Effect of miR-19b on the protective effect of Exendin-4 on islet cells in non-obese diabetic mice

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Abstract. This study analyzed the effect of miR-19b on the protective effect of Exendin-4 on islet cells in non-obese diabetic (NOD) mice. Twenty-four NOD/LT mice were randomized, according to the random number table, into a control group (4 μg/kg·day), a low-dose group (2 μg/kg·day Exendin-4), a medium-dose group (4 μg/kg·day Exendin-4) and a high-dose group (8 μg/kg·day Exendin-4) (n=6), with miR-19b expression interfered (an interference group) except for the control group. RT-qPCR was used to detect interference results and different doses of Exendin-4 were given for 8 weeks of intervention after the interference. $CD4^+$ and $CD8^+$ cell levels were detected by flow cytometry, IL-2 and IL-10 levels in the peripheral blood by enzyme-linked immunosorbent assay, and the apoptosis rate of islet cells in the pancreatic tissue by TUNEL. After 4 and 8 weeks of Exendin-4 intervention, mice in the high-dose group had lower blood glucose level than the medium-dose group ($P<0.05$). The medium-dose group had lower $CD4^+$ cell level than the high-dose group ($P<0.05$), while the medium-dose group had higher $CD8^+$ cell level than the high-dose group ($P<0.05$). After 8 weeks of intervention, compared with the medium-dose group, the high-dose group had lower IL-2 level ($P<0.05$), but higher IL-10 level ($P<0.05$). After 8 weeks of intervention, the medium-dose group had a higher apoptosis rate than the high-dose group ($P<0.05$). In conclusion, the decrease in miR-19b expression can improve the therapeutic effect of Exendin-4 on NOD, control blood glucose effectively and improve inflammatory response and immune function, as well as reduce islet cell injury. The increase in the dose of Exendin-4 can further improve its therapeutic effect on NOD.

Introduction

As a metabolic disorder of sugar, fat and protein with polydipsia, polyuria, more food and weight loss as main clinical features, diabetes is divided into type I and type II diabetes (1). With the improvement of living standards, the increase in the aging population and the changes in life structure, the incidence of diabetes continues to increase and diabetes is expected to become the seventh leading cause of death in the world by 2030, seriously endangering human health (2). Most patients with type II diabetes are caused by obesity, however, type I diabetes is not related to it, because of insulin resistance, absolutely insufficient insulin and genetic factors, most patients with type I diabetes are non-obese diabetic (NOD) patients (3).

Insulin is secreted by islet β cells, which are the main targets for the clinical treatment of diabetes (4). Exendin-4, a polypeptide that promotes islet cell regeneration and proliferation and stimulates insulin secretion from islet β cells, has a strong hypoglycemic effect and a long duration and half-life. Many studies have shown that Exendin-4 has good prospects for the treatment of type II diabetes (5,6). As a small, non-coding, endogenous RNA that is widely expressed in eukaryotic cell organisms, microRNAs regulate gene expression by causing mRNA degradation or translational suppression following the formation of RNA-induced silencing complex with proteins. There is increasing evidence that microRNAs are important for the production, secretion and function of insulin, as well as the proliferation, development and apoptosis of islet cells (7,8). A recent study reported that Exendin-4 inhibits miR-192 secretion from injured tubular epithelial cells and improves high glucose-induced tubular epithelial cell fibrosis, thereby inhibiting the downregulation of glucagon-like peptide-1 (GLP-1) and protecting renal cells (9). In addition, Exendin-4 regulates glucose metabolism, which may improve the function and quality of islet β cells and protect the cells by downregulating miR-7, miR-9 and miR-375 (10). Those studies indicate that microRNAs are effective for the treatment of diabetes with Exendin-4. miR-19b is also a molecule closely related to diabetes. It has been reported that GLP-1 regulates cholesterol homeostasis by inhibiting the downregulation of miR-19b-induced ATP-binding cassette transporter A1 (ABCA1) that plays an important role in regulating cholesterol efflux to apolipoprotein A-I and is the major maintenance mechanism of cellular cholesterol homeostasis (11). Exendin-4 has high affinity and homology with GLP-1 (12). Therefore, it is speculated that miR-19b also affects the role of Exendin-4.

