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Oral Delivery of a Therapeutic Gene Encoding Glucagon-like Peptide 1 to Treat High Fat Diet-induced Diabetes

Md Nurunnabi a,b,†,1, Seung-Ah Lee c,1, Vishnu Revuri a, Yong Hwa Hwang c, Sung Hun Kang a, Minhyung Lee c, Sungpil Cho d, Kwang Jae Cho c, Youngro Byun f, You Han Bae b,* Dong Yun Lee c,* and Yong-kyu Lee a,*

a Department of Chemical and Biological Engineering, Korea National University of Transportation, Chungju 380-702, Republic of Korea
b Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, College of Pharmacy, University of Utah, Salt Lake City, 84112, USA
c Department of Bioengineering, College of Engineering, and BK21 PLUS Future Biopharmaceutical Human Resources Training and Research Team, and Institute of Nano Science & Technology (INST), Hanyang University, Seoul 04763, Republic of Korea
d KB BioMed Inc., Chungju 380-702, Republic of Korea
e Department of Otolaryngology, Head & Neck Surgery, The Catholic University of Korea, College of Medicine Uijeongbu St. Mary’s Hospital, Kyunggi-Do 480-717, Republic of Korea
f WCU Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, and College of Pharmacy, Seoul National University, Seoul 151-742, South Korea
† Current address: Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129, United States of America
1 Contributed equally to this study as first authors
*Correspondence to: you.bae@utah.edu (Y.H. Bae), dongyunlee@hanyanag.ac.kr (D.Y. Lee), or leeyk@ut.ac.kr (Y. Lee)
Abstract

The number of people suffering from insulin-independent type 2 diabetes mellitus (T2DM) is ever increasing on a yearly basis. Current anti-diabetic medications often result in adverse weight gain and hypoglycemic episodes. Hypoglycemia can be avoided with glucagon-like peptide (GLP)-1 receptor agonists, which are expensive and require daily injections that may result in immune activation. This study demonstrates the use of non-viral vector based oral delivery of GLP-1 gene through enterohepatic recycling pathways of bile acids. Oral administration of the plasmid DNA (pDNA) encoding GLP-1 decreased diabetic glucose level to the normoglycemic range with significant weight reduction in a high-fat diet (HFD) induced diabetic mouse model and a genetically engineered T2DM rat model. This novel oral GLP1 delivery system is an attractive alternative to treat late stage T2DM conditions that require repeated insulin injection and can potentially minimize the occurrence of hypoglycemic anomalies.

KEYWORDS: Diabetes; Peptide nanoparticle; Oral Delivery; Hypoglycemia; Anti-obesity
1. Introduction

Glucagon like peptide-1 (GLP-1), an incretin, is a biological insulin secretagogue that helps to stimulate insulin secretion by pancreatic β-cells [1,2]. GLP-1 secretions can improve both insulin sensitivity and glucose tolerance in type 2 diabetes mellitus (T2DM) patients, helping them to avoid hypoglycemic episodes [3]. GLP-1 is also known as an agonist on body weight through loss of appetite thus food consumption. Nevertheless, the short half-life (< 5 min) and rapid clearance of GLP-1 limit its therapeutic activity and have resulted in development of the exogenous analogue, Exendin-4 (Exenatide), and chemically modified GLP-1s (e.g., Liraglutide and Taspoglutide) [4–6]. However, occasional development of antibodies against GLP-1 analogs from repeated subcutaneous (SC) administration somehow limits their therapeutic applications [7,8].

Non-viral gene delivery of a GLP-1 pDNA complex with a cationic polymer (polyplex) through intravenous (IV) or oral (PO) routes has shown potent therapeutic effects on diabetic animal models [9–11]. However, cationic polymers, which are widely used to condense pDNA, have both acute systemic side effects and cellular toxicity after IV administration [12–14]. Progress in oral gene therapeutics has also been plagued by the harsh environment of the GI tract, mucosal barriers, and tightly connected intestinal epithelial cells, all of which contribute to the degradation and/or poor permeability of orally administered biotherapeutics [15,16]. Although chitosan, a cationic polysaccharide, has been used for oral delivery of pDNA, their delivery mechanism into the blood remains unknown. Moreover, passive delivery of pDNA through temporally interrupted tight junction by chitosan makes the outcomes difficult to reproduce due to interactions of the cationic carrier with anionic components in the GI tract [17].
Endocytotic, transcytotic, and exocytotic transporters on the surface, in the cytosol of enterocytes and hepatocytes play a central role in recycling bile acids from the GI tract to the liver with an efficiency of >90% [18–20]. Bile acid binding protein in cytosol also known as cytosolic ileal bile acid binding protein (IBABP) helps the bile acid for transcellular movement to the basolateral movement. Studies have shown that conjugation of macromolecules to bile acids can facilitate their oral absorption, particularly in the ileum, where apical sodium-dependent bile acid transporters (ASBT) are predominantly expressed [21,22]. Selective interactions between bile acids and ASBT and import proteins like iBAT (ileal bile acid transporter) assist the formation of large vesicles and avoid lysosomal pathway [23].

