butyrate modestly enhanced NO production in endothelial cells, whereas intermediate doses suppressed NO generation and high doses returned NO to baseline, indicating a concentration-dependent effect (Fig. 7). Consistent with our findings, previous studies have shown that the vascular actions of butyrate are not purely linear with dose. Very low, tissue-level concentrations of sodium butyrate promote endothelial migration and tube formation, whereas higher concentrations lose this pro-angiogenic effect or even become anti-angiogenic [39]. Together with reports that butyrate can directly modulate endothelial NO production and improve Ang II-induced endothelial dysfunction [40,41], these data support the concept that low, microbiota-derived levels of butyrate may promote eNOS activity and endothelial homeostasis, whereas higher, pharmacological doses exert distinct, inhibitory effects.

These results are compatible with prior studies showing that SCFAs influence endothelial physiology by engaging G-protein–coupled receptors and intracellular signaling pathways that regulate eNOS phosphorylation and oxidative balance [41–43]. Thus, restoration of SCFA-producing taxa following liraglutide therapy may contribute to improved vascular function in diabetic mice.

Taken together, our findings provide insight into how liraglutide confers vascular protection in diabetes beyond its glucose-lowering effects. The concurrent improvement in endothelial NO signaling and restoration of SCFA-producing gut microbial populations suggest that liraglutide-induced changes in the gut microbiome are closely associated with improved endothelial function. This microbiome-associated modulation represents an additional regulatory context that may support endothelial homeostasis under diabetic conditions. Given the central role of endothelial dysfunction in the development of diabetic vascular complications, these results highlight the therapeutic relevance of considering gut microbiota modulation in conjunction with GLP-1RA therapy.

This study has several limitations. The sample size for microbiome analysis was modest, and we did not directly test causality between liraglutide-induced microbial changes and vascular outcomes. In addition, circulating SCFA levels were not measured, and therefore the link between microbial metabolites and endothelial function remains associative. Nevertheless, the convergent *in vivo*, *in vitro*, and microbiome findings provide consistent evidence supporting a microbiome-associated contribution to the vascular benefits of liraglutide. Importantly, despite these limitations, this study is the first to demonstrate a clear association between liraglutide-induced improvements in endothelial function and the restoration of gut microbial composition in a diabetic model, thereby offering novel mechanistic insight into a GLP-1RA–microbiome–vascular axis.

In conclusion, our findings indicate that liraglutide improves endothelial function in diabetic mice in association with marked remodeling of the gut microbiome toward a composition enriched in beneficial, SCFA-producing taxa. These results provide a conceptual framework for understanding how GLP-1RA therapy may exert vascular benefits beyond glycemic lowering and highlight the potential therapeutic relevance of targeting gut microbial ecosystems to alleviate endothelial dysfunction in diabetes. Future studies investigating microbial metabolites, receptor signaling, and transplantation-based approaches will be valuable for establishing causal relationships and further defining this microbiome–vascular interface

CRediT authorship contribution statement

Eun Yi Oh: Methodology, Investigation, Software. **Soo Hwan Suh:** Investigation, Software, Validation, Writing- Original draft preparation. **Seonhee Byeon:** Investigation, Software, Validation. **Jooyong Lee:** Investigation. **Young-Ho Lee:** Validation and Reviewing. **Soo-Kyoung Choi:** Conceptualization, Writing- Original draft preparation, Writing-Reviewing and Editing, Supervision.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85–23, 2011) and were approved by the Animal Experimental Committee of Yonsei University College of Medicine (Approval No. 2024–0203, Seoul, Korea).

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Declaration of Competing Interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2026.119042.

Data availability

Data will be made available on request.

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