Oral GLP-1 Analogue Ameliorates Obesity-Induced Diabetes In Db/Db Mouse

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Research Article

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Abstract

Type 2 diabetes is currently experiencing an outbreak worldwide. GLP-1 effectively lowers blood glucose level as an emerging target for the treatment of type 2 diabetes. However, the application of GLP-1 is limited by short half-life and too expensive cost in clinic. In this study we employed the food-grade probiotics as delivery system to express human GLP-1 and its analogue. Recombinant *Lactococcus lactis* could express GLP-1 and analogue in vitro and modified GLP-1 analogue was more resistant to DPP-4 degradation. Oral administration of GLP-1 analogue could reduce the fat mass. More importantly, GLP-1 analogue improved hyperglycemia and insulin resistance in Db/Db mouse although the insulin secretion is not observed in vitro. Our study demonstrates that *Lactococcus lactis* genetically modified with single amino acid mutation could prolong half-life of GLP-1 and increase insulin sensitivity in Db/Db mouse model as an oral drug delivery system driving the development and innovation of drug therapy for type 2 diabetes.

Introduction

Diabetes is a metabolic disease characterized by chronic hyperglycemia caused by multiple causes. According to the global report of World Health Organization (2016), more than 420 million adults were living with diabetes in 2014. Not only in high-income countries, but diabetes spread very quickly in low- and middle-income countries. Due to the high calorie diets and increasingly sedentary lifestyles prevalent in modern society, fat accumulation has become a major risk factor for type 2 diabetes[1]. Unlike the type 1, type 2 diabetes has been certain that the disease process could be halted with balanced diet to correct the poor fat metabolism[2]. Insulin resistance and islet dysfunction are the major pathophysiologic features of type 2 diabetes. The necessary therapeutic interventions are also helpful in accelerating the course of the diabetes, for example, bariatric surgery [3, 4], insulin administration [5, 6], sulfonamides [7, 8] or metformin treatment [8]. However, there are still problems with the aforementioned treatments. For those patients whose condition is not improved by oral medication, bariatric surgery can only be used in a small number of people [9–11], and interventional administration of insulin injection also brings endless torment. Reverse-engineer would replace the surgery to find less invasive and more equivalent therapeutics. Moreover, some Prudent advice have been offered that combining either pre- or probiotic approaches with gut-peptide-based therapies may ultimately achieve efficacy similar to bariatric surgery[12].

GLP-1 is a very short gut-peptide from L cells which could induce glucose-dependent insulin secretion, reduce glucagon secretion, delay gastric emptying, increase β -cell survival[13–16] and decrease blood glucose and food intake [17]. Thus as a potential drug therapy, the development of GLP-1 preparations has been highly sought after. Considering of lower half-life of wildtype GLP-1 for less than 2 min [17], GLP-1 analogues is been investigated to remedy their deficiency, such as restriction enzyme mutation, unnatural amino acid substitution, covalent complex and Exendin family from Glia monster [18]. Another problem, however, is that GLP-1 analogues are too expensive to achieve widespread use as first-line therapy compare with oral anti-diabetes drugs.

As a food grade engineering bacteria, *Lactococcus lactis* is emerging as a potential candidate for foreign protein delivery [19–21]. Genetically modified *Lactococcus lactis* has been utilized in many disease model including inflammatory bowel disease [22, 23], anti-tumour [24], anti-obesity [25], anti-oxidation [26], indicating that industrial bacteria could offer a prospective living biotherapeutics for the treatment of human disease.

Nevertheless, a new amino acid mutation of GLP-1 expressed in *Lactococcus lactis* hasn't been investigated to confirm the anti-diabetes effect in Db/Db mouse. In this study, we combined *Lactococcus lactis* and functional GLP-1 analogue to construct an anti-hyperglycemia probiotic expression system and the process of protein production is transferred to gut. GLP-1 Mutant could prolong half-life in vitro and have been verified the hypoglycemic effect in Db/Db mouse. This design solves the problems of short half-life and high cost of injectable GLP-1. In summary, feasibility design of GLP-1 mutation has a potential prospective in clinical application furtherly more improvement needs to be performed in the future.