In this study, we report a non-viral oral GLP-1 pDNA delivery system to treat T2DM. To accomplish oral delivery of the therapeutic gene, we synthesized a nano-sized gene complex (HTCA-GLP1) by initially electrostatic interaction of the plasmid DNA (pDNA) with bPEI (PP) followed by coating the surface of PP with Heparin-Taurocholic acid (HTCA) conjugates. Studies showed that molecules conjugated with bile acids like Taurocholic acid (TCA) can be exported by cellular exocytosis [24,25]. The therapeutic efficacy of HTCA-GLP1 complex was evaluated in T2DM animal models. The results were promising; oral administration of HTCA-GLP1 sustained blood glucose level in the normal range without any noticeable side effects. The oral gene delivery system described here has the potential to prevent from hypoglycemic episodes over a prolonged period in T2DM by the periodic intake of this gene capsule.

2. Materials and methods

2.1. Monitoring of blood glucose after administration of HTCA-GLP1. Six-week-old female ZDF rats (Orient Bio Inc., Seoul, Republic of Korea) were purchased and housed in a metal cage with free access to food and water. All animal experiments were approved by the Institutional
Animal Care and Use Committee (IACUC) of Hanyang University, and Korea National University of Transportation and all protocols were performed in accordance with NIH guidelines. Blood glucose levels were >300 mg/dl as measured by a portable blood glucose monitoring device (Accu-check, Roche Diagnostics, Basel, Switzerland). Rats were divided into two groups for oral delivery of the gene complex: one group was administered pβ-SP-GLP1 (pGLP1) only (n=3), while the other group was administered the HTCA-GLP1 gene complex (n=5). Rats were fasted overnight (12 hr) before oral gavage delivery of 100 µg of pGLP1 or HTCA-GLP1. Blood glucose levels were monitored by collecting blood at predetermined time intervals, and blood glucose meters (Accu-check, USA) were used to measure non-fasting blood glucose levels. After 21 days, rats were sacrificed, and certain organs were isolated. Tissues were fixed in 10% formalin and embedded in paraffin to slice the tissue into 15-µm-thick sections. Sections were subjected to indirect immunohistochemical staining. Mouse monoclonal (8G9) primary antibodies were used to detect GLP-1 (Abcam ab26278), and the process was conducted according to the manufacturer’s instructions. GLP-1 expression was directly observed by confocal laser microscopy.

2.2. Glucose monitoring in a type II diabetes model after administration of the pGLP-1 formulation. Seven-week-old male C57BL/6J mice were purchased from Orient Bio Inc. (Seoul, Republic of Korea) and fed high-fat diet food (rodent diet with 60% of kcal contributed by fat) for 3 months to develop insulin resistance. Mice were housed in metal cages with free access to food and water, and body weight was continuously monitored until it measured at least 50 g and blood glucose level measured at least 250 µg/dL for 3 consecutive days.

Mice were then divided into two groups (control and oral groups). HTCA-GLP1 complex (equivalent to 100 µg of the pGLP1 gene/mouse) was delivered orally after a 6 hr fast to mice in
the oral group once every 4 days for a total of 32 days (eight total administrations). Non-fasting blood glucose level, fasting blood glucose level (6 hr fast), body weight, and food consumption of the mice were monitored for 32 days. Blood samples were collected from each group at day 4, -3, -1, 0, 2, 5, 7, 10, 12, 15, 17, 19 and 22 for single dose and at day 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 for multiple dosage (administered for 4 or 8 times) to investigate insulin and GLP1 concentrations after administration of the HTCA-GLP1 gene complex (100 µg equivalent of the pGLP1 gene/mouse). Insulin was measured using a mouse insulin ELISA assay kit (Alpco, Keewaydin Drive, Salem, NH 03079 USA) according to the manufacturer’s instructions. GLP1 was measured using a GLP-1 ELISA assay kit (EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer’s instructions.

