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Laminaria Japonica polysaccharide promotes the expression of insulin receptor and reduces insulin resistance in diabetes mellitus mice

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ABSTRACT

The aim of experiment was to investigate the effect of *Laminaria Japonica* polysaccharide (LJPS) on the expression of insulin receptor (InsR) and its hypoglycemic activity in type 2 diabetes mellitus mice. Type 2 diabetes mellitus models were established by feeding high fatty forage and injecting alloxan in 40 healthy male mice. The LJPS was applied as additive in physiological saline to treat the mice by intragastric administraton. The levels of fasting blood glucose (FBG) were detected by automatic blood glucose device. The tissue morphology of brainstem, liver and pancreas were stained by Hematoxylin-eosin (HE) assay and observed under light microscopy. The expression of InsR was determined by immunohistochemisty and Western blot, and the expression of InsR mRNA was detected by RT-PCR. The results indicated after treated with LJPS, the serum fasting (FBG) levels were significantly decreased than those in model group ($P<0.05$). The morphology and structure of liver and pancreas tissue improved than those in model group. The expressions of InsR mRNA and protein were significantly higher than those in model group ($P<0.05$). These results suggest that LJPS could play a hypoglycemic effect by promoting the expression of InsR in liver and pancreatic tissue to lessen insulin resistance (IR).

KEY WOEDS: LJPS; Type 2 Diabetes; Insulin; Insulin receptor; Mice

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Introduction

Inaunlin is a peptide hormone secreted by the β cells of the pancreas and composed of 51 amino acids [1]. Insulin receptor (InsR) belongs to the class of the tyrosine kinase receptors and consists of α and β subunits [2]. The α submit is extracellular which the insulin binding sites, while the β subunits has a transmembrane domain as well as extra-and intracellular domains which responsible for the subsequent intracellular and membrane signal pathways induced by insulin. Thus, the insulin binds to the α submit at the membrane which triggers the tyrosine kinase activity in the β subunit which causes phosphorylation of the receptor, mediating and regulating intracellular enzyme system activity, eventually control the metabolism. InsR is a transmembrane glycoprotein that is activated by insulin, located in the target cell membrane of the specific parts, such as liver, adipose, skeletal muscle [3-4] as well as central nervous system[5], pancreas bulble [6] and red cells [7], etc. It is reported by Havrankova that insulin receptor exits extensively in central nervous system, which number varies in different parts of brain [8], and only could specially integrate with Insulin or insulin-molecular proinsulinto to regulate the function of β cell in islets [9-10]. When the insulin binds to the InsR, it could induce structural changes within the receptor and promote the downstream processes of glucose metabolism, and eventually decrease the blood glucose. Kelp, a traditional Chinese medicine, which main activating component is *Laminaria japonica* polysaccharides (LJPS) and has the efficacy of cold-natured, salty flavor, soft lump loosen knot, dissolving phlegm and diuresis[11]. LJPS mainly consisted of sodium alga acid and fucoidan [12], it played roles of anti-oxidant [13], anticoagulation [14-15], Hypolipidemic and hypoglycemic effects [16] etc. The previous researches shown that LJPS could promote islet cell secretion function to play hypoglycemic activity by enhanced anti-oxidation [17-19] , but rarely reported about InsR expression and insulin resistance (IR) [20]. In this study, we aim to investigate the influence of LJPS on the expression of InsR and to explore the hypoglycemic effect and mechanism of LJPS in type 2DM.

MATERIALS AND METHODS

1. Mouse Model

Forty healthy male Kunming mice weighing 23-27g were purchased from the Experiment Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 20110010). Animals were acclimatized to feed with normal forage for 7 days. Ten mice were randomly selected as a control group and given general forage. The remaining 30 mice were fed with high fatty forage. After 4 weeks of dietary manipulation, alloxan (50mg/kg body weight) was injected intraperitoneal ly once every other day for 3 times to establish type 2 MD models [20]. Mice in the control group were administered with equivalent amounts of normal saline. Fasting blood glucose (FBG) was measured third day after the final injection. The type 2 DM animal model as the successful markers for establishing model was when FBG differed by more than two standard deviations from the control group. Ten experimental mice were excluded because they did not satisfy the standard. The remaining 20 type 2 DM model mice were divided randomly into model group (n=10) and treatment group (n=10).

