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Modulation of Intestinal and Systemic Inflammation by Liraglutide and Lactobacillus Plantarum Ameliorates Intestinal Dysfunction and Glucolipid Abnormalities in Diabetic Rats

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Abstract

Background: Enteric inflammation contributes todiabetic gastrointestinal dysfunction. Enteric dysbiosis induced by high fat diet (HFD) increases intestinal inflammation, permeability and absorption of bacterial lipopolysacharide (LPS). LPS promotes systemic inflammation. This results in development of diabetes. Enteric inflammation also impairs the gut-vagus nerve brain axis, responsible for insulin secretion by glucagon-like peptide-1 (GLP-1), resulting in resistance to GLP-1 agonists. GLP-1 receptors in intestinal immune cells modulate pro-inflammatory Cytokines. Probioticsenrich gut with beneficial bacteria, counteracting dysbiosis-induced inflammation. We investigated whether, modulation of intestinal and systemic inflammation by GLP-1 agonist liraglutide and/or lactobacillus plantarum (LP) probiotic, might ameliorate the intestinal dysfunction and glucose/lipid abnormalities indiabetic rats.

Aim of Study: The aim of this study was to study the effect of Lactobacillus Plantarum alone and in combination with liraglutide to ameliorate intestinal dysfunction and glucolipid abnormalities in diabetic rats.

Material and Methods: Rats were divided into: Chow fed (control, liraglutide and LP) and HFD/streptozotocin groups (control, liraglutide, LP, liraglutide/LP). Effects of4-week treatment on glucose and lipid abnormalities, intestinal permeability and visceral hypersensitivity induced by HFD/ streptozotocinwere determined. Changes ininterleukin (IL)-6, total antioxidant capacity, LPS and neuronal nitric oxide synthase (n NOS) were investigated.

Results: HFD/streptozotocin-induced changes in glucose and lipid metabolism, intestinal permeability and visceral hypersensitivity were improved by liraglutide and LP. The drugs reduced intestinal and systemic inflammation, oxidative stress, plasma LPS and plasma/colonic IL-6. LPincreasedcolonic nNOS and enhanced anti-diabetic and intestinal effects of liraglutide.

Conclusion: Modulation of intestinal and systemic inflammation by liraglutide, especially when combined with probiotics, reduces diabetic metabolic and gastrointestinal dysfunction and represents a unique antidiabetic mechanism for GLP-1 agonists, besides their insulinotropic effect.

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Significance statement: Modulation of intestinal and systemic inflammation by liraglutide, especially when combined with probiotics, reduces diabetic metabolic and gastrointestinal dysfunction and represents a unique antidiabetic mechanism for GLP-1 agonists, besides their insulinotropic effect.

Key Words: *Liraglutide – Probiotics – IL-6 – LPS – n NOS – Diabetes – Visceral hypersensitivity – Intestinal permeability.*

Introduction

MUCOSAL inflammation, oxidative stress and visceral hypersensitivity may contribute to the gastrointestinal dysfunction in diabetes **[1-3]**. Several factors suggest that glucagon-like peptide-1 (GLP-1) agonists might have beneficial effects against gastrointestinal dysfunction associated with diabetes. GLP-1 receptors are co-expressed with neuronal nitric oxide synthase (nNOS) in the enteric neurons **[4]** and are also densely expressed on intestinal immune cells, including intra-epithelial lymphocytes **[5]** and monocytes/macrophages **[6]**. In addition, GLP-1 and its receptors have been implicated in the development of visceral hypersensitivity in experimental models ofirritable bowel syndrome **[7]**. Furthermore, the GLP-1 analog liraglutide was reported to reduce inflammation-induced visceral hypersensitivity and pain **[8,9]**.

Enteric microbiota dysbiosis has been proposed as a keylink between gastrointestinal dysfunction and diabetes/metabolic syndrome. Enteric dysbiosis, commonly associated with high-fat diet and diabetes, is an imbalance between beneficial and harmful bacteria in favor of the harmful species. Enteric dysbiosis leads to intestinal inflammation andincreased permeability with absorption of bacterial lipopolysaccharide (LPS). Increased serum LPS results in low grade systemic inflammation which may result in the development or worsening of diabetes **[10,11]**. Indeed, systemic inflammation

due to enteric dysbiosis has been implicated in increased risk of diabetes and metabolic syndrome [11-13]. Dysbiosis-induced intestinal inflammation is also implicated in enteric degeneration with down regulation of GLP- 1 receptors and impairment of the gut-vagus nerve brain axis, responsible for insulin secretion by GLP-1 agonists. This results in resistance to the antidiabetic and gastrointestinal effects of GLP-1 agonists [14,15].

Probiotics enrich the gut with beneficial bacteria thus counteracting the effects of harmful bacteria and reducing dysbiosis-induced inflammation. Probiotics were reported to effectively decrease flatulence and pain in irritable bowel syndrome by reducing the dysbiosis-induced intestinal inflammation [16]. Probiotics were also reported to improve glucose as well as lipid metabolism [17-19].

This study was done to determine theeffects of liraglutide and the probiotic lactobacillus plantarum (LP) onintestinal dysfunction (visceral hypersensitivity and increased intestinal permeability)and metabolic disturbances in diabetic rats on high-fat diet. The involvement of interleukin 6 (IL-6), oxidative stress, LPS and nNOS in the effects of the drugs was investigated.

Material and Methods

1- Diet:

- Rat chow: Obtained from Meladco for Animal Food, Egypt and composed of (20% proteins, 10% fat and 70% carbohydrates).
- High Fat Diet (HFD): Obtained from Meladco for Animal Food, Egypt and composed of (72.5% rat chow, 2% cholesterol, 0.2% bile salts and 25% commercial lard) [20].

This study was conducted at the Pharmacology Department in Ain Shams University. Animals were brought from the Animal House in the Faculty of Medicine and the study was conducted during the period of March – July 2020.

2- Bacterial strain:

Lactobacillius plantarum (LP) EMCC-1039 EMCC-1039 was provided by Cairo MIRCEN, Egypt. LP EMCC-1039cultured for 24h in MRS broth (de Man, Rogosa and Sharpe), Oxoid, UK.at 37ºC. Prepared of skim milk flask (100mL) and was sterilized at 115ºC for 10min.