2.3. Immunohistochemistry. Mice were euthanized, dissected, and selected organs were removed on day 3 after oral administration of the HTCA-GLP1 gene complex. Tissues were fixed in 10% formalin and embedded in paraffin to obtain serial sections of tissue that were 5 µm thick. The sections were deparaffinized with xylene and rehydrated through gradient ethanol immersion. The pancreas was treated with mouse monoclonal antibody to insulin (1:200; Abcam) and rabbit polyclonal antibody to GLP1 (1:200; Abcam) overnight at 4°C after antigen retrieval and blocking of the sections with 20% goat serum. Other organ samples were treated with rabbit polyclonal antibody to GLP1 primary antibodies (1:200; Abcam) overnight at 4°C after antigen retrieval and blocking with 20% goat serum. Then, secondary AlexaFluor® 574 goat anti-mouse (1:1,000; Invitrogen) and AlexaFluor® 488 goat anti-rabbit (1:1000; Invitrogen) were added, and the sections were kept at room temperature for 1 h in the dark. Samples were then washed with PBS and counterstained with DAPI medium (Vectashield H-1200; Vector Laboratories Inc.,
Burlingame, USA). Insulin and GLP1 expression was assessed by fluorescence microscopy (Eclipse TE2000-S, Nikon) with the emission filter of 575 and 488 nm respectively.

2.4. In vivo toxicity. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of The Catholic University of Korea College of Medicine, and all protocols were performed in accordance with NIH guidelines. SD rats (Orient Bio Inc., Seoul, South Korea) were fasted overnight before oral administration of the formulations. Different dosages of PP (100, 200, and 400 µg/rat) and HTCA-GLP1 (230, 460, and 920 µg/rat) were administered trans-orally or intravenously to the rats. One week after administration, CBC and serum biochemistry was analyzed by collecting 5 mL of whole blood from heart puncture at predetermined intervals. EDTA was added to 2 mL of blood to a final concentration of 10% EDTA in an anticoagulant-coated bottle for CBC. An automated hematology analyzer (Sysmex XE-2, 100, Japan) was used to count white blood cells, hemoglobin, hematocrit, and number of platelets. The supernatant obtained from centrifugation (3,000 rpm for 7 min) of the remaining 3 mL of blood was used to determine various parameters of liver and kidney function such as total protein (TP), albumin (ALB), globulin (GLOB), glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), blood urea nitrogen (BUN), creatinine (CRE), and alkaline phosphatase (ALP). To measure TP, 200 µL of serum was mixed with 800 µL Bradford reagent, and absorbance was measured at 600 nm after 10 min of incubation in the dark. BUN, CRE, GLOB, ALB, GPT, and GOT were assayed using an enzyme assay kit, and data were obtained using a spectrophotometer (Hitachi 7,600).

Hematoxylin/eosin (H&E) staining was performed to determine the toxicity of the gene complex to various organs. Briefly, harvested organs (heart, liver, lung, kidney, and spleen) were fixed with 4% paraformaldehyde for 4 hr. After paraffin embedding of the fixed organs, tissue
sections were placed on a glass slide and stained with H&E. Slides were examined under a microscope, and pathological assessment was then performed.

CBC studies were also performed in animals fed relatively high doses of HTCA-GLP1. SD rats (n=6) were orally administered with HTCA-GLP1 gene complex (15 mg/kg and 30 mg/kg), and blood was collected serially after 7, 14, 28 and 56 days to determine the CBC profiles of these rats.