Materials And Method

Materials and animals

Lactococcus lactis cremoris NZ3900 (Cat# VS-ELS03900-01) and plasmids pNZ8149 (Cat# VS-ELV00300-01) were purchased from MoBiTec GmbH, Germany. The standard substance of GLP-1 and GLP-1 Mutant used in this study was artificial synthesis by Genscript Biotech Corporation. All reagents are from Sigma unless otherwise specified.

Genetic obesity model C57BLKS/J-Lepr^o/Lepr^o (Db/Db) male (7-week-old) was purchased from Model Animal Research Center of Nanjing University. After a week of adaptive breeding, mice were divided into three groups according to body weight and oral administration daily for 4 weeks with 1.0×10^8 CFU frozen-dried powder of vehicle, GLP-1 and GLP-1 Mutant probiotics, respectively. During this phase, body weight and blood glucose would be recorded to assess the function of GLP-1 analogue.

Animals were housed under a 12/12 hr light/dark cycle with free access to food and water. Before sacrifice, animals were given an anesthetic and cervical dislocation rapidly to relieve pain. All animal studies followed the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and were approved by the Institutional Animal Care and Use Committee of Institute of Zoology, Chinese Academy of Sciences.

Plasmid cloning and transformation

The human GLP-1 (7-37aa) followed by USP45-LEISS, 6 $\times$ his-tag and enterokinase restriction site (EK) was sub-cloned into pNZ8149. Recombinant vector above was transferred into home-made NZ3900 competent cell through Gene PulserXcell™ electroporation system (BioRad, California, USA). That goes for preparation of GLP-1 analogue and these two freeze-dried powders were registered under GLP-1 and GLP-1 Mutant for short, respectively.

DNA sequence of USP45-LEISS-His-EK-GLP-1:

ATGAAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACTTTCTGCTGCAGCCCCGTTGTGAGGTGTTTACGCTGATACTAATTCTGATTGGAAATATCGTCG,

DNA sequence of USP45-LEISS-His-EK-GLP-1 Mutant:

ATGAAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATACTTTCTGCTGCAGCCCCGTTGTGAGGTGTTTACGCTGATACTAATTCTGATTGGAAATATCGTCG,

In vitro expression and mass spectrum analysis

The pure culture of GLP-1 and GLP-1 Mutant was transferred into 10 ml fresh M17 broth for stationary culture until OD600 around 0.4–0.6, 25ng/ml nisin was added to induce the target protein expression. Given the very small peptide chain of GLP-1 and its mutant, the supernatant of the pure culture was enriched by his-tag affinity chromatography and detected by tandem high performance liquid chromatography and mass spectrometry (HPLC-MS, Orbitrap Elite, Thermofisher) after enrichment.

Stability detection of analogues

In the 96 well plate, 1.5ng DPP-4 was mixed with 10 ug GLP-1 or GLP-1 Mutant under sodium phosphate buffer (0.1 M, pH 7.4), respectively. After incubation for 60 min in 37°C incubator, trifluoroacetic acid (TFA, 0.1%) was added in well to terminate reaction. Then sample mixture was purified by Oasis HLB extraction cartridges (Waters, WAT094225). Following the elution of methanol and vacuum drying, purified protein was re-dissolve with 0.1% methanoic acid. At the end, samples through 0.45um centrifugal filters (Millipore, UFC30HV00) could be tested by mass spectrometry.

Body composition analysis

The total fat and lean masses of mice after a 4-week-treatment with either vehicle or GLP-1 analogue were assessed with the Small Animal Body Composition Analysis and Imaging System (MesoQMR23060H-I, Nuimag Corp., Suzhou, China).

Glucose homeostasis analysis

Mice would be fasted for 12 hours (21:00–9:00) with free access to water. Then refeed glucose was measured after cafeteria feeding for 2 hr and random glucose could be detected at any time of maintenance feeding.

