Coagonist of GLP-1 and glucagon receptors ameliorates non-alcoholic fatty liver disease
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Abstract

Nonalcoholic fatty liver disease (NAFLD) is often associated with obesity and type 2 diabetes. Coagonist of glucagon-like peptide-1 receptor (GLP-1R) and glucagon receptor (GCGR) are under clinical investigation for the treatment of obesity and type 2 diabetes. In this study, we have demonstrated the effect of a balanced coagonist in the treatment of NAFLD using mice models. GLP-1R agonist exendin-4, glucagon, and coagonist (Aib2 C24 Chimera2) were administered to C57BL6/J mice, in which NAFLD was induced by carbon tetrachloride (CCl4) treatment after in high fat diet (HFD) feeding, and CDAHFD (choline-deficient, L-amino acid-defined, HFD). Repeated dose administration of coagonist significantly attenuated liver inflammation and steatosis induced by acute and long-term treatment with CCl4 in HFD-fed mice. Coagonist markedly attenuated the CDAHFD-induced expression of TIMP-1, MMP-9, TNF-α, MCP-1, COL1A1 and α-SMA. It also inhibited progression of hepatic steatosis and fibrosis in mice. Exendin-4 was better than glucagon, but coagonist was most effective in reduction of hepatic inflammation as well as steatosis. Coagonist of GLP-1R and GCGR improved NAFLD in C57BL6/J mice. This effect is mediated by reduction in lipotoxicity and inflammation in liver.

Key words: Coagonist of GLP-1 and glucagon receptors, GLP-1, Glucagon, Inflammation, NAFLD.
**Introduction**

Nonalcoholic fatty liver disease (NAFLD) is caused by deposition of lipids in liver (Brea and Puzo 2013). NAFLD may start from only steatosis, and can worsen to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. If left untreated, NAFLD can lead to total liver dysfunction and carcinoma (Bellentani et al. 2010). NAFLD is closely associated with obesity and insulin resistance, and the mortality due to NAFLD is mainly ascribed to cardiovascular complications (Younossi et al. 2016; Dyson et al. 2015). NAFLD is mainly treated with lifestyle changes, followed by pharmacotherapy that targets different aspects of metabolic syndrome like obesity, dyslipidemia and insulin resistance. Despite these multiple approaches, the efficacy of pharmacotherapy for NAFLD is minimal (Del et al. 2014; Schwenger and Allard 2014; Filozof et al. 2015).

Glucagon-like peptide-1 receptor agonists (GLP-1RA) are used for the treatment of type 2 diabetes. In addition, they demonstrate a significant weight loss in overweight and obese adults (Patel et al 2014). GLP-1RA, liraglutide treatment showed histological resolution of liver abnormality in NASH patients (Armstrong et al. 2016). Another proglucagon derived hormone, glucagon is a functional antagonist of insulin. It increases thermogenesis, lipolysis and fatty acid oxidation (Habegger 2010). Development of coagonist of GLP-1R and GCGR is a recent therapeutic strategy for achieving optimum control of type 2 diabetes associated with obesity. Indeed, studies using Aib2 C24 chimera2, a balanced Glucagon-like peptide-1 receptor (GLP-1R)/Glucagon receptor (GCGR) coagonist have demonstrated that, balancing the opposing effects of GLP-1R and GCGR activation reduce diabetes and obesity in animal models (Day et al. 2009). Oxyntomodulin endogenous coagonist of GLP-
1R and GCGR showed weight loss, and increased energy expenditure in humans (Cohen et al. 2003).

Hepatic lipogenesis and insulin resistance cause lipotoxicity, oxidative stress and induce proliferative and apoptotic changes typically observed in NAFLD (Bechmann et al. 2012). Earlier, we have observed that GLP-1R and GCGR coagonist treatment improved insulin sensitivity, and reduced hepatic and circulating lipids in HFD fed C57BL6/J mice (Patel et al. 2013). This effect was partially independent of food intake or body weight lowering effect of the coagonist (Patel et al. 2013). It suggests that a direct hepatic effect of coagonist that can be exploited for treatment of NAFLD. Hence, in this study we investigated the effect of coagonist, exendin-4 (a GLP-1RA), and glucagon in NAFLD induced by carbon tetrachloride (CCl₄) and high fat diet (HFD), and choline-deficient, L-amino acid-defined high fat diet (CDAHFD).