3. RESULTS

3.1. Preparation and characterization of HTCA-pDNA. Taurochoic acids (TCA) were linked with low molecular weight heparin through covalent chemistry by formation of amide bond between carboxyl group of heparin and hydroxyl group of TCA. The hydroxyl group of TCA was modified with ethylenediamine to introduce primary amine group, which was further modified with NHS and EDC. A targeted oral GLP-1 gene delivery system was synthesized to treat T2DM. pDNA (pEGFP-N1 or pβ-SP-GLP-1) were initially condensed with branched polyethyleneimine (bPEI) at an N/P ratio of 5:1 to form a stable polyplex (PP; Figs. 1a-b). HTCA was synthesized and wrapped around the surface of PP to synthesize anionic gene complex, HTCA-pDNA (Figs. S1 and S2; Fig. 1c). Wrapping of HTCA conjugates over the PP was performed to mask the cationic surface, protect the pDNA from gastrointestinal degradation, and facilitate intestinal absorption of pDNA. Figure S3 represents the structure of the GLP-1 cDNA encoded in the furin cleavage site, next to signal peptide. Transmission electron microscopy (TEM) images depict the formation of a thin corona of HTCA surrounding the dark core of the PP (Fig. 1a). The developed HTCA-pDNAs possessed a negative surface charge of -35 mV and size around 160 nm (Fig. 1c). The shielding of pDNA within the cores was confirmed by the absence of pDNA bands in the PP and HTCA-pDNA lanes on agarose gels (Fig.
Moreover, EtBr displacement assay showed a similar degree of pDNA shielding in both HTCA-pDNA (~99%) and the PPs (~96%) (Fig. S4b). Furthermore, HTCA-pDNA maintained their stability for up to 24 hr, under gastric pH without significant change in size or zeta potential as shown in Fig. 1d, while PPs displayed changes in size and zeta potentials within 2 hr of incubation (Fig. S4a). Stability of the NP and HTCA-GLP1 in SIF and SGF were conducted over 24 hr of incubation. PP was not stable in either SIF neither SGF as the PP aggregates or dissociate demonstrate from the size analysis. However, HTCA-GLP1 was stable in both SIF and SGF as their particle size diameter were constant for while. The results indicate that the PP undergone excessive interaction with the gastric juice as the surface of the PP was highly cationic prior condensed with GLP1. The interaction of PP with the SIF and SGF solution results protonation of on the surface of PPs and that results either aggregation or dissociation. On the other hand, cationic charge of the PP reduced after condensed with the anionic GLP1 and that resulted less interaction with SIF and SGF (Fig. S5). We have also observed release profile of DNA from the PP and HTCA-GLP1 for 12 hr of incubation. DNA release was about 20% from both PP and HTCA-GLP1 in SIF solution where as DNA release was counted less than 10% in SGF solution as shown in figure S6.
Figure 1. Synthesis and analysis of the HTCA-pDNA gene complex. (a) Scheme depicting the synthesis of the HTCA-pDNA gene complex. The FE-TEM image (Left) confirms the formation of nano-sized PP (N/P 5:1). The FE-TEM image (Right) shows the presence of a HTCA coat on the surface of PP (scale=200 nm). The DNA bands in the agarose gel shows the absence of pDNA bands before and after coating with HTCA. (b) Analysis of size and surface charge of PP at different N/P ratios. (c) Effect of weight ratio of HTCA on the size and zeta potential of the HTCA-pDNA gene complex. (d) Time-dependent changes in the size and surface charge of HTCA-pDNA under gastric pH conditions (pH 2.0).

3.2. Cellular uptake, epithelial transportation, and gene expression. Sodium/taurocholate co-transporting polypeptides (NTCP) in the liver and ASBT receptors in the small intestines play a crucial role in the transport and uptake of TCA modified nano-carriers. Because HepG2 cells possess abundant number of TCA sensitive NTCP transporter on their surface, they were used
for evaluating the transfection studies in vitro [26]. GFP plasmid, pEGFP-N1 was used to compare their transfection efficacy of the carriers PP and HTCA- pEGFP-N1 in HepG2 cells. Cells treated with HTCA- pEGFP-N1 (PP with HTCA coating) displayed enhanced green fluorescence compared to the cells treated with PP (PP without HTCA coating). The presence of NTCP on the surface of HepG2 improved the cellular uptake and thereby improved the transfection efficacy of HTCA-pEGFP-N1 (Fig. 2a).

We have also studied effect of glucose level in GLP1 expression in vitro. In absence of glucose GLP1 expression was 2 and 6 pM from PP and HTCA-GLP1, respectively. However, in presence of 250 mM of glucose the expression of GLP1 increased to ~ 200 and 300 pM, from PP and HTCA-GLP1, respectively (Fig. S7). ASBT receptors in the ileal regions of the small intestine play an important role in the transport of TCA linked nanocarriers into the enterohepatic circulation [21,22]. To confirm that enhanced cellular uptake of HTCA-pDNA was mediated by interaction of TCA with the surface transporter, we assessed in vitro cellular uptake profiles of heparin-pDNA and HTCA-pDNA in ASBT over expressed MDCK cells (MDCK-ASBT). Rhodamine B (RB) was conjugated to the carriers to compare and distinguish their cellular uptake in vitro. The cells treated with RB-HTCA displayed a remarkable enhancement in RB red fluorescence compared to the cells treated with RB-heparin (Fig. 2b top). The absence of TCA on the surface of heparin limited the uptake of RB-heparin in MDCK-ASBT cells. Moreover, MDCK-ASBT cells transfected with RB-HTCA-pEGFP-N1 gene complex showed strong green fluorescence along with RB fluorescence, suggesting TCA mediated enhanced cellular uptake and transfection of RB-HTCA-pEGFP-N1 gene complex (Fig. 2b bottom; Fig. S8a).
Therapeutic activity of the orally administered drugs/bio macromolecules can be achieved only if they traverse through the transepithelial monolayers of the small intestines. To evaluate intestinal transport of HTCA-pDNA, we tested RB-HTCA-GLP1 using a Caco-2 transwell system, which closely mimics the securely packed intestinal epithelial monolayers (Fig. 2c top). RB-HTCA-GLP1 presented less RB red fluorescence than RB-PP in the monolayer of Caco-2 transwell system (Fig. 2c, bottom), suggesting that the TCA component facilitated the transport of RB-HTCA-GLP1 from the apical to the basolateral side on the monolayer of Caco-2 cells.