3- Animals and grouping:

The protocol was approved by Institutional Ethical Committee at Ain Shams University, Faculty of Medicine. This work was conducted in 2020 at the department of pharmacology Ain Shams University. Animal care and the time of sacrifice following the principles of the UK Animals Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Eighty rats were acclimatized to laboratory conditions for one week and body weight was estimated. Rats were randomly divided into two main groups: Normal chow diet (NCD; n=30) and high fat diet- streptozotocin (HFD-STZ; n=50) groups. After two weeks of feeding, NCD rats received vehicle citrate buffer (pH 4.4) in a dose volume of 1 ml/kg, intraperitoneally (i.p), whereas rats in the HFD-STZ group were i.p injected with a low dose of STZ (35mg/kg). Serum glucose was measured just before and 7 days after the vehicle or STZ injection, i.e, after 3 weeks of dietary manipulation in rats. The rats with fasting serum glucose level of 300mg/dl 21 were considered diabetic and selected for further pharmacological studies. The feed and water intake of the animals were also measured. The rats were allowed to continue to feed on their respective diets until the end of the study. Six rats died after STZ injection and 4 failed to develop diabetes.

The 30 rats from NCD group and remaining 40 diabetic rats from the HFD-STZ group were assigned to the following groups (10 rats each): Ia. saline NCD (control), Ib. liraglutide NCD, Ic. probiotic NCD and IIa. saline HFD-STZ (diabetic model), IIb. liraglutide HFD-STZ, IIc. probiotic HFD-STZ, and IId. liraglutide probiotic HFD-STZ groups. Drugs were given daily for 4 weeks.

4- Drug dosing:

- Liraglutide (0.8mg/kg, s.c.) in the last 4 weeks of experiment [22].
- LP EMCC-1039 (1.2×10^9 cfu/ml) daily by oral gavage in the last 4 weeks of the experiment [23].

5- Activated and cultivated bacterial strain [24]:

LP EMCC-1039 was cultured in MRS broth and incubated anaerobically (Gas Generating Kit Anaerobic System, Oxoid, UK) at 37°C for 24h. After incubation period, the skim milk flask was inoculated with 5% LP EMCC-1039 (~108cfu/ml) and incubated at 37ºC for 24h. After the incubation period, the count of LP EMCC-1039 was determined using MRS agar medium. Serial dilutions of 1ml of the previously skim milk flask was prepared in 9 ml sterile saline and 1ml from each dilution 10^{-7} and 10^{-8} was placed in Petri plate (triplicate plates for each dilution), then MRS agar medium was poured into the previous prepared

Petri plates. The pour plates were incubated at 37°C for 48h. After incubation, the most countable plate was counted according to 25. Live cells (colony-forming units [CFUs/ml]) = Number of colonies x dilution factor.

At the end of the study, body weight was estimated, measurement of visceral hypersensitivity and intestinal permeability were performed then on the following day blood samples were collected fromthe lateral tail vein (tail tip) for biochemical parameters and rats were sacrificed by decapitation. The samples were stored at –80°C until processed for biochemical estimations.

6- *Outcome measurements:*

I- Measurement of visceral hypersensitivity ***[26]*** *:*

Colorectal distension (CRD) is the most widely used method to assess visceral sensation both preclinically and clinically. This technique involves insertion of a silicon urethral catheter lubricated with liquid paraffin oil intra-rectally 8cm from the anus, and was then fixed to the tail with adhesive tape. The balloon of the catheter was distended with pre-warmed (37ºC) water. Ascending-limit phasic distension (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml) was applied for 30 s. Each volume was repeated three times at intervals of 5min to obtain an accurate estimate. During the CRD, abdominal hypersensitivity was measured by the abdominal withdrawal reflex (AWR). AWR is a semiquantitative scored test for IBS models to assess hypersensitivity. The score was assigned as follows:

0 = no behavioral response to distension; 1 = brief head movements followed by immobility; 2 = contraction of abdominal muscle without lifting of abdomen; 3 = lifting of abdomen; and 4 = body arching and lifting of pelvic structure.

II- Measurement of intestinal permeability:

Colonic permeability measurement was performed according to **[8]**.

The rats were anesthetized, and laparotomy was performed. The colon was ligated at the junction with the cecum, and an open tipped catheter was inserted in the proximal colon and secured by a ligature. Using a catheter, the colon was gently flushed with phosphate-buffered saline (PBS) until all stools were washed out. Then, another ligation was added on the colon at approximately 4cm from the junction with the cecum, and 1mL of 1.5% Evans blue (Sigma-Aldrich) in PBS was instilled into the colon. After 15min, the rats were killed, and the colons were excised and washed with PBS. Then, the colons were opened and placed in 2mL of N, N-dimethylformamide for 12h. Permeability was calculated by measuring the Evans blue concentration in the supernatant using a spectrophotometer at 610nm.

III- Body weight of animals:

Body weight was measured at the beginning and at the end of the study.

IV- Biochemical estimations:

Measurement of Fasting serum glucose level:

Measured according to the method described by **[27]** using Glucose Coloriometric PAP Detection Kit (Greiner Diagnostic Gmbh, Germany).

Measurement of Fasting serum insulin level:

Determined by using rat insulin ELIZA kits SPI BIO (MEDGENIX-INS-EASIA, Biosource, Europe S.A).

Insulin resistance index:

Homeostasis Model Assessment insulin resistance (HOMA-IR) index derived by the following equation: HOMA-IR = Fasting serum glucose (mg/dl) X fasting serum insulin (Uu/ml)/405 **[28]**.

Measurement of Serum lipid profile:

The serum levels of Total cholesterol (TC), Triglycerides (TG), and high density lipoprotein (HDL) were measured by automated spectrophotometric method using Synchron cx5 autoanalyzer (Beckman, USA).

The LDL low density lipoprotein serum level was calculated by Friedewald Formula: LDL = TC - HDL - TG/5.0 (mg/dL) **[29]**.

Measurement of total antioxidant capacity (TAC):

The total antioxidant capacity of intestine homogenate was determined by measuring the ability of the sample to reduce Fe3+ to Fe2+ established as the Fluorescence Recovery After Photo bleaching (FRAP) test. Briefly, in this test, the medium is exposed to Fe3+ and the antioxidants present in medium start to produce Fe2+ as an antioxidant activity. The reagent included 300mmol/l acetate buffer, pH 3.6 and 16 ml acetaldehyde/l of buffer solution, 10mmol/l 2,4,6-tripyridyl-S-triazine (TPTZ) in 40mmol/l HCl, and 20 mmol/l $FeCl_3.6H_2O$. Working FRAP reagent was prepared as required by mixing 25ml acetate buffer, 2.5ml TPTZ solution and 2.5ml $FeCl_3.6H_2O$ solution. 10µl of H_2O-diluted sample was then added to 300µl freshly prepared reagent warmed at 37°C. The complex between Fe2+ and TPTZ gives a blue colour with absorbance at 593nm.