**Materials and methods**

**Chemicals and Reagents**

Aib² C24 Chimera2 (H¹SQGT⁵FTSDY¹⁰SKYLD¹⁵EQAAK²⁰EFIAW²⁵LMNT-NH²) the coagonist of GCGR and GLP-1R, exendin-4 and glucagon were synthesized at Zydus Research Centre, Ahmedabad, India. All other chemicals and reagents were purchased from Sigma- Aldrich chemicals, USA, unless stated otherwise. Coagonist, exendin-4 and glucagon were dissolved in normal saline. CCl₄ was formulated in corn oil (5%v/v).
Animals

Male C57BL6/J mice (6-8 weeks old) were obtained from the Animal Research Facility of Zydus Research Centre, Ahmedabad, India. They had free access to food and water and were kept on a 12 h light–dark cycle. The procedures for animal use and experimentation were reviewed and approved by Institutional Animal Ethics Committee (as per CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India) of Zydus Research Centre, which is an AAALAC accredited facility. All the procedures were done in accordance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health, USA. Chow-fed control animals were maintained on chow diet. All the animals were anesthetized using 2% isoflurane. Animals were bled by retro-orbital puncture under anesthesia. Animals were sacrificed under 5% isoflurane anesthesia.

Induction of hepatic inflammation after carbon tetrachloride administration in high fat diet-fed mice

Animals were fed with a HFD (60% fat by kcal, D12492; Research Diets, Inc., New Brunswick, NJ) for 20 weeks. We have used CCl₄ induced hepatic inflammation in DIO mice as it resembles key distinguishing characters of human liver fibrosis, i.e. inflammation, regeneration, fibre-formation and potentially fibrosis regression (Liedtke et al. 2013; Allman et al. 2010). Owing to this similarity, carbon tetrachloride induced liver inflammation is most commonly used approach to induce experimental liver fibrosis in preclinical models.
In acute experiment, HFD-fed mice were randomized based on body weight. Hepatic inflammation was induced by a single dose of CCl$_4$ (0.5 mL.kg$^{-1}$, i.p.). Treatment of either exendin-4 (50 µg.kg$^{-1}$, s.c.) or glucagon (150 µg.kg$^{-1}$, s.c.) or coagonist Aib2 C24 chimera 2 (150 µg.kg$^{-1}$, s.c.) was given twice a day starting two days prior to the administration of CCl$_4$, and continued for next three days after CCl$_4$ dose. The doses of exendin-4, glucagon, and coagonist selected using pilot studies based on literature reports (Day et al. 2009; Wang et al. 2014; Patel et al. 2017b). All animals were bled through retro-orbital puncture under isoflurane anesthesia 72 h after CCl$_4$ administration and plasma samples were separated for estimation of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and triglycerides (RCFL, Delhi, INDIA).

In chronic experiment, HFD–fed mice for 20 weeks were randomized based on body weight. Chronic hepatic inflammation was induced by twice a week intraperitoneal administration of CCl$_4$ (0.5 mL.kg$^{-1}$, i.p.) in corn oil for six weeks. Treatment of either vehicle or exendin-4 (50 µg.kg$^{-1}$, s.c.) or glucagon (150 µg.kg$^{-1}$, s.c.) or coagonist Aib2 C24 chimera 2 (150 µg.kg$^{-1}$, s.c.) was given twice a day for six weeks. Animals were bled and sacrificed 72 h after last dose of CCl$_4$. Plasma samples were separated for estimation of AST, ALT, glucose and triglycerides (RCFL, Delhi, INDIA). Thereafter, mice were sacrificed and liver samples were isolated and stored at -70ºC for further analysis.