For oral glucose tolerance test, 0.75 g/kg D-glucose (Sigma) was oral administered and blood glucose level was measured with Accu-Chek Performa test strips (Roche Diagnostics Corp) at each indicated time point. If the blood glucose value is over the limit of detection, then blood would be diluted immediately into saline and repeated this test. The individual glucose concentration would be calculated and recorded by dilution proportion itself. The situation of insulin resistance would be reflected by HOMA-IR (Formula by Fasting glucose (mM) × Fasting insulin (μU/mL)/22.5. The serum insulin was measured by Rat/Mouse Insulin ELISA (Merck Millipore, EZRMI-13K)

Isolation of islets

Common bile duct perfusion method has been referenced for islet separation. Detailed, the liver-pancreatic junction was found to reveal the position of common bile duct after cervical vertebrae dislocation. Clamping the junction of common bile duct and duodenum with tweezers, digestive solution with 1 mg/ml collagenase P was injected into common bile duct with 31G needle and complete infiltration of the pancreas. Then the whole pancreas was rapidly stripped and digested in vitro at 37°C for 15 min. At the end, the islets were washed twice and manual picked under stereoscope for quantitative statistics.

Cell culture

INS-1 were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (GIBCO, Grand Island, NY), 10 mM HEPES, 2 mM L-glutamine (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Sigma, St. Louis, MO) and 50 mM mercaptoethanol (Sigma, St. Louis, MO), at 37°C in 5% CO₂.

For glucose stimulated insulin secretion, continued stimulus of glucose on INS-1 cell could reflect the ability of insulin secretion. Briefly, when cell confluence of INS-1 cell reaches 80%, incubate the cell with KRBB solution (118.5mM NaCl, 2.54mM CaCl₂, 1.19mM KH₂PO₄, 4.74mM KCl, 25mM NaHCO₃, 1.19mM MgSO₄, 10mM HEPES and 1% BSA) for 30 min. Then INS-1 cells were incubated with KRBB solution containing 2.8 mM and 16.7 mM glucose for 1 hr and the blending supernatant was collected, respectively. Insulin in the culture media was detected by Rat/Mouse Insulin ELISA Kit (EZRMI-13K, Millipore).

Statistical Analysis

All data are presented as means ± SEM. Data from multiple groups were analyzed by one-way ANOVA. Data from two groups were analyzed by unpaired Student's t-test. P < 0.05 was considered to indicate a statistically significant difference.

Results

The design and construction of GLP-1 analogue

GLP-1 analogues, such as exendin-3, have almost exactly the same biological function and resistant to DPP-IV degradation with a longer half-life. Thus we referred to the structure of exendin-3 and eliminated the DPP-4 recognition site by transferring alanine to serine (Fig. 1A). According to the construction of oral protein expression system, GLP-1 and GLP-1 Mutant were sub-cloned into pNZ8149 following the signal peptide Usp45, enhancer sequence LEISS, his tag and enterokinase recognition site under nisin induced promoter (Fig. 1B). We successfully harvested the recombinant *Lactococcus lactis* with GLP-1 or GLP-1 Mutant (Fig. 1C).

GLP-1 analogue expression in vitro by HPLC-MS assay

Due to very short peptide chain of GLP-1 and its analogue and the difference of individual amino acid, the western blotting is not adoptable for GLP-1 analogue. Here we made a synthesis of GLP-1 and analogue in vitro as standard substance. After comparison, GLP-1 and analogue could be detected in culture supernatant consistent with fingerprint of standards (Fig. 2A). In addition, the sequence of peptide matched design that recombinant *Lactococcus lactis* could express the GLP-1 and analogue in vitro (Fig. 2B).

Stability study of GLP-1 analogue and insulin secretion in vitro

In order to assess the stability of GLP-1 analogue, we employed HPLC-MS to detect the degradation of GLP-1 and its analogue in vitro with adding of DPP-4. After 1 hr treatment, GLP-1 content quickly dropped to 60% due to carrying enzyme cleavage sites of DPP-4. On the other hand, GLP-1 analogue only decreased by 15% (Fig. 3A). Therefore, this mutant of GLP-1 extended half-life of wildtype GLP-1 in some degree.