Measurement of colonic and plasma IL6 level:

The colon segments were homogenized in phosphate buffer containing 0.05 % Tween 20, 0.1mM phenyl methyl sulfonyl fluoride, 0.1 mM benzethonium chloride, 10mM EDTA and 20 IU aprotinin A. These homogenates, and blood samples, were centrifuged at 3,000xg for 10min. The supernatants were assayed for IL-6 using ELISA system (Cytoscreen, Biosource International, Camarillo, CA). The assay is a solid-phase s and wich-type system that utilizes specific anti-rat IL-6 antibody coated onto the wells of microtiter plates. The samples (50µl) and standards were pipetted in triplicate into appropriate microtiter wells, and the assay was performed according to manufacturer's instructions.The sensitivity of this IL-6 ELISA system is 0.7 pM, and the upper limit of detection is 150 pM.

Measurement of plasma LPS level:

Serum lipopolysaccharide (LPS) was determined using ELISA kits (Westang Bio-Tech, Shanghai, China) according to the manufacturer's instructions.

Measurement of colonic neuronal Nos gene expression by Quantitative RT-PCR analysis:

Total RNA was isolated from tissue homogenate using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity were assessed by a spectrophotometer (Smart Speplus, Bio-Rad Laboratories, Inc., Hercules, CA, USA based on an OD260/OD280 ratio. The total RNA reverse-transcription procedure was performed according to the manufacturer's instruction (Promega, USA).

Real-time PCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (Step OneTM, USA). The reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs which were shown in Table (1) and were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from the gene bank. Data from real-time assays were calculated using the v1 ·7 sequence detection software from PE Biosystems (Foster City, CA).

Table (1): The primer sequence of the studied gene.

nNos	Forward primer: 5´TTCAGATCCCGAAACGCTACAC 3´ Reverse: 5´ACAATCCACAACTCGCTCCAAG 3´
Beta actin	Forward primer: 5'-GGTCGGTGTGAACGGATTTGG -3 Reverse primer: 5'- ATGTAGGCCATGAGGTCCACC-3

Statistical analysis:

All values in the results will be expressed as means ± standard deviation (S.D.). Statistical difference among groups was determined using one way analysis of variance; ANOVA followed by Tukey's multiple comparison test. p-values <0.05 will be considered statistically significant. Statistical analysis was carried out using Graphpad prism, software program, version 5.0. (2007). Inc., CA, US.

Results

Effects on abdominal with drawal reflexes:

Administration of HFD-STZ resulted in significant increase in AWR at distension volumes 0.8 (p<0.01), 1 and 1.2 (p<0.001) compared to control NCD group F (8,75=0.09; p<0.001). Treatment of HFD-STZ rats with liraglutide or probiotic resulted in a significant decrease in AWR compared to HFD-STZ group at distension volumes 1, 1.2 (p<0.001). The combination of both drugs significantly decreased AWR at distension volume 1, 1.2 (p<0.001) relative to HFD-STZ and at distension volume 1.2 (p<0.05) relative to liraglutide/ HFD-STZ treated group (Fig. 1). Treatment with Liraglutide or probiotic in control NCD rats showed results that were similar to control untreated NCD group.

Effects on intestinal permeability:

Administration of HFD-STZ produced significant increase in intestinal permeability in comparison to control NCD group F. (6,35) = 81.09; p<0.001). Treatment of HFD-STZ rats with either liraglutide or probiotic significantly decreased (p<0.05) colonic permeability compared to HFD/STZ treated group (Fig. 2). Combination treatment produced more significant decrease in intestinal permeability compared to HFD-STZ untreated (p<0.001) and to liraglutide/HFD-STZ treated group (p<0.05).

Table (2): Effect of liraglutide and probiotic on diabetes-induced changes in body weight.

Animal groups N=10	Body weight week 1 (g)	Week 8 (g)
Control NCD	159.2±13.43	269.2± 14.22
Lira NCD	167.8±10.48	210±12.02###
Prob NCD	157.2±11.53	278.4±14.4
HFD/STZ	157±10.67	353.2±18.8###
Lira/HFD/STZ	158.6±10.45	274±9.5***
Prob/HFD/STZ	154.7±8.32	345.7±13.2
Comb/HFD /STZ	160.4±6	226.7±10.4***^

NCD: Normal chow diet. Lira: Liraglutide. Prob: Probiotic. HFD-STZ: High fat diet/streptozotocin, combined liraglutide plus probiotic.

# p<0.05, ## p<0.01, ### p<0.001 vs. control NCD group; * p<0.05, ** p<0.01, *** p<0.001 vs. HFD-STZ , ^ p<0.05 vs. liraglutide HFD-STZ treated.

- Data is expressed as mean and standard deviation, n=10 per group.

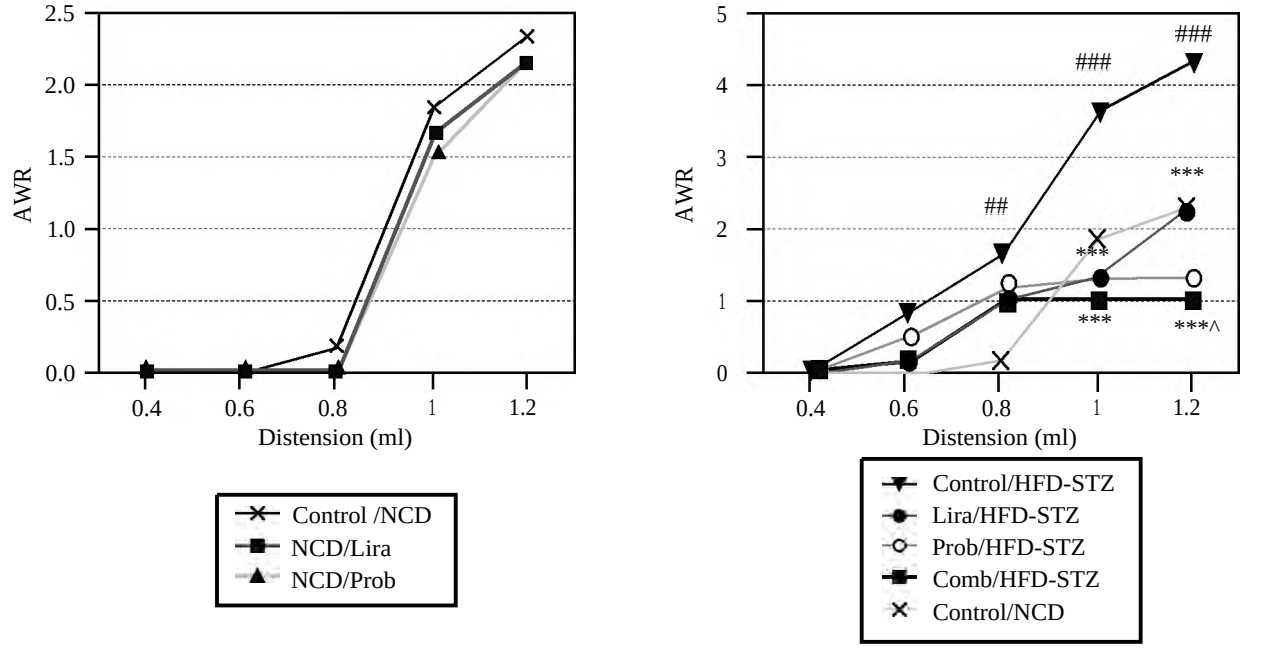


Fig. (1): Effect of liraglutide and probiotic on high-fat diet streptozotocin (HFD-STZ)-induced changes in abdominal withdrawal reflexes (AWR).