**Choline-deficient, amino acid-defined, high-fat diet (CDAHFD) -induced NASH**

Animals were fed with CDAHFD (0.1% methionine without choline and 60% fat, A06071302, Research Diet, New Brunswick, NJ, USA) for 8 weeks. Following which, they were randomized based on plasma AST and ALT and assigned to either vehicle or exendin-
4 (50 µg.kg\(^{-1}\), s.c.) or glucagon (150 µg.kg\(^{-1}\), s.c.) or coagonist Aib2 C24 chimera 2 (150 µg.kg\(^{-1}\), s.c.) treatment twice a day for next 8 weeks. We use this model because it is presented with steatosis and inflammation caused due to lipid accumulation, and not due to inflammation *per se*, as observed with CCL\(_{4}\)-induced toxic insult (Matsumoto et al. 2013). Hence, CCl\(_{4}\)-induced and CDAHFD-induced models were complimentary to each other, encompassing the pathology of clinically observed NAFLD. At the end of treatment, animals were bled and plasma samples were separated for estimation of AST, ALT, glucose and triglycerides (RCFL, Delhi, INDIA). Thereafter, mice were sacrificed and liver samples were isolated and stored at -70°C for further analysis.

**Hepatic lipid estimation**

Liver samples were homogenized using a Polytron PT3100 tissue homogenizer (Kinematica, Bohemia, NY, USA) in heptane-isopropanol-Tween mixture (3: 2: 0.01 v/v/v) and centrifuged at 1,500 \(\times\) g for 15 min at 4°C. The supernatant (the upper phase contained extracted triglycerides) was collected and evaporated using a nitrogen evaporator. The residue was solubilized in tritonX100:t-butyl alcohol (1:1). The triglyceride content was determined using a triglyceride kit.

**Hepatic hydroxyproline estimation**

Hydroxyproline content in the liver was measured using a modified colorimetric method (Reddy and Enwemeka 1996). Briefly, 50 mg of liver tissue was homogenized in 500 µL of phosphate buffered saline (PBS), and centrifuged (10000 r.p.m., 10 min at 4 °C). Supernatant (200 µL) was hydrolyzed in 200 µL of 4N NaOH and incubated for 30 min at 37 °C. Methyl red solution (5 µL) was added to each tube, and pH was adjusted to 7.0 using
2N HCl. Samples (400 µL) were diluted in PBS and oxidized with 200 µL chloramine-T at room temperature for 20 min. Ehrlich’s aldehyde reagent (200 µL) was added to and tubes were incubated at 60 °C for 30 min. Absorbance was measured at 550 nm. The concentration of hydroxyproline was determined using a standard curve prepared from known concentrations of hydroxyproline.

**Histological Examination**

Hepatic fibrosis and macrophages infiltration were examined in 10% formaldehyde-fixed paraffin embedded tissue sections. Hepatic fibrosis was assessed by Masson’s trichome staining method. Activated macrophages in liver i.e. kupffer cells, were visualized in liver sections stained with periodic acid–schiff–diastase (PAS-D). Fat deposition, was assessed using oil red O (ORO) staining technique in frozen tissue samples. NASH scoring was applied to the severity of hepatocellular steatosis, ballooning, inflammation and fibrosis in liver sectioned stained using hematoxylin and eosin (H&E) stain (Klener et al. 2005). The scoring for Masson’s trichome, ORO and PAS-D stain were graded as 0-4 (0: No abnormalities detected, 1: Minimal abnormality, 2: Mild abnormality, 3: Moderate abnormality and 4: Severe abnormality).

**Quantification of mRNA expression by real-time polymerase chain reaction**

Expressions of monocyte chemoattractant protein 1(MCP-1), matrix metallopeptidase-9 (MMP-9), tissue inhibitor of metalloproteinases-1 (TIMP-1), alpha-smooth muscle actin (α-SMA), collagen type 1 α 1 (COL1A) and tumor necrosis factor-α (TNF-α) and β-actin mRNA in liver tissue were assessed by RT-PCR at the end of the study. Total RNA was extracted with TRIzol reagent (Applied Biosystem, USA) according to the manufacturer's
instructions. Thereafter, first strand cDNA synthesis was performed using the High-Capacity cDNA reverse transcription kit (Applied Biosystem, USA). The resulting cDNAs were used for quantitative PCR using the QIAGEN Quanti Fast SYBR Green kit (Cat. No. 204052, Qiagen, Germantown, MD, USA). The qPCR was run in an ABIprism-7300 (Applied Biosystems, Foster City, CA, USA). Quantitation of the mRNAs was performed using the $2^{-\Delta\Delta Ct}$ method using β-actin as a housekeeping gene. All primers and sequence details are given in Table 1.