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The aim of the study was to determine the effect of miR-19b on the protective effect of Exendin-4 on islet cells in non-obese diabetic (NOD) mice using ELISA and TUNEL. The results showed that, an increase in the dose of Exendin-4 can further improve its therapeutic effect on NOD.

Materials and methods

Subjects. Twenty-four NOD/LT mice were purchased from the Institute of Medical Laboratory Animals, Chinese Academy of Medical Sciences, and fed with ordinary nutritional feed (Beijing Zhecheng Technology Co., Ltd., Beijing, China) with acidified water with a pH between 2.5 and 3.0 after autoclaving as drinking water. The average age of the mice was 22.6±2.4 days, and the average body weight was 24.1±1.5 g, with a temperature of 18-22˚C and a relative humidity of 40-70%. The mice were kept separately in the terrarium, and the pads were changed every morning and evening. The environmental noise was <85 decibels (dB), ammonia concentration did not exceed 20 ppm, and the ventilation was 8-12 times per hour; the nests were changed 1-2 times and cleaned and disinfected each week; the noise did not exceed 60 dB, ammonia concentration was less than 14 ppm, and the ventilation per hour was not less than 15 times, with a 12-h day/night cycle under a fluorescent lamp. The mice were divided into the control group, the low-dose group, the medium-dose group and the high-dose group (n=6 each group), according to the random number table.

The study was approved by the Ethics Committee of Zhangzhou Affiliated Hospital of Fujian Medical University (Zhangzhou, China).

Intervention methods. All mice were adaptively fed with ordinary nutritional feed for one week and then interfered with miR-19b expression (the interference group) except for the control group. The lentivirus was used to mediate the targeted miR-19b gene, shRNA (Thermo Fisher Scientific, Inc., Waltham, MA, USA), in order to silence mouse miR-19b gene expression, with RT-qPCR detecting the interference results and the blood glucose level (BGL) (blood glucose meter) (OneTouch UltraVue). The mice were tail intravenously injected with different doses of Exendin-4 at 2 μg/kg·day (the low-dose group), 4 μg/kg·day (the medium-dose group) and 8 μg/kg·day (the high-dose group) for intervention. Exendin-4 reagent was purchased from Kangtai Biology (Beijing, China), and mice in the control group were given Exendin-4 at a dose of 4 μg/kg·day. After 8 weeks, the mice were intraperitoneally injected with sodium pentobarbital (100 mg/kg; Shanghai Rongbai Biological Technology Co., Ltd., Shanghai, China) for anesthesia, and then the peripheral blood was collected from the tail vein before the intervention, and after 4 and 8 weeks of intervention. The mice were sacrificed via cervical dislocation to isolate the pancreatic tissue under aseptic conditions.

Detection of T cell subsets. The isolated pancreatic tissue was washed twice with Hank's solution, cut with ophthalmic scissors and repeatedly beaten to free islet cells that were filtered with 300-mesh net and collected, and then prepared into a cell suspension of not less than $1x10^6$/ml. Flow cytometry was used to detect T cell subsets in the pancreatic tissue and the peripheral blood, which was performed by instrument engineers. FITC-anti mouse CD4 (catalog no. AB133616; diluted, 1:100) and Per CP-Cy5.5 anti-mouse CD8 (catalog no. AB22378; diluted, 1:100) (both from Abcam, Cambridge, MA, USA); and flow cytometry (Beijing Kexue Yingye Science and Technology Development, Beijing, China) were used to detect T cell subset.