NCD: Normal chow diet, Lira: Liraglutide, Prob: Probiotic, comb: Combined liraglutide plus probiotic.
#p<0.05, ##p<0.01, ###p<0.001vs. control NCD; *p< 0.05, **p<0.01, ***p<0.001 vs HFD-STZ,
^p<0.05 vs liraglutide HFD-STZ treated group.
- Data is expressed as mean and SEM; n=6 per group.

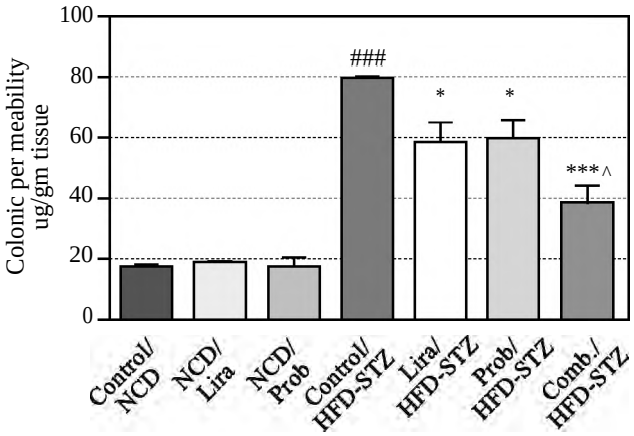


Fig. (2): Effect of liraglutide and probiotic on diabetes-induced changes in intestinal permeability.

- NCD: Normal chow diet. Lira: Liraglutide, Prob: Probiotic, HFD-STZ: High fat diet/streptozotocin, Comb: Combined liraglutide plus probiotic
- ###p<0.001vs. control NCD; *p<0.05, ***p<0.001 vs. HFD-STZ ,^p<0.05 vs liraglutide HFD-STZ treated.
- Data is expressed as mean and SEM; n=6 per group.

Effects on body weight of animals:

Repeated measures ANOVA showed a significant increase in weight in all groups between week 1 and week 8 (F=1,62)=1777.7; p<0.001 and a significant difference between groups (F=7,62) =93.57; p<0.001; as well as a significant group x time interaction (F(7,62)=82.15; p<0.001). Post hoc analysis showed that liraglutide treatment in NCD group resulted in significantly (p<0.001) less increase in body weight at week 8 compared to control untreated chow fed rats at week 8 (Table 2).

HFD-STZ resulted in a significant increase in body weight compared to control naive chow fed rats. Liraglutide HFD/STZ treated rats showed a significant (p<0.001) reduction in body weight compared to HFD-STZ treated group. Combination of both drugs resulted in a significant reduction in body weight compared HFD-STZ untreated (p< 0.001) and to liraglutide HFD-STZ treated group (p<0.05).

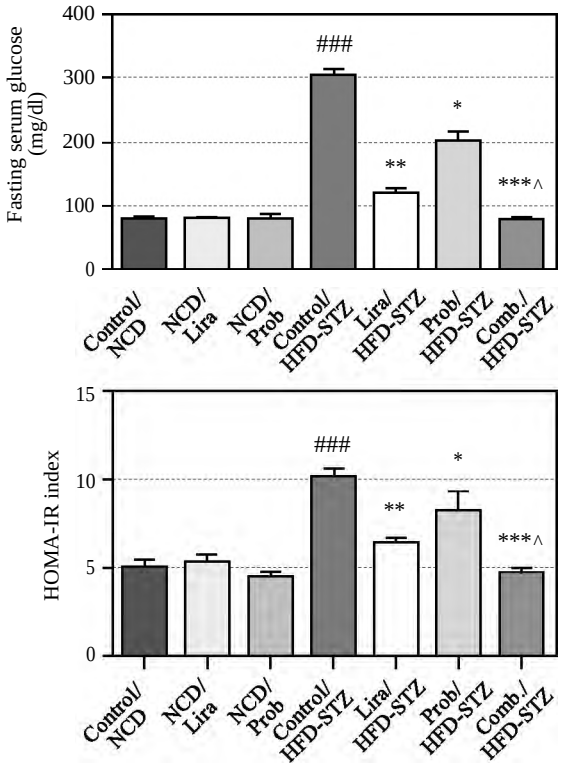
Effects on glucose homeostasis:

HFD-STZ resulted in a significant increase in fasting serum glucose (F(6.3 5=2.2 1; p<0.00 1), IR index (F(6.35=690; p<0.001) with a decrease in fasting serum insulin level (F(6.35=46.4; p<0.001) compared to controlNCD group (Fig. 3). Treatment of HFD-STZ rats with either liraglutide or probiotic

resulted in a significant reduction in fasting serum glucose and IR index and increasein fasting serum insulin compared to HFD-STZ group. Combination treatment produced more significant decrease in fasting blood glucose and HOMA IR index compared to HFD-STZ untreated (p<0.001) and to liraglutide HFD-STZ treated group (p<0.05).

Effects on lipid profile:

HFD-STZ resulted in a significant increase in triglycerides (F(6,35)=176.2; p<0.001), total cholesterol (F(6,35)=93.3; p<0.001), LDL (F(6,35)= 527; p<0.001) and significant decrease in HDL (F(6,35)=37.03; p<0.001) compared to control/ NCD group (Fig. 4).



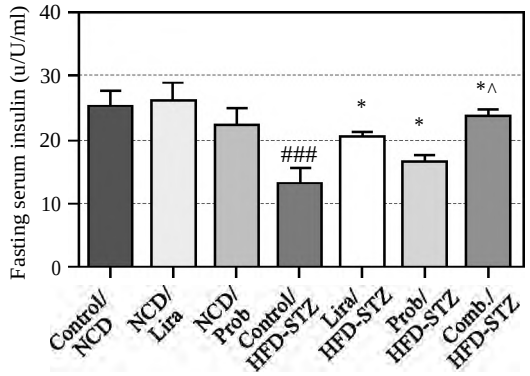


Fig. (3): Effect of liraglutide and probiotic on diabetes-induced changes in glucose homeostasis.