**Data analysis**

Data are represented as mean ± SD. Statistical significance was determined by one-way ANOVA for multiple comparisons with post hoc Dunnett’s test. P<0.05 was considered as significant. Histological score was evaluated using Kruskal–Wallis test followed by Dunn’s test for multiple comparisons. All data were analyzed by using GraphPad Prism version 7.03 (GraphPad Software, San Diego, CA, USA).

**Results**

**Coagonist reversed CCl₄-induced hepatic steatosis in mice**

A significant increase in plasma triglycerides, AST and ALT was observed after HFD with CCl₄ treatment in C57BL6/J mice, when compared to chow-fed mice (Fig. 1). Treatment with exendin-4 reduced plasma AST by 22.1±10.8% (Fig. 1B) and plasma ALT by 18.4±8.8 % (Fig. 1C). Glucagon treatment showed 12.6±15.3% reduction in AST and 10.9±12.4% reduction in ALT, against vehicle control (Fig. 1B, C). The coagonist treatment reduced plasma AST by 62.1±6.2% and ALT by 34.3±7.2% against vehicle control (Fig. 1 B,C).
Since the short-term treatment of the coagonist improved AST and ALT after single dose of 
CCl₄, we evaluated the effect of coagonist for a longer duration. Exendin-4, glucagon and 
coagonist reduced plasma triglycerides, and body weight against vehicle control (Fig. 2 A, 
C). Exendin-4 and coagonist reduced plasma glucose by 11.8±11.0 % and 29.6±12.4%, 
while glucagon did not alter glucose levels (Fig. 2B). Exendin-4, glucagon and coagonist 
treatment reduced plasma AST by 21.4±5.5%, 11.1±8.4% and 48.9 ± 7.4%, respectively 
and plasma ALT by 22.5±10.5%, 13.7±16.0% and 52.1± 8.4%, respectively, when 
compared to vehicle control group (Fig. 2D,E). A marked increase in NASH score was 
observed in vehicle control (6.3±1.2) against chow-fed controls (0 score). Treatment by 
exendin-4, glucagon, and coagonist caused 27.4±9.3%, 14.7±19.4% and 54.4±11.7% 
reduction in NASH score (Fig. 3A,D). Lipid accumulation (as observed using oil red O 
stain) and triglyceride levels in liver were significantly increased in vehicle control group 
when compared to chow-fed mice (Fig. 3B,E). Treatment with exendin-4, glucagon and 
coagonist reduced the lipid accumulation scores by 50.0±15.1%, 24.0±22.5%, and 
75.0±23.7% and hepatic triglyceride levels by 33.3±13.2%, 25.5±14.4%, and 50.0 ± 10.2%, 
respectively, when compared with vehicle control group (Fig. 2 B,E). Exendin-4, glucagon, 
and coagonist treatment reduced fibrosis score (as observed by Masson’s trichome stain) by 
33.3±13.2%, 16.7±19.9%, and 50.0±15.1%, respectively, when compared to vehicle control 
group (Fig. 3C,F).

**Coagonist ameliorated CDAHFD-induced hepatic inflammation in mice**

Sixteen weeks exposure to CDAHFD significantly reduced plasma triglycerides, while 
increased plasma ALT and AST in C57BL6/J mice (Fig. 4A,C). Exendin-4 and glucagon
treatment did not change body weight, while coagonist treatment reduced weight gain by 28.5±2.4% when compared to vehicle control (Fig. 4A). Exendin-4, glucagon and coagonist did not alter plasma glucose (Fig. 4B). Exendin-4 and glucagon treatment reduced plasma AST by 42.6±13.9% and 24.9±11.5%, and plasma ALT by 36.6±15.8% and 42.6±13.9%, respectively (Fig. 4D,E). Coagonist treatment caused 53.2±6.1% decrease in plasma AST and 44.3±12.2% decrease in plasma ALT, respectively against vehicle control (Fig. 4D,E). Exendin-4, glucagon and coagonist did not change plasma triglycerides (Fig. 4C).