Next, we quantified GLP-1 expression from HTCA-GLP1 in both HepG2 and intestinal L cells (NCI-H716), the latter of which secrete GLP-1 in vivo. Both HepG2 and NCI-H716 cells treated with HTCA-GLP1 showed enhanced GLP-1 peptide expression (6-fold and 3-fold, respectively) compared to the expression of GLP1 in control cells (Fig. 2d). These results indicate the importance of HTCA in improving cellular uptake as well as gene transfection of pDNA in biological environments.

3.3. Oral uptake and biodistribution of HTCA-pDNA. The HTCA-pDNA was designed to target ASBT receptors in the small intestine and enhance cellular transfection and therapeutic efficacies. In this context, we assessed the oral absorption profiles and real-time biodistribution of HTCA-eGFP in vivo by conjugating the fluorescence dye Cy5.5 to the nano-carrier HTCA-eGFP. Biodistribution of Cy5.5 labelled HTCA-eGFP in a mouse was monitored for 24 hr. The results indicate that HTCA-eGFP accumulated in the ileum at 3 hr and reached saturation by 7 hr, followed by intermittent fluorescence for up to 19 hr (Fig. 3a). The ex vivo carrier distribution profiles showed strong fluorescence of Cy5.5 in the ileal region of the small intestine (Fig. 3b), along with fluorescence in the liver. The fluorescence from the liver is due to the enterohepatic circulation of bile acids, which allowed HTCA to traverse from the small intestines and
accumulate in the liver. From these studies, we inferred that the gene complex was taken up by enterocytes in the ileum, supporting ASBT-mediated transport of the gene complex.

Figure 2. Cellular uptake, epithelial transport, and GLP1 expression of HTCA-GLP1. (a) Confocal overlay, bright field, and fluorescence images of HepG2 cells depicting the transfection efficacy of eGFP plasmid pEGFP-N1. The cells treated with HTCA-pEGFP-N1 displayed enhanced cellular transfection with brighter green fluorescence from the cells (Scale=50 μm). (b) Cellular uptake in the ASBT receptor-overexpressing MDCK cell lines (MDCK-ASBT). Cells treated with Rhodamine B-HTCA displayed enhanced cellular uptake compared and pEGFP-N1 transfection (Scale=50 μm). (c) Transepithelial transport of the nanocarriers across the Caco-2 transwell monolayers. Rhodamine B-labeled HTCA-GLP1 exhibited higher transportation and thus displayed lower cellular fluorescence compared to Rhodamine B-labeled PP (Scale=100 μm). (d) GLP-1 ELISA assay was used to quantify the GLP-1 expression in HepG2 and NCI-
H716 cells; very high expression was observed in HTCA-GLP1 treated cells compared to PP or GLP1-1 alone.

Later, we studied the transfection efficacy of orally administered HTCA-eGFP in different tissues to investigate the ability of HTCA in shielding the orally delivered therapeutic gene from GI degradations in vivo. We further compared the transfection efficacy and eGFP gene expression from PP, heparin-eGFP, and IV HTCA-eGFP with PO HTCA-eGFP. The animals treated with PP displayed non-specific eGFP expression in the duodenum and jejunum (Fig. 3c). This non-specific absorption and expression of eGFP from PP were likely due to electrostatic interactions of PP with negatively charged glycocalyx in mucin and result in destabilizing the gene complex.
Figure 3. Biodistribution of HTCA-eGFP. (a) *In vivo* optical imaging depicting the time-dependent biodistribution of Cy5.5-labeled HTCA-eGFP; (b) *ex vivo* images show the high accumulation of Cy5.5-labeled HTCA-eGFP in the ileum. (c) eGFP expression of HTCA-eGFP (oral and intravenous), demonstrating higher uptake in the liver and ileum (scale=100 µm), and (d) quantitative fluorescence analysis by ELISA for understanding comparative distribution.