- NCD: Normal chow diet. Lira: Liraglutide, Prob: Probiotic, HFD-STZ: High fat diet/streptozotocin, Comb: Combined liraglutide plus probiotic
- ###p<0.001vs. control NCD; *p<0.05, **p<0.01, ***p<0.001 vs. HFD-STZ group, ^p<0.05 vs liraglutide HFD-STZ.
- Data is expressed as mean and SEM; n=6 per group.

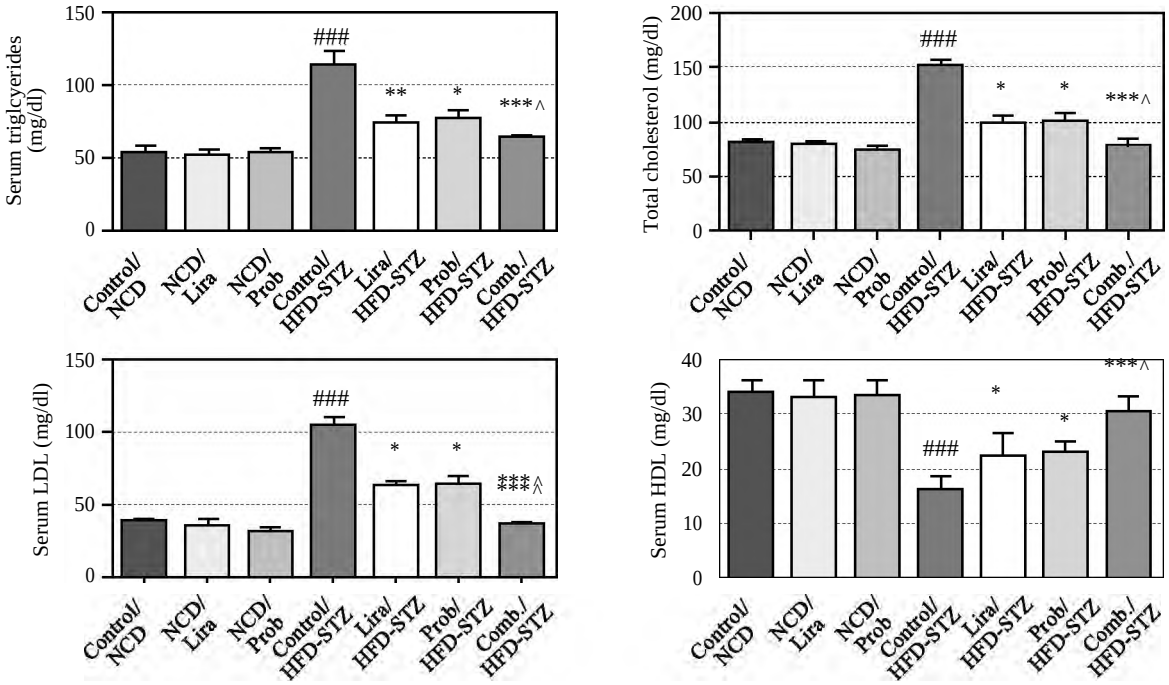


Fig. (4): Effect of liraglutide and probiotic on diabetes-induced changes in lipid profile.

- NCD: Normal chow diet. Lira: Liraglutide, Prob: Probiotic, HFD-STZ: High fat diet/streptozotocin, Comb: Combined liraglutide plus probiotic
- ###p<0.001 vs. control NCD; *p<0.05, **p<0.01, ***p<0.001 vs. HFD-STZ group, ^p<0.05 vs liraglutide HFD-STZ.
- Data is expressed as mean and SEM; n=6 per group.

Treatment of HFD-STZ rats with liraglutide or probiotic resulted in a significant decrease in triglycerides, total cholesterol, and LDL and increase in HDL compared to HFD-STZ group. Treatment with the combination of both agents resulted in an even more significant improvement in lipid profile compared to HFD-STZ untreated (p<0.001) and to liraglutide/HFD-STZ treated group (p<0.05).

Effects on total antioxidant capacity (TAC):

HFD-STZ produced significant decrease in TAC compared to control NCD group (F(6,35) =22.13; p<0.001). Treatment of HFD-STZ rats with either liraglutide alone and probiotic alone significantly (p<0.05) increased TAC (Fig. 5). Combination therapy produced more significant increase relative to HFD-STZ untreated group (p<0.001) and to liraglutideHFD-STZ treated group (p<0.05).

Effects on plasma IL6 level:

HFD-STZ produced significant increase in plasma IL6 level compared to control/NCD group (F(6, 35)=49.22; p<0.001). Treatment of HFD-STZ rats with either liraglutide alone and probiotic alone significantly (p<0.01) decreased IL6 level (Fig. 6). Combination therapy produced more significant (p<0.001) decrease relative to HFD-STZ untreated group and to liraglutide/HFD-STZ treated group (p<0.05).

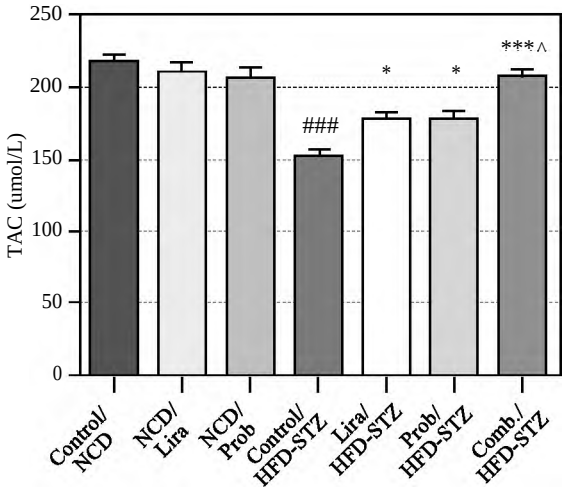


Fig. (5): Effect of liraglutideand probiotic on diabetes-induced changes in total anti-oxidant capacity (TAC).

- NCD: Normal chow diet. Lira: Liraglutide, Prob: Probiotic, HFD-STZ: High fat diet/streptozotocin, Comb: Combined liraglutide plus probiotic.
- ###p<0.001 vs. control NCD group; *p<0.05, ***p<0.001 vs. HFD-STZ group, ^p<0.05 vs liraglutide HFD-STZ.
- Data is expressed as mean and SEM; n=6 per group.