Detection of T cell-related cytokines. IL-2 and IL-10 levels in the peripheral blood of mice were detected by ELISA in strict accordance with the kit instructions. The IL-2 and IL-10 ELISA detection kits were purchased from Shanghai Biotechnology (Shanghai, China).

Detection of islet cell apoptosis. The pancreatic tissue was cut into paraffin sections that were stained with TUNEL, and the specific method referred to protocol. Image analysis software (Image-proPlus 5.0) was used to calculate the number of TUNEL-positive cells and the total number of islet cells in five 40x10x visual fields in order to calculate the apoptotic rate of islet cells. The TUNEL detection kit was purchased from Xiamen Huijia Biotechnology Co., Ltd. (Xiamen, China; item no. 4815-30-K).

RT-qPCR. Mouse peripheral blood and TRIzol lysate (Shanghai Ying Gong Reagent Co., Ltd., Shanghai, China) were added at a ratio of 3:1 for extracting total RNA, the purity and concentration of which were detected after RNA was purified by miRNeasy Mini Kit. The quality was determined using a 2100 bioanalyzer (Agilent Technology Co., Ltd., New York, NY, USA) with A260/A280 values between 1.8 and 2.1 considered to meet the experimental requirements. Reverse transcription reaction was carried out after the RNA extraction, and PCR amplification was performed after the first-strand cDNA was synthesized by RT^2 Easy First Strand Kit. The PCR amplification system was as follows: 1 μl of cDNA template, 12.5 μl of 2X RT^2 SYBR-Green qPCR Master Mix, each 0.4 μl of upstream and downstream primers, and double distilled water added to 25 μl; pre-denaturation at 95˚C for 10 min, denaturation at 95˚C for 15 sec, annealing at 60˚C for 1 min and extension at 72˚C 30 sec, for a total of 40 cycles. Melting curve analysis was performed after the experiment during which GAPDH was used as a reaction internal reference. Experiments were repeated 3 times and the results were analyzed by $2^{-\Delta Cq}$ (13). The ABI 7500 real-time fluorescence quantitative PCR was purchased from Shanghai Ke Hua Experimental Systems Co., Ltd. (Shanghai, China). The primer sequences were designed and produced by Wcgene Biotechnology Co., Ltd. (Shanghai, China) (Table I).

Statistical methods. SPSS 21.0 statistical software (IBM SPSS, Armonk, NY, USA) was used. Measurement data were expressed as %, and χ^2 test was used for the comparison of ratio. Count data were expressed as mean ± standard deviation (mean ± SD). The t-test was used for comparison between the two groups, analysis of variance (ANOVA) for comparison between groups, LSD test for post hoc test, ANOVA with repeated measures for comparison at different time-points in the group. $P<0.05$ indicates the difference is statistically significant.

Table I. Primer sequences.

Gene name	Upstream primer	Downstream primer
miR-19b	5'-TTGCAGATTTGCAGTTCAGCGT-3'	5'-TCCCACAATCAGTTTTGCATGG-3'
GAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

Table II. Analysis of mouse BGL detection (μg/l).

Time period	Control group	Low-dose group	Medium-dose group	High-dose group	F-value	P-value
Before intervention	23.83±3.52	22.01±3.56	21.94±3.54	21.89±3.53	0.426	0.736
After 4 weeks of intervention	19.12±2.14[a]	17.33±1.89[a]	14.06±1.42[a,c,d]	11.81±1.34[a,c,d,e]	21.477	<0.001
After 8 weeks of intervention	16.24±1.84[a,b]	14.24±1.64[a-c]	12.26±1.37[a-d]	10.39±1.21[a-e]	16.207	<0.001

[a]P<0.05 compared with before intervention in the same group; [b]P<0.05 compared with after 4 weeks of intervention in the same group; [c]P<0.05 compared with the control group at the same time-point; [d]P<0.05 compared with the low-dose group at the same time-point; [e]P<0.05 compared with the medium-dose group at the same time-point. BGL, blood glucose level.