On the functional verification, we used INS-1 cell to check the insulin secretion under low and high glucose stimulation of physiological conditions. Consistently with GLP-1, GLP-1 Mutant also induced insulin secretion whatever 2.8 mM or 16.7 mM of glucose (Fig. 3B).

GLP-1 analogue reduce the fat mass without changing body weight

Db/Db mouse is a very good model of obesity and diabetes which is suitable for verifying the function of GLP-1 analogues. Here we employed Db/Db mouse to verify the function of GLP-1 analogue (Fig. 4A). During four weeks of continue oral administration, there was no significant change in body weight between the treatment group and the control group (Fig. 4B). However, it was found that only fat mass of GLP-1 Mutant was decreased by 6.6% significantly, but the lean mass remained unchanged (Fig. 4C, D). Tissue weight is also shown that GLP-1 Mutant significantly reduced the weight of adipose tissue (Fig. 4E).

GLP-1 analogue improved the glucose homeostasis in Db/Db mice

The function of GLP-1 is to promote glucose dependent insulin secretion and reduce blood glucose. Firstly we tested random blood glucose and found that the blood glucose of GLP-1 Mutant gradually down-regulated after 4 weeks treatment, and GLP-1 group has a significant hypoglycemic effect in the early stage of treatment (Fig. 5A). Then we investigated the change of blood glucose before and after cafeteria feeding. Whatever GLP-1 or GLP-1 Mutant didn't influence the 12 hr-fasting glucose, but GLP-1 and GLP-1 Mutant indeed mitigated the surge in postprandial blood glucose after 2 hour-cafeeteria feeding (Fig. 5B). At posttreatment of 2 week, glucose tolerance test was also confirmed that GLP-1 and GLP-1 Mutant could significantly reduce the fasting glucose and enhance the capability of tolerance for oral glucose comparing with control group (Fig. 5C).

GLP-1 analogue improved the insulin resistance without changing insulin secretion in Db/Db mice

Given the GSIS function in INS-1 cell in vitro, we tried to confirm that insulin secretion in Db/Db mouse. The recombinant *Lactococcus lactis* didn't change the intestinal length in three groups (Fig. 6A). The GLP-1 and GLP-1 analogue could up-regulate the islet number which means insulin secretion might be enhanced (Fig. 6B). Combined with the results of insulin secretion experiment in vitro, we hypothesized that GLP-1 also increases insulin production in vivo and detected the insulin level in tail tip blood glucose of Db/Db mouse. Unexpectedly, we didn't observe insulin concentration increasing in peripheral blood due to abnormal fluctuations (Fig. 6C). But more powerful than GLP-1, GLP-1 Mutant indeed improved the insulin resistance in Db/Db mouse depending on the careful calculation of glucose and insulin concentration (Fig. 6D).

Discussion

The number of patients with type 2 diabetes increases year by year worldwide. Although diabetes does not directly cause death, the pain brought by long-term medication seriously reduces the quality of life of patients because of inconvenient operation and poor patient compliance, especially injection of insulin and other protein drug [27]. GLP-1 and its analogues are one of the most popular drugs for type 2 diabetes. Long acting GLP-1 analogues have been developed to reduce the number of injections needed recently like amino acid sequence modification, chemical modification, fusion protein polymers and special material package, and mode of administration for peptide drugs and higher cost from complicated preparation process still not be changed. Therefore an appropriate oral drug delivery system has long been the ultimate goal of scientists. Several studies have attempted to use lactic acid bacteria for mucosal drug delivery [28]. It has been widely approved that food-grade *Lactococcus lactis* is safe enough to be mucosal delivery therapeutic drugs [29–31]. Herein we combined *Lactococcus lactis* with functional GLP-1 analogue to construct an anti-hyperglycemia probiotic. For the sake of prolongation of GLP-1 half-life, we referred to the structure of exendin-3 and mutated the DPP-4 enzyme restriction site to serine. By mass spectrometry, it was found that GLP-1 Mutant was indeed resistant to enzymatic hydrolysis and significantly prolonged the action time of GLP-1.