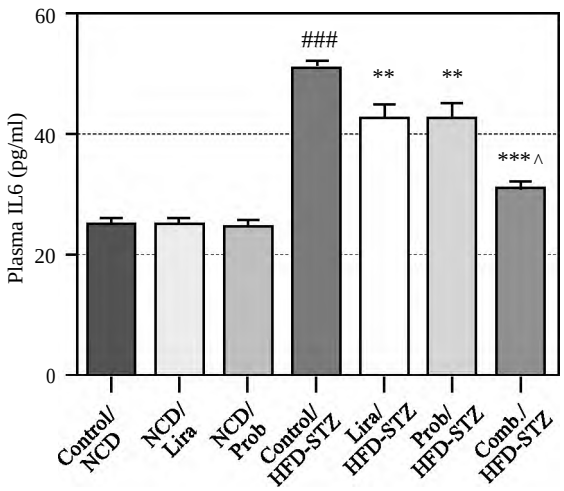


Fig. (6): Effect of liraglutide and probiotic on diabetes-induced changes in plasma IL-6 level.

- NCD: Normal chow diet. Lira: Liraglutide, Prob: Probiotic, HFD-STZ: High fat diet/streptozotocin, Comb: Combined liraglutide plus probiotic.
- ###p<0.001 vs. control NCD group; *p<0.05, ***p<0.001 vs. HFD-STZ group, ^p<0.05 vs liraglutide HFD-STZ.
- Data is expressed as mean and SEM; n=6 per group.

Effects on colonic IL6 level:

HFD-STZ produced significant increase in colonic tissue IL6 level compared to control/NCD group (F (6, 35)=64.88 ; p<0.001). Treatment of HFD-STZ rats with either liraglutide alone or probiotic alone significantly (p<0.01) decreased IL6 level (Fig. 7). Combination therapy produced more significant (p<0.001) decrease relative to HFD-STZ untreated group and to liraglutide/HFD-STZ treated group (p<0.05).

Effects on plasma LPS level:

HFD-STZ produced significant increase in plasma LPS level compared to control/NCD group (F(6, 35)=17.7; p<0.001). Treatment of HFD-STZ rats with liraglutide alone or probiotic alone significantly (p<0.01) decreased LPS level (Fig. 8). Combination therapy produced more significant (p<0.001) decrease relative to HFD-STZ untreated group and to liraglutide/HFD-STZ treated group (p<0.05).

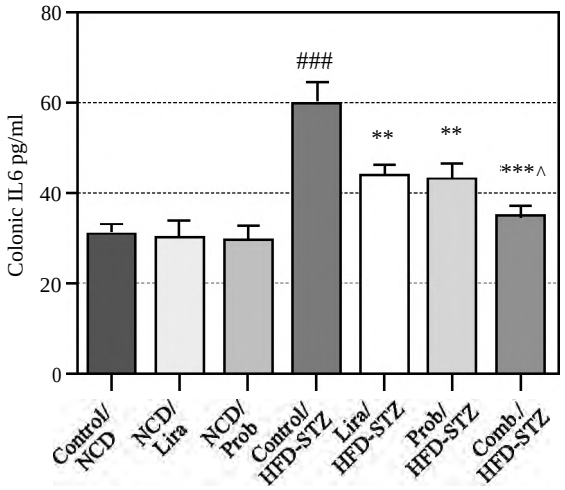


Fig. (7): Effect of liraglutideand probiotic on diabetes-induced changes in colonic tissue IL-6 level.

- NCD: Normal chow diet. Lira: Liraglutide, Prob: Probiotic, HFD-STZ: High fat diet/streptozotocin, Comb: Combined liraglutide plus probiotic.
- ###p<0.001 vs. control NCD group; **p<0.01, ***p<0.001 vs. HFD-STZ group, ^p<0.05 vs liraglutide HFD-STZ.
- Data is expressed as mean and SEM; n=6 per group.

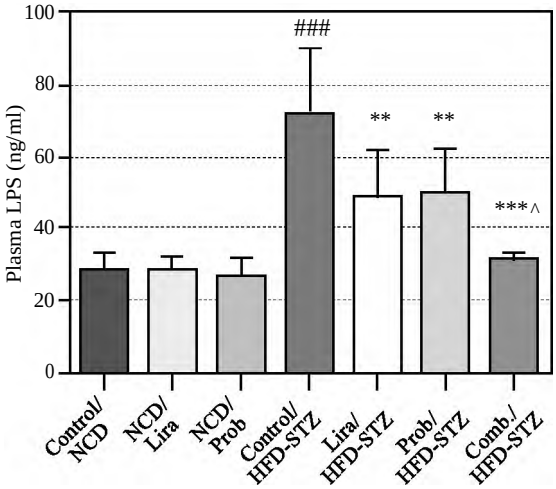


Fig. (8): Effect of liraglutide and probiotic on diabetes-induced changes in plasma LPS level.

- NCD: Normal chow diet. Lira: Liraglutide, Prob: Probiotic, HFD-STZ: High fat diet/streptozotocin, Comb: Combined liraglutide plus probiotic.
- ###p<0.001 vs. control NCD group; **p<0.01, ***p<0.001 vs. HFD-STZ group, ^p<0.05 vs liraglutide HFD-STZ.
- Data is expressed as mean and SEM; n=6 per group.

Effects on neuronal gene expression:

HFD-STZ produced significant (p<0.001) decrease in neuronal NOS gene expression in comparison to control/NCD group (F (6,35)=90.11; p<0.001). Treatment of HFD-STZ rats with probiotic alone significantly (p<0.05) increased neuronal NOS gene expression (Fig. 9). Combination treatment produced significant (p<0.001) increase in neuronal NOS gene expression relative to HFD-STZ untreated group and to liraglutide HFD-STZ treated group (p<0.05).

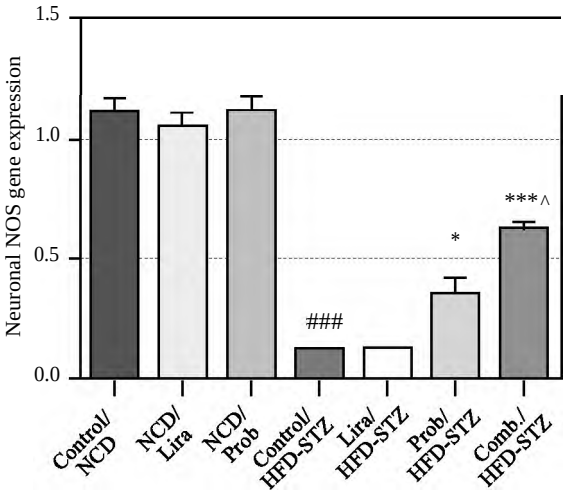


Fig. (9): Effect of liraglutide and probiotic on diabetes-induced changes in neuronal NOS gene expression.