Heparin-eGFP also displayed non-specific absorptions in the duodenum and liver. However, mice PO administered with HTCA-eGFP displayed enhanced eGFP expression in the ileum and liver (Fig. 3d). This specific expression of eGFP in the ileum reflects the affinity of HTCA for ASBT and correlating with the *in vivo* carrier mediated biodistribution of HTCA-eGFP. However, the liver distribution is contributed by the TCA mediated transport of HTCA-eGFP to the liver where NTCP receptors aid in the uptake and expression of eGFP from these carriers. IV administered HTCA-eGFP also showed enhanced eGFP expression in the liver but not in the intestinal tissues (Fig. 3c). This confirms the TCA mediated uptake of HTCA-eGFP in the liver. Together, these results confirm HTCA-guided active transport and tissue-specific (ileum, liver) expression of pDNA (here eGFP) from PO administered HTCA-eGFP.

3.4. Regulation of blood glucose by orally delivered HTCA-GLP1. We tested the regulation of blood glucose by orally delivering HTCA-GLP1 in a T2DM Zucker diabetic fatty rat model (ZDF). A single dose of orally delivered HTCA-GLP1 sustained normal blood glucose levels around 150 mg/dL for at least 21 days (Fig. 4a) with negligible changes in body weights (Fig. 4b). Immunohistochemistry studies revealed marked expression of GLP-1 in intestinal tissues after administration of HTCA-GLP1 compared to the control group treated with GLP1 plasmid.
Moreover, insulin secretion around pancreatic islets was observed in rats treated with HTCA-GLP1 (Fig. 4c).

Figure 4. Blood glucose level, body weight and immunohistochemistry study in ZDF rat. (a) Blood glucose levels in the ZDF rats after orally administering GLP1 and HTCA-GLP1. (b) Body weights of the ZDF rats after orally administering GLP1 and HTCA-GLP1. (c) Immunohistochemistry staining confirmed the enhanced expression of GLP1 in different organs and enhanced expression of insulin in pancreas after the oral administration of HTCA-GLP1 (Scale=100 µm).

High-Fat Diet (HFD) is known to induce insulin resistance in T2DM patients. We evaluated the regulation of blood glucose by HTCA-GLP1 in a high-fat diet (HFD)-induced diabetes mouse model (Figs. S9a, b). After a single oral administration of HTCA-GLP1, blood glucose levels of HFD mice reached a minimum level at day 3 (75.4 ± 5.5% vs. untreated control group) and gradually recovered to the hyperglycemic condition (Figs. S9c, d). Similar blood glucose-
lowering effects and body weights were observed after administration of either oral or IV HTCA-GLP1 (Figs. S9e, f). Immunohistochemistry analysis of intestinal tissues showed that enhanced GLP1 expression was presented in the mice orally administered with HTCA-pGLP1 than control or IV administered HTCA-GLP1. (Fig. S10a). Pancreas from the orally delivered HTCA-pGLP1 groups displayed higher number of GLP-1 positive and insulin secreting cells in the pancreatic islets than the mice administered with IV HTCA-GLP1 (Fig. S10b). These results conclude the potent efficacy of a single oral dose of HTCA-GLP1 in maintaining the blood glucose levels in a normal range. However, the temporal glucose-lowering effect of a single dose HTCA-GLP1 suggests that repetitive administrations are required to obtain an extended effect in HFD-induced diabetic animals.

3.5. Repetitive and long-term administration of HTCA-GLP1. To maintain normal blood glucose level over an extended time period, we repeatedly administered HTCA-GLP1 orally to HFD-induced diabetic mice. Normal non-fasting blood glucose levels were maintained in mice treated every four days (4 or 8 treatments) for 32 days by oral administration of HTCA-GLP1 (Fig. 5a).

The blood glucose levels in the mice administered with four doses of HTCA-GLP1 resulted in reverting to the hyperglycemic condition after about 7 days of discontinuation of oral HTCA-GLP1. Interestingly, the mice administered with eight doses of HTCA-GLP1 maintained a normal blood glucose level for an extended time period with a substantial reduction in body fat (Figs. 5b, e). However, the amount of food ingested by the two groups was similar (Fig. 4c).

Furthermore, long-term administration of HTCA-GLP1 (8 doses) displayed noticeable two-fold enhancement in plasma insulin and GLP1 secretion in the HFD mice (Fig. 5d). It is noteworthy to mention that plasma insulin levels were higher in the animals fed with HFD compared to the
animals fed with normal diet (ND). This could be due to the high fat content in the food, which might induce the pancreas to secrete higher amounts of insulin in order to overcome insulin resistance (Fig. 5d).