Because of the anti-obesity and anti-diabetes effect of GLP-1, Db/Db mouse are employed to verify the function of GLP-1 analogues. Db/Db mouse is a common obesity mouse model which exhibit type 2 diabetes, dyslipidemia and liver damage during the course of growth and development [32–34]. We found that the GLP-1 Mutant significantly remitted adiposity of Db/Db mouse after 4 week treatment without changing body weight. Unlike previous understandings of GLP-1 classical function, GLP-1 analogues could induce the body weight loss obviously [35]. But oral semaglutide is mostly attributable to body fat mass loss while causing weight loss [36, 37]. Consistently, recombinant *Lactococcus lactis* with natural GLP-1 still didn't affect the body weight and fat mass of chow-diet and high fat diet feed mouse [38]. Taken together, fat mass loss of GLP-1 Mutant is same as commercial GLP-1 analogues and prolonged, sustained and stable GLP-1 stimulation might lead to obvious changes in body weight.

The insulin secretion is another identify object for GLP-1 function. In our study, insulin secretion is modestly up-regulated by 11% under high glucose stimulation in vitro. This up-regulation is not enough to elevate the serum insulin production in Db/Db mouse. Thus serum insulin was not detected by any difference between three groups as expected. Interestingly, Arora and his colleagues didn't find changes in insulin secretion in chow-diet mouse [38], and the fluctuation of insulin levels was also violently in ZDF rats totally different from the cell experiments [39, 40]. On the other hand, the dosage and method of oral

delivery is different from previous studies (1.0×10^8 CFU frozen-dried powder in our method v.s. $\geq 1.0 \times 10^9$ fresh culture suspension), and this might be another factor contributing to insignificant increasing of insulin release. Next we will attempt to use higher dosage of GLP-1 Mutant to check the function of glucose-dependent insulin secretion.

For safety reasons, antibiotic resistance and labeled proteins are not suitable for clinical research. Recombinant *Lactococcus lactis* doesn't carry any antibiotics resistance gene and doesn't seriously affect flora balance in theory. His tag was linked before GLP-1 fusion protein for convenient purification and detection in this study. There is a possibility that the tagged proteins could induce antibodies production in animals and trigger an inflammatory response. Alternatively, the enterokinase recognition site was loaded between his tag and GLP-1 sequence to crack the mature GLP-1 analogue in the environment of digestive juices. Thus unlabeled fusion protein would be considered to facilitate clinical transformation. Safety evaluation would be executed to verify the possible side effects of oral GLP-1 analogue. In addition, pharmacodynamic studies are not sufficient to expand GLP-1 Mutant analogue. The influence of gut microbiota on type 2 diabetes should not be ignored completely. Abnormalities in the composition of the gut microbiota might contribute to the development of type 2 diabetes [41]. Chinese herbal formula may ameliorate type 2 diabetes via enriching beneficial bacteria with metformin [42]. Thus potential therapeutic strategies might relieve hyperglycemia from both molecular and microbial dimensions. Faecal metagenomic analysis should be carried out to reveal the exact molecular mechanism.

Unlike expensive eukaryotic cell culture situation and purification process, lactic acid bacteria production is relatively affordable. The feature of living biotherapeutics lies on the production process postposition that designed process flow are performed in gut. The advantage of this process is that it reduces production costs and side effect [43]. In partially, the problem of excessive economic burden of biological medicine has been solved and living biotherapeutics with versatility would provide us with more and more options [44].

In our study, we employed the food-grade probiotics as delivery system to express human GLP-1 and its analogue. Recombinant *Lactococcus lactis* could express GLP-1 and analogue in vitro and GLP-1 Mutant was more resistant to DPP-4 degradation. More importantly, GLP-1 analogue reduced the fat mass, improved hyperglycemia and insulin resistance in Db/Db mouse. Our study demonstrates that *Lactococcus lactis* genetically modified with single amino acid mutation could prolong half-life of GLP-1 and increase insulin sensitivity in Db/Db mouse model as an oral drug delivery system driving the development and innovation of drug therapy for type 2 diabetes. In summary, feasibility design of GLP-1 mutation has a potential prospective in clinical application furtherly more improvement needs to be performed in the future.