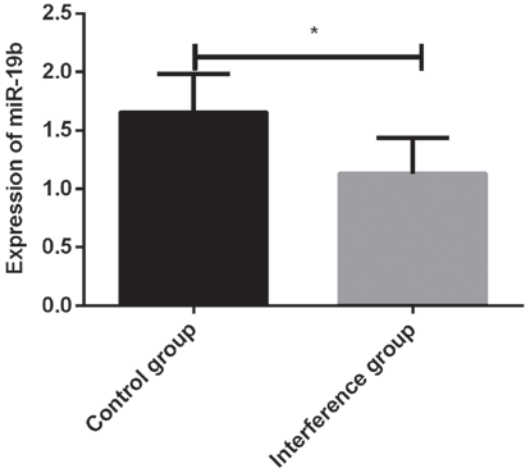

Figure 1. miR-19b expression level in the peripheral blood of mice in the control and interference groups. *P<0.05.

Results

Results of interference with miR-19b. The miR-19b expression level in the mouse peripheral blood was 1.653±0.329 in the control group, significantly higher than 1.128±0.306 in the interference group, which indicated a successful interference (Fig. 1).

Results of BGL detection. Before Exendin-4 intervention, there was no statistically significant difference in BGL between the four groups (P>0.05), whereas after 4 and 8 weeks of intervention, there was a significant difference (all P<0.001). Post hoc test showed that after 4 and 8 weeks of intervention, mice in the medium and high-dose groups had lower BGL than those in the control group (all P<0.05), whereas the high-dose group had lower BGL than the medium-dose group (P<0.05). After 4 weeks of intervention, there was no difference in BGL between the control and low-dose groups (P>0.05), whereas after 8 weeks of intervention, the low-dose group had lower BGL than the control group (P<0.05). The comparison at different time-points in the group showed that the mouse blood glucose in the four groups continued to decrease (all P<0.05) (Table II).

Results of T lymphocyte subset detection. After 8 weeks of Exendin-4 intervention, there were statistically significant differences in $CD4^+$ and $CD8^+$ cell levels in the pancreatic tissue and the peripheral blood of mice between the four groups (P<0.05). Post hoc test showed that the $CD4^+$ cell level in the control group was lower than that in the medium and high-dose groups (P<0.05), but not different from that in the low-dose group (P>0.05), and the medium-dose group had lower $CD4^+$ cell level than the high-dose group (P<0.05); the $CD8^+$ cell level in the control group was higher than that in the medium and high-dose groups (P<0.05), but lower than that in the low-dose group (P>0.05), and the medium-dose group had higher $CD8^+$ cell level than the high-dose group (P<0.05) (Tables III and IV).

Results of T cell-related cytokine detection. After 8 weeks of Exendin-4 intervention, there were significant differences in IL-2 and IL-10 levels between the four groups of mice (all P<0.001). Post hoc test showed that after 8 weeks of intervention, compared with the control group, the medium and high-dose groups had lower IL-2 level (both P<0.05), but higher IL-10 level (P<0.05), whereas compared with the medium-dose group, the high-dose group had lower IL-2 level (P<0.05), but higher IL-10 level (P<0.05). Compared with the low-dose group, the medium-dose group and the high-dose group had lower IL-2 level (P<0.05), but higher IL-10 level (P<0.05). After 8 weeks of intervention, there was no difference in IL-2 level between the control and low-dose groups (P>0.05), whereas after 8 weeks of intervention, the low-dose group had higher IL-10 level than the control group (P<0.05) (Table V).

Results of islet cell apoptosis detection. After 8 weeks of Exendin-4 intervention, the apoptosis rate of islet cells

Table III. Results of T lymphocyte subset detection in the mouse pancreatic tissue (%).