Immunohistochemistry analysis confirmed the interaction of insulin and GLP1 in pancreatic islets (Fig. 5f). While insulin resistance in HFD mice resulted in weaker expression of insulin-positive cells in pancreatic islets, mice administrated HTCA-GLP1 showed enhanced secretion of insulin around pancreatic islets, due to expression of GLP1 from HTCA-GLP1.

Moreover, the PO administered HTCA-GLP1 displayed enhanced expression of GLP1 in the small intestine, liver, and kidneys, which is significantly higher than that of normal diet rat (Fig. 5g and Fig. S10). The presences of GLP1 expression in kidney and spleen also demonstrate that the secreting proteins circulate through the systemic circulation of the mice. Together, these results demonstrate that continuous administration of HTCA-GLP1 can maintain a normal glucose level in a high-fat diet-induced T2DM mouse model.

3.6. Toxicology profiles of HTCA-GLP1. To compare the cellular toxicity of PP and HTCA-GLP1 polyplex, in vitro cytotoxicity assays were performed using three different cell lines: HepG2, Caco-2, and MDCK-ASBT. In HepG2 and Caco-2 cells, HTCA-GLP1 had negligible cellular toxicity regardless of the N/P ratio, compared to the cellular toxicity of PPs (Figs. S11a, b). Though HTCA coated with lower N/P ratios (<10) show much less cellular toxicity than uncoated in MDCK-ASBT cells, HTCA-GLP1 synthesized using a higher N/P ratio (≥ 10) caused significant cell death (Fig. S11c), perhaps due to imperfect layering of HTCA around the PPs, which could result in toxicity due to exposure to cationic bPEI.
Figure 5. Repeated administration of HTCA-GLP1 to HFD-induced diabetic mice. (a) Non-fasting blood glucose profile in HFD mice after multiple administrations of HTCA-GLP1. (b) Body weight profile of HFD mice after HTCA-GLP1 administration. (c) Food uptake profile in HFD mice after either 4 or 8 administrations of HTCA-GLP1. (d) Plasma insulin (top) and GLP1 (bottom) levels in mice fed a normal diet, HFD, or HFD treated with HTCA-GLP1. (e) Representative image of a HFD mouse (top) and HFD mouse treated with HTCA-GLP1 (bottom) (f) Immunohistochemical analysis of insulin and GLP1 expression in pancreatic islets. (g)
Immunohistochemical comparison of small intestine components, kidney, liver, and spleen among mice fed a normal diet, HFD, or HFD treated with HTCA-GLP1. Scale=100 μm.

To further examine the toxicity of our HTCA-GLP1 oral gene delivery system, we performed *in vivo* survival analysis, complete blood count (CBC), tissue histopathology, and evaluated serum biochemistry parameters. The survival rate of SD rats treated with orally delivered HTCA-GLP1, even at the high dose of 920 µg/rat, was 100%, while no animals administered with equivalent dose of free bPEI survived. Dose dependent survival after IV administration of HTCA-GLP1 was observed (Fig. 6a, Fig. S11d). This could be due to the unpacking of the HTCA-GLP1 in the blood stream resulting the bPEI mediated toxicity. The liver and kidney activity studied by serum biochemistry parameters (Fig. 6b, c), and CBC (Figs. 6d, f) were similar between control and experimental groups, indicating that HTCA-GLP1 had negligible toxicology effects in the biological system. Histopathology studies revealed acute inflammation in the lung tissues of rats administered with non-coated GLP1 complex (IV) and a high dose of HTCA coated GLP1 complex at (920 µg/rat). However, no inflammation was observed for lower doses of HTCA-GLP1 (Fig. 6e). No significant changes in CBC profile were observed after oral administration of HTCA-GLP1 at doses of 15 mg/kg or 30 mg/kg for 56 days (Fig. S12).
Figure 6. *In vivo* toxicity, complete blood count test, serum biochemistry and histopathological analysis. (a) Kaplan-Meier survival plot of PP and HTCA-GLP1 (both oral and IV) in SD rats. (b & c) Total protein and albumin test results (Units: BUN (mg/dL) CRE (mg/dL) T-P (g/dL) ALB (g/dL) GLOB (g/dL) GOT (unit/L) GPT (unit/L) ALP (unit/L) TB (mg/dL)) to determine the liver and kidney activity after oral administration of PP and HTCA-GLP1. (d&f) Complete blood count profile (Units: WBC (10⁹/L) Hb (g/dL) Hct (%)PLT (10⁹/L)) of mice treated with PP and HTCA-GLP1 (oral and IV). (e) Immunohistology patterns in different organs after administration of PP and HTCA-GLP1 through either oral or IV routes (scale=100 μm).