Hepatocellular steatosis, hepatocyte ballooning, inflammation and moderate peri-sinusoidal or peri-portal fibrosis was observed in liver of CDAHFD-fed animals, after H&E staining (Fig. 5A,E). Exendin-4 and glucagon treatment reduced inflammatory and fibrotic changes by 17.9±17.3%, 9.0±7.4%, respectively, when compared to vehicle control group (Fig. 5A,E). Coagonist treatment showed 40.3±12.1% reduction in NASH score against vehicle control (Fig. 5 A,E). Oil red O staining showed significant fat accumulation in liver of CDAHFD-fed animals, which was reduced by exendin-4, glucagon, and coagonist by 21.9±14.1%, 14.1±12.2% and 41.4±9.3% respectively, against vehicle control (Fig. 5B,F). CDAHFD-fed animals showed an increase in intrahepatic triglycerides by 6.0±0.96 fold and cholesterol by 2.9±0.4 fold against chow-fed animals (Fig. 4F,G). Glucagon and exendin-4 treatment reduced hepatic triglycerides by 16.5±11.1 and 30.5±9.1%, respectively, and cholesterol by 13.5±10.9% and 27.0±11.8%, respectively (Fig. 4F,G). Coagonist showed a stronger effect by reducing hepatic triglycerides by 45.0±11.7% and cholesterol by 47.1±15.3%, when compared to vehicle control (Fig. 4F,G).
CDAHFD-fed animals showed increased collagen deposition and presence of activated macrophages, when examined using Masson’s trichome and PAS-D staining (Fig. 5 C,D,G,H). Glucagon treatment did not reduce the fibrosis score (Fig. 5C,G) or PAS-D score (Fig. 5D,H). Exendin-4 and coagonist treatment reduced fibrosis score by 21.3±9.1% and 45.7±13.2%, respectively (Fig. 5C,G), and PAS-D score by 20.0±15.1% and 35.5±11.8%, respectively (Fig. 5D,H), against vehicle control. Increased collagen deposition in the liver of CDAHFD-fed animals was also confirmed by increase in hydroxyproline content (Fig. 4I). Treatment with exendin-4 and coagonist reduced hepatic hydroxyproline by 28.6±11.7% and 44.3±8.8% respectively, while glucagon treatment did not alter it (Fig. 4I). CDAHFD control showed increase in hepatic expression of COL1A1 by 2.1±0.47 fold and α-SMA by 1.8±0.24 fold against chow-fed control (Fig. 6E,F). Exendin-4, glucagon and coagonist treatment reduced COL1A1 expression by 45.4±5.5%, 25.8±8.4% and 66.9±14.6%, respectively, against vehicle control (Fig. 6E). Expression of α-SMA was also reduced by treatment with exendin-4, glucagon and coagonist in liver by 40.5±5.5%, 21.3±9.4% and 61.8±17.8%, respectively, against vehicle control (Fig. 6F).

CDAHFD exposure caused as increase in expression of pro-inflammatory genes, namely TIMP-1, MMP-9, TNF-α, and MCP-1 in liver (Fig. 6A-D). Exendin-4 treatment decreased expression of all these four pro-inflammatory genes, and glucagon treatment was only halfway effective as that of exendin-4 treatment (Fig. 6 A-D). Coagonist treatment demonstrated a significant reduction in the expression of TIMP-1, MMP-9, and MCP-1 by more than 50% and reduced TNF-α by 48.0±11.0%, when compared to vehicle control (Fig. 6A-D). The inflammatory and fibrotic changes were associated with increased liver weight.
in CDAHFD-fed animals. Exendin-4, glucagon and coagonist treatment reduced liver weight (Fig. 4H).

**Discussion**

In this study, we investigated the effect of GLP-1R and GCGR coagonist on hepatic steatosis and fibrosis in mice models of NAFLD. Carbon tetrachloride causes hepatic injury by inducing oxidative stress. Fibrosis induced by CCl$_4$ is a reversible process, similar to the symptoms of inflammation-induced hepatic fibrosis in clinic (Lassailly et al. 2015; Neuschwander-Tetri et al. 2015). HFD prolongs the inflammatory and necrotic phase of NAFLD induced by CCl$_4$ (Allman et al. 2010; Wang et al. 2011). However, HFD feeding in mice induced obesity, insulin resistance, hyperlipidemia which mimics NAFLD in humans (Nakamura and Terauchi 2013). HFD feeding creates calorie overload in mice and leads to development of obesity and insulin resistance involving multiple factors such as mammalian target of rapamycin (mTOR). Hence, HFD was used in combination with CCl$_4$ in the current work.