Cell	Control group	Low-dose group	Medium-dose group	High-dose group	F-value	P-value
$CD4^+$	11.42±1.02	10.23±1.03	13.45±1.02[a,b]	15.33±1.05[a-c]	28.622	<0.001
$CD8^+$	13.83±1.04	14.62±1.01	11.45±1.04[a,b]	9.33±1.05[a-c]	31.774	<0.001

[a]P<0.05 compared with the control group at the same time-point; [b]P<0.05 compared with the low-dose group at the same time-point; [c]P<0.05 compared with the medium-dose group at the same time-point.

Table IV. Results of T lymphocyte subset detection in the mouse peripheral blood (%).

Cell	Control group	Low-dose group	Medium-dose group	High-dose group	F-value	P-value
$CD4^+$	25.33±1.45	24.47±1.38	26.75±1.46[a,b]	29.17±1.53[a-c]	16.352	<0.001
$CD8^+$	31.19±1.77	32.43±1.69	28.62±1.68[a,b]	25.74±1.57[a-c]	18.696	<0.001

[a]P<0.05 compared with the control group at the same time-point; [b]P<0.05 compared with the low-dose group at the same time-point; [c]P<0.05 compared with the medium-dose group at the same time-point.

Table V. Results of T cell-related cytokine detection in the mouse peripheral blood.

Cytokine	Control group	Low-dose group	Medium-dose group	High-dose group	F-value	P-value
IL-2 (pg/ml)	57.25±8.36[a]	53.24±7.24	44.33±6.56[c,d]	35.42±5.25[c-e]	11.777	<0.001
IL-10 (pg/ml)	204.47±20.93[b]	223.56±21.47	258.38±22.31[c,d]	294.21±23.25[c-e]	17.946	<0.001

[a]P>0.05 compared with the low-dose group after 8 weeks of intervention; [b]P<0.05 compared with the low-dose group after 8 weeks of intervention; [c]P<0.05 compared with the control group at the same time-point; [d]P<0.05 compared with the low-dose group at the same time-point; [e]P<0.05 compared with the medium-dose group at the same time-point.

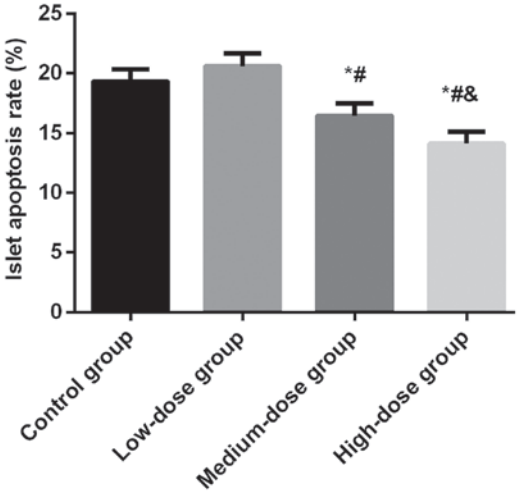

Figure 2. Results of islet cell apoptosis rate detection. *P<0.05 compared with the control group at the same time-point; #P<0.05 compared with the low-dose group at the same time-point; &P<0.05 compared with the medium-dose group at the same time-point.

was 19.33±1.04% in the control group, 20.59±1.06% in the low-dose group, 16.45±1.07% in the medium-dose group and 14.12±1.02% in the high-dose group, with a significant difference between the four groups (P<0.05). The apoptosis rate in the control group was significantly higher than that in the medium and high-dose groups (P<0.05), but not different from that in the low-dose group (P>0.05). In addition, the medium-dose group had a higher apoptosis rate than the high-dose group (P<0.05) (Fig. 2).

Analysis of adverse reactions. No significant adverse reactions were found in the mice during the study.

Discussion

Diabetes, a global public health problem with a high incidence, is difficult to cure, and the number of diabetic patients worldwide is estimated to exceed 440 million by 2030 (14,15). At different stages of NOD, pancreatic tissue morphology changes generally. The current traditional treatment cannot fundamentally prevent the reduction of islet cell number and improve functional impairment in patients, but normal islet structure and tissue form is the basis for ensuring the normal function of the body's islet physiological function (16).