3.7. Cellular transport mechanism of HTCA-GLP1 on the surface of the ileum. Interaction between ASBT and TCA facilitates the transport of nanomaterials conjugated with TCA to the
ileum [27,28]. Al-Hilal et al. reported that transport of low-molecular weight heparin-tetraDOCA through interactions with ileal bile acid binding proteins (IBABP) resulted in the formation of vesicular structures and the evasion of endo/lysosomal maturation [23]. We therefore conjugated quantum dot with TCA (QD-TCA) to investigate the importance of TCA in the mode of uptake and transport of TCA conjugated nanoparticles in the ileum. Tissue TEM images presented in Fig. S13 depicts the formation of vesicles in the ileum after the oral delivery of QD-TCA. Moreover, the microvilli were intact and did not induce tight junction openings (Fig. S13, mid). These results indicate that the TCA conjugated nano-carriers could possibly traverse through the receptor mediated transcytosis but not paracellular or intercellular transport.

4. DISCUSSION

GLP-1 gene therapy is a promising solution for treating T2DM because GLP-1 secretions can assist insulin secretion based on the quantity of food ingested and thereby avert glycemic and hyperinsulinemic anomalies [9,29–32]. In pursuit of this goal, we developed a targeted oral GLP-1 gene delivery system that can maintain blood glucose level in the normoglycemic range for an extended period of time by periodic administration. By engineering our non-viral vector with HTCA, we were able to prevent GI degradation and also take advantage of the biological bile acid-based dietary lipid transport system to achieve active transport of HTCA-GLP1 [33–35]. Though positively-charged bPEI interacts strongly with subcellular moieties and initiates systemic toxicity [36,37], a layer of HTCA stabilized the gene complex and masked the net positive surface charge, thereby preventing non-specific interactions, as reflected in negligible systemic toxicity (Fig. 6). Because the rate of sub-mucosal diffusion and transport of negatively-charged particles are higher than those of positively-charged particles, the negative surface zeta
potentials of the gene complex may have prevented the gene complex from non-specific interactions with the mucosa and accelerated TCA-assisted active transport in the GI tract (Figs. 1d and 4) [38].

Compared to the passive transport mechanisms that are currently being investigated for oral gene therapy, we believe that active transport of the non-viral vectors will improve the bioavailability of therapeutic genes and result in therapeutic efficacy at low doses (Fig. 4c). Preferential uptake of the HTCA-GLP1 gene complex by MDCK-ASBT, HepG2, and Caco-2 cells and detection of vesicular structures in the ileum highlight the major role of ASBT in the active transport of the HTCA-GLP1 gene complex (Fig. 2 and Fig. S10).

After confirming the expression of the GLP1 peptide by intestinal L cells ad HepG2 cell lines, we evaluated the therapeutic efficacies of HTCA-GLP1 in ZDF- and HFD-induced T2DM animal models. Orally administered HTCA-GLP1 reduced the blood glucose to normal levels without inducing toxicity. However, the regulation period varied between models (ZDF, 21 days versus HFD, 3 days). This stark contrast might be due to differences in the shedding and turnover times of intestinal epithelia among animal models [39]. Repetitive short-term and long-term administration of HTCA-GLP1 reduced blood glucose level and improved plasma insulin and GLP1 secretion without inducing any abnormalities.

5. Conclusions

In summary, we developed an HTCA-mediated targeted oral gene delivery system for GLP-1 that can maintain normal blood glucose level for 21 days. We performed quantitative and qualitative analyses of our HTCA-GLP1 gene complex both in vitro and in vivo to determine the oral absorption mechanism, organ expression, and therapeutic efficacy. Based on our findings, we conclude that HTCA enhanced the stability of PPs and stimulated oral absorption through
ASBT in the small intestine. We were able to avoid the so-called “toxic bPEI” effect through first-pass mechanism and improve the bioavailability of the target gene. However, we also aim to explore our study on finding an safer cationic polymer to replace bPEI. Our non-viral oral GLP1 gene delivery system holds great promise for the treatment of T2DM. We continue investigation for understanding molecular biology and mechanism of action in details.

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Author contributions

M. N., D.Y. L., Y. L., and Y. H. B. designed the studies. M.N. conducted the synthesis, analysis, optimization, and in vitro studies. M.N., V. R., and S. H. K performed the in vitro cell experiments. M. N., S.-A. L., and Y.H.H. performed the in vivo experiments unless otherwise indicated. Data were analyzed by M. N., S. C., M.L., Y. B., and D.Y.L. D.Y.L., Y. L., and Y. H. B. supervised the project. All authors discussed the results and commented on the manuscript.

Competing financial interests

The authors declare no competing financial interests

References


Graphical abstract