CDAHFD prevents secretion of hepatic lipids in circulation due to defective incorporation of triglycerides into Apo B100. As a result, lipids get accumulated in hepatocytes, causing steatosis, inflammation, oxidative stress, and fibrosis (Rinella et al. 2008). CDAHFD shuts down the hepatic β-oxidation and triglyceride production, which leads to increase in liver and causes subsequent cellular damage resulting in fibrosis (Matsumoto et al. 2013). This model shows rapid onset and progression of hepatic fibrosis due to excess accumulation of ectopic fat in the liver. Further, CDAHFD mouse model is useful for investigating multiple hits leading to the treatment of NAFLD (Matsumoto et al. 2013).
Acute and long-term treatment of exendin-4 caused decrease in liver inflammation, accompanied by a reduction in hepatic lipids and inflammation in NAFLD mice models. Our results are in line with previous studies, which reported a reduction in plasma triglycerides by exendin-4 treatment (Varanasi et al. 2010; Lee et al. 2012). The effect of exendin-4 on hepatic steatosis in ob/ob mice or HFD-induced obese mice is reported in literature (Lee et al. 2012; Ding et al. 2006). Exendin-4 reduces food intake by its action on ventromedial hypothalamus and arcuate nucleus (Yang et al. 2014; Burmeister et al. 2017). GLP-1 receptors in brain cause anorexia by activating AMP-activated protein kinase (AMPK) and mTOR (Burmeister et al. 2017). We have reported earlier that lipid lowering effect of GLP-1 was also mediated by activation of central GLP-1R, which was partially independent of anorexia (Patel V et al. 2015). Exendin-4 has protective role in cardiac hypertrophy which is mediated by activation of mTOR signaling (Zhou et al. 2015). Though we have not measured either mTOR or AMPK signalling after exendin-4 treatment in these models, their involvement cannot be ruled out. In the current study, we have observed the effect of exendin-4 in CCl$_4$- or CDAHFD-induced hepatic injury, in a chronic dosing regimen. It is interesting to note that acute and long-term treatment with exendin-4 caused a substantial reduction in plasma AST and ALT in CCl$_4$-treated animals. This data supports the anti-inflammatory properties of exendin-4, which was demonstrated in other mice models of lipotoxicity (Ceriello et al. 2014). We have observed that the anti-inflammatory effect of exendin-4 is mediated by a reduction in pro-inflammatory genes, namely $TIMP-1$, $MCP-1$, $MMP-9$ and $TNF-\alpha$. A potential role of exendin-4 in the treatment
of hepatic steatosis and fibrosis is thus indicated by demonstration of its efficacy in animal models for NAFLD employed in current study.

We have observed that glucagon reduces plasma ALT and AST in CCl$_4$-treated mice, an effect that was inferior to that of exendin-4. Glucagon’s effect in CCl$_4$-induced hepatic injury model was associated with reduction in body weight, hepatic lipids and hepatic inflammation. Reduction in hyperlipidemia is a known effect of glucagon (Jaya and Kurup 1987; Patel et al. 2017a). Hence, it can be concluded that glucagon has a weak anti-inflammatory effect that can be utilized in the treatment of hepatic inflammation and fibrosis.