Due to the islet cells being destroyed by autoimmune cells and then absolutely insufficient insulin secretion, patients with type I diabetes need exogenous insulin supportive treatment for life (17). GLP-1 is one of the most central mediators of glycemic control after gastric bypass in diabetic patients (18).

Exendin-4 is a GLP-1 analogue and not inactivated by dipeptidyl peptidase-4, which has an incretin effect, controls blood glucose and protects islet β cells (19,20), but its specific mechanism of action remains unclear. Previous findings have shown that improving pancreatic inflammation and fibrosis and changing microRNA expression level in the pancreatic tissue and blood, Exendin-4 intervention in NOD mice upregulates miR-1 level, downregulates miR-19a, miR-19b and miR-22 levels and inhibits the differentiation and maturation of islet β cells, as well as promotes the recovery of insulin secretion function (21). This is speculated to be one of the mechanisms of action of Exendin-4. In recent years, studies have verified some microRNA. Additionally, miR-204 enhances the effect of GLP-1 receptor, promotes insulin secretion and maintains glucostasis (22). The effect of miR-19b on the role of Exendin-4 was analyzed in this study.

Insulin-dependent NOD mice were studied, and then a lentiviral vector was constructed to interfere with miR-19b expression in some of the mice. RT-qPCR results showed a successful intervention. First, the effect of interference with miR-19b expression on the role of Exendin-4 was analyzed. The analysis of the mice in the control and medium-dose groups showed that by increasing the hypoglycemic effect of Exendin-4 and effectively improving $CD4^+$ and $CD8^+$ cell levels in the mouse pancreatic tissue and blood, the decrease in miR-19b expression inhibited the release of IL-2, promoted that of IL-10 and reduced islet cell apoptosis. Our results show that reducing miR-19b expression improves the therapeutic effect of Exendin-4. Then, the therapeutic effect of Exendin-4 at different concentrations was analyzed when miR-19b expression was inhibited. The results showed that the therapeutic effect of Exendin-4 was improved with the rise in its concentration, suggesting that Exendin-4 exerts a therapeutic effect on NOD through multiple targets. The mechanism of action of Exendin-4 remains to be explored more extensively. Previous studies have reported the improvement of Exendin-4 on glycemic control, inflammatory response and immune function in diabetic patients (23,24), and its protective effect on islet cells has also been confirmed in an animal experiment (25). However, there are few studies directly related to the effect of miR-19b on the treatment of diabetes with Exendin-4. It has been reported that GLP-1 regulates cholesterol homeostasis by inhibiting the downregulation of miR-19b-induced ABCA1 (11). Cholesterol homeostasis is the key to normal cell function, the improper distribution or metabolism of which has serious consequences for cells and the body, and the increase in cholesterol is also one of the important causes of diabetes (26). Exendin-4, which shares 53% homology with human GLP-1, binds to GLP-1 receptor and produces a physiological effect similar to human GLP-1 (20). This also verifies the correctness of our results to some extent.

However, there are shortcomings in this study. Although NOD mice are a common model for studying diabetes, there are differences between mice and humans, so the results of this study still require more validation of clinical trial data. The study time was short, and research on the safety of adenovirus vectors and different doses of Exendin-4 in long-term treatment is still required. Although no significant adverse reactions were found in the mice during the study, the exploration concerning the safety still cannot be ignored.

In conclusion, the decrease in miR-19b expression can improve the therapeutic effect of Exendin-4 on NOD, control blood glucose effectively and improve inflammatory response and immune function, as well as reduce islet cell injury. The increase in the dose of Exendin-4 can further improve its therapeutic effect on NOD.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JH wrote the manuscript. JH, YK and CL performed RT-qPCR and ELISA. JW and HZ were responsible for flow cytometry. XY contributed to statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Zhangzhou Affiliated Hospital of Fujian Medical University (Zhangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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