- NCD: Normal chow diet. Lira: Liraglutide, Prob: Probiotic, HFD-STZ: High fat diet/streptozotocin, Comb: Combined liraglutide plus probiotic.
- ###p<0.001 vs. control NCD group; *p<0.05, ***p<0.001 vs. HFD-STZ group, ^p<0.05 vs liraglutide HFD-STZ.
- Data is expressed as mean and SEM; n=6 per group.

Discussion

In this study, we show that liraglutide and the probiotic lactobacillus plantarum have beneficial and synergistic effects on intestinal dysfunction as well as on glucose and lipid abnormalities in diabetic rats. Consistent with earlier studies, diabetic rats in the present study exhibited hyperglycemia and hyperlipidemia, which were associated with increased intestinal permeability and visceral hypersensitivity to colorectal distension **[3,30,31]**. This was also associated with increase incolonic and plasma interleukin(IL)-6 as well as plasma lipopolysacharide (LPS). HFD induces inflammatory signaling and increases cytokines,such as IL-6, that disrupt the intestinal barrier, increasing gut permeability **[32]** with subsequent increase in visceral hypersensitivity. IL-6also affects nociceptor terminals, and stimulates intracellular signaling pathways, inducing pain hypersensitivity **[33]**. Indeed, IL-6 has been shown to increase [Ca2+]i in the submucosal plexus via T-type calcium channel, which is linked to visceral pain **[33]**. HFD also negatively modulates intestinal mucus composition and induces gut dysbiosis with increase in barrier-disrupting bacterial species **[32]**. LPS released from gram negative bacteria increases intestinal permeability and is a marker for in vivo assessment of intestinal permeability **[34]**.

The increase in visceral hypersensitivity, observed in our study, may be related to the increase inoxidative stress and reduction in nNOS noted in the diabetic rats. Advanced glycationend product-

sand oxidative stress, associated with diabetes and HFD,result in enteric inflammation and degeneration **[35-37]**. The enteric degeneration mainly affects the inhibitory nitrergic myenteric neuronswith reduction in nNOS and subsequent increase in colonic motility and visceral hypersensitivity **[1,38]**.

In the present work, diabetic rats treated with liraglutideshowed an improvementinthe HFD/STZ-induced glucose and lipid abnormalities and the associatedincrease inintestinal permeability and visceral hypersensitivity.This was associated with intestinal and systemic anti-inflammatoryeffects as evidenced by a reduction in plasma and colonic IL-6, oxidative stressandplasma LPS levels.

GLP-1 receptors are densely expressedon intestinal intraepithelial lymphocytesandmonocytes/macrophages **[5,6]**. Thus GLP-1 receptor agonists may modulate enteric immune responses and mucosal integrity and permeability. Indeed, inhibitory effects of liraglutide on gut permeability and visceral allodynia induced by LPS in rats wasreported to be associated with a reduction in IL-6 levels in colonic mucosa **[8]**. Liraglutide was reported to reduce cytokine level and colonic inflammation in mice **[39]**. The GLP-1 receptor agonist exendin-4 suppressed pro-inflammatory cytokines production by activated intestinal intra-epithelial lymphocytes **[5]** and in peripheral blood mononuclear cells in type 2 diabetes **[40]**.

The antioxidant effects of liraglutide have also been reported by several studies **[41,42]**. Liraglutide improved endothelial function and increasedeNOS level in mice aortic endothelium **[43]** and renal endothelium in diabetic/obese Zucker rats **[21,44]**. In human umbilical vein endothelial cells, liraglutide, prevented endoplasmic reticulum stress induced by hyperglycemia **[45]**. The drug alsoreduced oxidative stress induced by TNF-a, and increased anti-oxidant enzymes, superoxide dismutase-1 and –2 **[46]**.

Reduction in blood level of LPS by liraglutide has also been previously reported. The drug has been shown to induce significant changes in gut microbiota with increase in beneficial probiotic bacteria and reduction in serum LPS **[47,48]**. LPS-induced systemic inflammation has been implicated in the development and worsening of diabetes and the metabolic syndrome **[12,13,49]**.

In the current study, diabetic rats treated with lactobacillus plantarum (LP) showed an improvement inthe glucose and lipid abnormalities as well as intestinal dysfunction. LP also enhanced the antidiabetic and intestinal effects of liraglutide in rats receiving combined treatment. The beneficial effects of LP on the glucose and lipid abnormalities and intestinal dysfunction may be due to its ability to counteract STZ/HFD-induced intestinal and systemic inflammation.The reduction in oxidative stress, plasma and colonic IL6, and plasma LPS was noted in rats treated with LPand to a greater extent with those receiving combined treatment.

LP enriches the gut with beneficial bacteria. Such bacteria provide short chain fatty acid butyrate that counteracts the intestinal inflammatory effects of harmful bacteria. Butyrate preserves intestinal epithelial barrier and reduces intestinal permeability by increasing mucin and suppressing activation of interleukin IL-6 **[50]**. Improvement of intestinal permeability decreases absorption of LPS. This in turn reduces the systemic inflammation which isimplicated in the high fat diet-induced metabolic abnormalities **[12,13,49]**.

The improvement of glucose and lipid metabolism by LP, observed in our study, was similarly reported with probiotics in elderly people with type 2 diabetes Moroti, et al., **[50]**, in streptozotocin induced diabetic rats **[18]**, and in male rats fed on cholesterol-enriched diet **[19]**. The beneficial effects of LP on visceral hypersensitivity and intestinal permeability,noted in our study, are also consistent with previous studies. Probiotics improve visceral hypersensitivity and intestinal permeability in irritable bowel syndrome with predominant diarrhea **[16,51]**. Beneficial effects of probiotics on intestinal permeability were also reported in human epithelium in vitro **[52]**. Similar results were reported in young piglets with E-coli-induced diarrhea **[53]**. LP promoted intestinal barrier function in weaned piglets by strengthening the epithelium via increase in tight junction proteins, reduction in IL-6 and antioxidant effects **[54]**. LP was also shown to reduce oxidative stress, and prevent tight junction disruption in biliary obstruction in rats **[55]**. Reduction in IL6 and oxidative stress in rats on high fat diet by LP was also reported by Li et al., **[56]**. Lactobacillus probiotic reduced oxidative stress in patients with type 2 diabetes **[57]**. It also increased antioxidant capacity in aging mice **[58]**. Moreover, the probiotic improved eNOS coupling and restored vascular redox state in mice with tacrolimus- induced endothelial dysfunction **[59]**.