The balanced coagonist of GLP-1 and glucagon used in the current work is reported to have significant anti-obesity, anti-diabetic, hypolipidemic effect that is partially independent of anorexia (Day et al. 2009; Patel et al. 2013). It has already been reported that the coagonist used in the current work has optimally balanced agonistic activity on GLP-1 and glucagon receptors (Day et al. 2009). Previously, we and others have observed that coagonist activates both GLP-1R and GCGR, by which the pharmacological effect in glycemic and lipid metabolism control is observed (Patel et al. 2013; Valdecantos et al. 2017; Pocai et al. 2009). Since these properties of coagonist can be used to alleviate NAFLD and related disorders, we investigated the effect of the coagonist of GLP-1R and GCGR in the two mice models of NAFLD. G49, a dual agonist of GLP-1 and glucagon receptors has been reported to reduce inflammation, steatosis, oxidative stress, and apoptosis in NAFLD induced by partial hepatectomy with methionine and choline-deficient (MCD) diet in mice (Valdecantos et al. 2017). Treatment with another dual agonist of GLP-1 and glucagon
receptors has also been reported to reduce lipid accumulation in liver of DIO mice (Zhou et al. 2017). The models used in these studies are intended to induce obesity and not NAFLD. Hence, in our work we have used carbon tetrachloride along with long term exposure of high fat diet to induce overt NAFLD, as confirmed by histological markers (Allman et al. 2010; Nakamura and Terauchi 2013). Hence, ours is the first report in which a balanced coagonist of GLP-1 and glucagon receptors has been shown to reduce hepatic inflammation induced by carbon tetrachloride. This model of NAFLD is presented by inflammation, insulin resistance and fibrosis, which resemble clinical NAFLD. Typically, for induction of NAFLD, mice are fed with methionine- and choline-deficient diet. However, exposure to this diet causes weight loss (Chiba et al. 2016). Since coagonist of GLP-1R and GCGR also cause reduction in body weight, it can reinforce the weight loss inducing action of MCD, causing undue toxicity. To avoid this complication, we have used choline-deficient amino acid defined high fat diet, which induces NAFLD without body weight loss.

We have observed that acute and chronic treatment of coagonist prevented rise in plasma AST and ALT in CCl4-induced hepatic inflammation in diet-induced obese mice. NAFLD is a result of imbalance between lipogenesis and lipid disposal from liver (Kawano and Cohen 2013). Coagonist reduced body weight and plasma triglycerides in CCl4-induced hepatic steatosis in diet-induced obese mice. CDAHFD increased the hepatic macrophage content in mice, which is a characteristic feature of advanced NAFLD in humans (Vetelainen et al. 2007). Coagonist treatment suppressed liver weight and reduced signs of inflammation, collagen and fibrosis. PAS-D staining of liver tissue indicated a significant reduction in activated macrophage content by coagonist treatment in CDAHFD-fed
C57BL6/J mice. NAFLD is associated with chronic inflammation in liver. The adipose tissue is the primary source of inflammatory mediators, notably MCP-1 and TNF-α, in obesity and dyslipidemia. NAFLD is associated with increased inflammatory mediators in liver (Farrell et al. 2012), like TIMP-1 and MMP-9 and cause fibrosis (Okazaki et al. 2014). Stellate cells are the principal collagen-producing cells, α-SMA is a marker for its activation was upregulated in hepatic steatosis (Washington et al. 2000). Coagonist treatment reduced expression of α-SMA and COL1A1 in liver. Coagonist treatment reduced the liver lipid content, accompanied by a reduction in inflammatory markers and fat accumulation in liver. We have observed that coagonist treatment has reduced expression of TIMP-1, MMP-9, TNF-α and MCP-1 in liver. Earlier, we have observed that glucagon enhances cholesterol excretion in bile (Patel et al. 2017a). These properties of glucagon receptor agonism may also be responsible for the hepatoprotective effect of coagonist in lipotoxicity conditions. Interestingly, no significant changes in plasma triglycerides were observed after coagonist treatment in CDAHFD-fed mice. This indicates that the beneficial effects of coagonist in CDAHFD-induced NAFLD cannot be attributed to hyperlipidemia, but also to reduction ion inflammation in liver. Recently, we have observed that coagonist improves FGF21 sensitivity, which might be involved in the hepatoprotective effect by coagonist (Patel et al. 2017b). Signaling of mTOR is involved in the metabolic actions of exendin-4, and glucagon activates mTOR signaling (Kimball et al. 2004; Burmeister et al. 2017). NAFLD is an independent risk factor for cardiovascular disease, in which mTOR signalling has an important role (Tarantino and Capone 2013). Hence, in prospective, involvement of mTOR/AMPK signaling in amelioration of NAFLD by coagonist is worth
investigation. The balanced coagonist used in this study possesses potent GLP-1 agonist activity, and weaker glucagon agonist effect (Day et al. 2009). This combination of dual agonistic activity balances the opposing effects of GLP-1 and glucagon on glucose metabolism, while reinforcing the effects on body weight and lipid metabolism. In prospective, long term toxicity studies are required to understand the target-based and off-target toxic effects of the coagonists, which were beyond the scope of the current work.