Declarations

Ethical Statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors declare that they have no conflict of interest.

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Author Contributors

M. D. constructed the expression system of GLP-1 and GLP-1 Mutant. H. Z. and M. D. finished the cell experiment in vitro. H. Z., M. D. and X. W. together carried out the animal experiment. H. Z. and M. D. analyzed the results. H. Z. wrote the manuscript. M. D. and W. J. conceived the project. All authors were involved in editing the paper and had final approval of the submitted and published versions.

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Figures

A

GLP-1 His-**Ala**-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Gly **DPP-4**

GLP-1 Mutant His-**Ser**-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Gly

Exendin-3 His-**Ser**-Asp-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser

B

C

Figure 1

The design and construction of GLP-1 analogue (A) Amino acid sequence of GLP-1, GLP-1 Mutant and Exendin-3. (B) Construction of recombinant GLP-1 and GLP-1 Mutant vector. (C) Agarose gel electrophoresis results of PCR fragments and suspected positive clone.

A

B

Description	Sequence	Coverage(%)	AAs	MW(kDa)
GLP-1	HAEGTFTSDVSSYLEGQ AAKEFIAWLVKGRG	100	31	3.4
GLP-1 Mutant	HSEGTFTSDVSSYLEGQ AAKEFIAWLVKGRG	100	31	3.4

Figure 2

GLP-1 analogue expression in vitro by HPLC-MS assay (A) HPLC-MS assay of pure culture of GLP-1 and GLP-1 Mutant. (B) Peptide sequencing and coverage comparison by HPLC-MS between GLP-1 and GLP-1 Mutant.

Figure 3

Stability study of GLP-1 analogue and insulin secretion in vitro (A) The stability of GLP-1 and GLP-1 Mutant on DPP-4 enzyme digestion in vitro (n=3). *P<0.05 compared with GLP-1 group. (B) Insulin secretion of INS-1 cell under GLP-1 and GLP-1 Mutant (n=3). Multiple comparisons which were significant were represented with alphabetic notation, abc recorded as p value < 0.05.

Figure 4

GLP-1 analogue reduce the fat mass without changing body weight (A) Procedure design of animal experiment. (B) Body weight tracing, (C) fat mass, (D) lean mass and (E) tissue weight of control, GLP-1 and GLP-1 Mutant group (Control, n=8; GLP-1, n=8 and GLP-1 Mutant, n=8). Multiple comparisons which were significant were represented with alphabetic notation, abc recorded as p value < 0.05.

Figure 5

GLP-1 analogue improved the glucose homeostasis in Db/Db mice (A) Random glucose tracing every week of control, GLP-1 and GLP-1 Mutant group. (B) Fasting and refeeding glucose of control, GLP-1 and GLP-1 Mutant group. (C) Glucose tolerance test of control, GLP-1 and GLP-1 Mutant group (Control, n=7; GLP-1, n=7 and GLP-1 Mutant, n=8). Multiple comparisons which were significant were represented with alphabetic notation, abc recorded as p value < 0.05.

Figure 6

GLP-1 analogue improved the insulin resistance without changing insulin secretion in Db/Db mice (A) The intestinal length (Control, n=8; GLP-1, n=8 and GLP-1 Mutant, n=8), (B) islet number (Control, n=8; GLP-1, n=8 and GLP-1 Mutant, n=8), (C) serum insulin (Control, n=8; GLP-1, n=8 and GLP-1 Mutant, n=8) and (D) the situation of insulin resistance (Control, n=7; GLP-1, n=7 and GLP-1 Mutant, n=7) of control, GLP-1 and GLP-1 Mutant group. Multiple comparisons which were significant were represented with alphabetic notation, abc recorded as p value < 0.05.