The beneficial effects of LP on visceral hypersensitivity, in thepresent study,may be also related to the ability of LP to counteract the deleterious effects of HFD-STZ on the inhibitory nitrergic myenteric neurons with subsequent decrease in colonic motility. This was evident by the increase

in colonic nNOS in LP treated group compared to control diabetic group. This finding is consistent with other studies which showed that LP resulted in a significant increase in nNOS, in colonic tissues in mice with oxazolone-induced colitis **[60]**. Similar results were reported in mice with dextran sulphate sodium-induced colitis **[61]**. Beneficial effects of LP on nNOS have also been reported by Zhao et al., (2019) who showed that LP inhibited the d-galactose-induced oxidative aging and dysregulation of NOS in mice.

The findings of the current study have important clinical implications. Diabetes and obesityare commonly associated with gastrointestinal dysfunction **[62,63]**. The beneficial effects of liraglutide on the intestinal dysfunction, observed in the current work, may be an added advantage of the drug in diabetic and obese patients,be sides their anti-diabetic and body weight lowering effects.

The anti-inflammatory effects of liraglutide against the intestinal and systemic inflammation associated with diabetes and HFD, observed in our study, are also of clinical significance. Low grade systemic inflammation has been implicated in the increase risk of metabolic syndrome and diabetes **[12,13,49]**. Thus the anti-inflammatory effects of liraglutidemay represent a unique antidiabetic mechanism for GLP-1 agonists, besides their insulinotropic effect.

The potentiating effect of LP on the anti-inflammatory effects of liraglutideis also of significance. It emphasizes the importance of manipulation of dysbiosis-induced intestinal and systemic inflammation in management of diabetes and the associatedenteric neuropathy and intestinal dysfunction. This is especially important in patients resistant to GLP-1 agonists who require discontinuation of therapy.Most (79%) of GLP-1 receptor immunoreactive neurons in the colon are co-expressed with nNOS4.Gut microbiota dysbiosis induces down regulation of enteric GLP-1 receptors and nNOS, possibly through an immunogenic mechanism. Down regulation of enteric nNOS results inreduction in nitric oxide (NO) production by nNOS and loss of its down stream stimulation of vagus nerve. With the loss of vagal stimulation, the physiological effects of GLP-1 (gastric emptying, and insulin secretion), mediated by a functional gut-brain axis, are impaired. This results in resistance to the antidiabetic effects of GLP-1 agonists **[14,15]**. Counteracting the effects of dysbiosis on nNOS byprobiotics, might thus enhance the insulinotropic and gastrointestinal effects of GLP-1 agonists, reducing resistance to these agents **[64]**. In addition, probiotics have been reported to increase NO synthesis **[65]** and GLP-1 release **[50,66,67]**.

Conclusion:

Treatment of diabetic rats with either liraglutide or LP improved glucose and lipid abnormalities and the associated visceral hypersensitivity, and intestinal permeability. Agreater improvement was noted with the combination. Possible beneficial effects might involve a reduction in intestinal and systemic inflammation with reduction inplasma/ colonic IL-6, oxidative stressand plasma LPS by both agents and increase in colonicn NOS by the probiotic. These findings suggest that modulation of intestinal and systemic inflammation by liraglutide, especially when combined with probiotics, reduces diabetic gastrointestinal dysfunction and represents a unique antidiabetic mechanism for GLP-1 agonists, besides their insulinotropic effect.

Author contributions:

Amina Sedky and conceived the idea, designed the study, performed the rat experiments, analyzed the data and prepared the manuscript.

Competing interests: None.

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تعديل إلتهاب الأمعاء بواسطة ليراجلوتايد ولاكتوباسيلس بلانتاروم فى الفئران المصابة بالسكرى

يساهم الإلتهاب المعوى فى حدوث خلل وظيفى فى الجهاز الهضمى السكرى. النظام الغذائى عالى الدهون (HFD) يزيد من إلتهاب الأمعاء ونفاذية وإمتصاص السكاريد الدهنى البكتيرى (LPS). هذا يؤدى إلى تطور مرضى السكرى. يؤدى الإلتهاب المعوى أيضاً إلى إضعاف محور الدماغ العصبى المعوى، المسؤول عن إفراز الأنسولين بواسطة (GLP-1)، مما يؤدى إلى مقاومة مستقبلات GLP-1. تعدل مستقبلات GLP-1، فى الخلايا المناعية المعوية السيتوكينات المؤيدة للالتهابات. تعمل البروبيوتيك على إثراء القناة الهضمية بالبكتيريا المفيدة، مما يقاوم الإلتهاب.

لقد درسنا ما إذا كان تعديل الإلتهاب المعوى والإلتهاب عموماً الجهازى بواسطة محفز GLP-1 liraglutide و/أو بروبيوتيك (LP) Lactobacillus plantarum، قد يخفف من الخلل المعوى وتغييرات نسب الجلوكوز / الدهون فى الفئران المصابة بداء السكريز.

\الطريقة: تم تقسيم الفئران إلى: تغذية الطعام (مجموعة التحكم، الليراجلوتايد وLP) ومجموعات HFD/الستربتوزوتوسين (مجموعة التحكم، الليراجلوتايد، LP، الليراجلوتايد/LP). تم تحديد تأثيرات العلاج لمدة ٤ أسابيع على تغييرات الجلوكوز والدهون ونفاذية الأمعاء وفرط الحساسية الناجم عن HFD/الستربتوزوتوسين. تم فحص التغيرات فى الإنترلوكين 6-(LI)، السعة الكلية لمضادات الأكسدة، LPS وأكسيد النيتريك العصبى (nNOS).

\النتائج: تم تحسين التغيرات التى يسببها HFD/الستربتوزوتوسين فى استقلاب الجلوكوز والدهون ونفاذية الأمعاء وفرط الحساسية بواسطة الليرجلوتايد وLP. قللت الأدوية من الإلتهابات المعوية والعامة، والاجهاد التأكسدى، والبلازما LPS والبلازما/القولون IL-6. زاد LP من nNOS القولون وتعزيز التأثيرات المضادة السكرى والأمعاء من liraglutide.

\الخلاصة: تعديل الالتهاب المعوى بواسطة الليراجلوتيد، خاصة عندما يقترن بالبروبيوتيك، يقلل من التمثيل الغذائى للسكرى واختلال وظائف الجهاز الهضمى، ويمثل آلية فريدة مضادة لمرض السكر لمنبهات GLP-1، إلى جانب تأثير الأنسولين.