Taken together these results suggest that coagonist can be a therapeutic option for ameliorating metabolic abnormality associated with NAFLD and can prevent NAFLD in susceptible population.
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Disclosure

No author of this manuscript has any conflicting potential interest.
References


Table

Table 1. Primer sequences used for real time PCR reactions.

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<td>MMP9 Forward</td>
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</table>
**Figure legends**

Figure 1. Effect of acute treatment of exendin-4, glucagon and coagonist in carbon tetrachloride-induced hepatotoxicity in diet-induced obese mice. A. Triglycerides, B. Aspartate Aminotransferase (AST) and C. Alanine Aminotransferase (ALT). Data in graph are presented as mean ± SD, n=6. *P<0.05 when compared with vehicle control.

Figure 2. Effect of chronic treatment of exendin-4, glucagon and coagonist in carbon tetrachloride-induced hepatotoxicity in diet-induced obese mice. A. Body weight, B. Plasma glucose, C. Plasma triglycerides, D. Plasma AST, E. Plasma ALT, F. Hepatic triglycerides. Data in graph are presented as mean ± SD, n=6. *P<0.05 when compared with vehicle control. TG, Triglycerides, AST, Aspartate transaminase, ALT, Alanine transaminase.

Figure 3. Effect of chronic treatment of exendin-4, glucagon and coagonist in carbon tetrachloride-induced hepatotoxicity in diet-induced obese mice. A. NASH score (H&E score), B. Fat accumulation score (oil red O stain), C. Fibrosis score (Masson’s trichome stain), D. Liver sections stained with H&E, E. Liver sections stained with oil red O, F. Liver sections stained with Masson’s trichome, Data in graph are presented as mean ± SD, n=6. *P<0.05 when compared with vehicle control. Liver histology slides are representative of each group. H&E, hematoxylin and Eosin.

Figure 4 Effect of exendin-4, glucagon and coagonist in CDAHFD-induced NASH in mice. A. Body weight, B. Plasma glucose, C. Plasma triglycerides, D. Plasma AST, E. Plasma ALT, F. Hepatic cholesterol, G. Hepatic triglycerides, H. Relative liver weight, I Hepatic
hydroxyproline, Data in graph are presented as mean ± SD, n=6. *P<0.05 when compared with vehicle control. AST, Aspartate transaminase, ALT, Alanine transaminase.

Figure 5: Effect of exendin-4, glucagon and coagonist on hepatic gene expression in CDAHFD-induced NAFLD in mice. A. TIMP-1, B. MMP-9, C. TNF-α, D. MCP-1, E. COL1A1 and F. α-SMA Data in graph are presented as mean ± SD, n=6. *P<0.05 when compared with vehicle control. TIMP-1, tissue inhibitor of metalloproteinases-1, MMP-9, Matrix metallopeptidase-9, MCP-1, Monocyte chemoattractant protein-1, TNF-α, Tumour Necrosis Factor alpha, α-SMA, alpha-smooth muscle actin; COL1A1, collagen type 1 α 1.

Figure 6: Effect of exendin-4, glucagon and coagonist in CDAHFD-induced NAFLD in mice. A. NASH score (H&E stain), B. Fat accumulation score (oil red O stain), C. Fibrosis score (Masson’s trichome stain), and D. PAS-D score (PAS-D stain), E. Liver sections stained with H&E, F. Liver sections stained with oil red O, G. Liver sections stained with Masson’s trichome stain, H. Liver section stained with PAS-D stain, Data in graph are presented as mean ± SD, n=6. *P<0.05 when compared with vehicle control. Liver histology slides are representative of each group.
Figure 1

258x78mm (300 x 300 DPI)
Figure 2

156x97mm (300 x 300 DPI)
Figure 3

248x213mm (300 x 300 DPI)
Figure 4

176x154mm (300 x 300 DPI)
Figure 5

184x121mm (300 x 300 DPI)
Figure 6

195x276mm (300 x 300 DPI)