2 Intervention

According to our previous research confirmed LJPS effective therapeutic dose 3.00g/kg body weight [21], LJPS was diluted with normal saline to desired concentrations (300mg/ml) with intragastric administration once a day for 2 weeks. In control group and model group, mice were orally administrated an equivalent amount of saline. Meanwhile, all of animal model mice were fed normal forage for 2 weeks.

3. Specimen collection

3.1. Blood Sampling: Blood samples 1.0ml for each mouse were collected from heart and centrifuged for 10 min at 4000r/min to separate serum and then stored at -20℃.

3.2 Paraffin section: Five mice form each group were perfused and fixed form heart with 0.9% saline 45ml and 4% paraformaldehyde 45ml. Then brainstem, liver and pancreas tissues were collected and fixed in 4% paraformaldehyde for 2h and distilled water for 4h. The sample tissues were subjected conventional ethanol dehydration, transparent of xylene, paraffin embedding, and cut into serial section with 5 mm thickness by microtome (Leica 2015, Shanghai) and stored at room temperature.

3.3 Total protein extraction: Five mice from each group were perfused from heart with 0.9% saline 45ml, and then collected brainstem tissue 50mg, liver tissue 100mg and pancreas tissue 100mg. Add RIPA lysis buffer (P0013B, Beyotime Co. Ltd.) to grind tissues into homogenizer on the ice, then centrifuged (Eppendorf 5801,Germany) at 12000r/min for 15 min to separate the supernatant, and determine the concentration o f protein by enhanced BCA protein assay kit (P0010, Beyotime Co. Ltd.), then stored at -20℃.

3.4 Total RNA extraction: Trizol extraction kit was purchased from Invitrogen Co. Ltd. Firstly, added 1ml Trizol solution into brainstem tissue 50mg, liver tissue 100mg and pancreas tissue 100mg, ground 5min 4℃, and centrifuged at 12000r/min for 15min to separate the supernatant into sterile EP tube. Secondly, added 0.2ml chloroform, mixed 15s at room temperature, and centrifuged at 12000r/min for 15min to separate the colorless aqueous phase into another sterile EP tube. Thirdly, added 0.5ml isopropanol, mix gently and centrifuged at 12000r/min for 15 min to discard the supernatant carefully, and added 75% precooling ethanol 1ml to wash RNA precipitation to , centrifuge (4℃) at 7500r/min for 5min. Then discard the supernatant carefully to dry about 20min in cupboard (RNA precipitate becomes transparent). At last, added 0.1% DEPC·H_2O 100μl to dissolve RNA for 10min at 57°Cwater bath. Micro-spectrophotometer (K5500, Beijing Kaiao Tech. Co., Ltd) was used to detect RNA abundance and stored at -80℃.

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4. Detection index

4.1. Fasting blood glucose (FBG): Using automatic blood glucose meter (Johnson & Johnson Medical Ltd) and Onetouch Ultra test strips to detect FBG (mmol/L).

4.2. Hematoxylin-eosin (HE) staining: The paraffin section of brainstem,liver and pancreas tissues were general hydration of dewaxing with hematoxylin semen infection for 2 min, saturated lithium carbonate to blue. After 1% hydrochloric acid in ethanol differentiation, the nucleus becomes blue and the cytoplasm showed different degrees of red.

4.3. Immunohistochemistry staining: Rabbit anti-mouse InsR antibody (Ab75998, Abcam). SABC mmunohistochemistry kit (SP-90001, ZSGB-BIO). Firstly, paraffin sections were de-waxed and incubated 3%H_2O_2 10 min, soaked by PBS with 5min. Dropped reagent A on the section to incubate at room temperature for 10min, and washing with PBS for 5min×3times. Dropped primary InsR antibody (1:50) to incubate at 37℃ for 1h and then PBS washing 5min×3times. Secondly, dropped reagent B to react at 37℃ for 15min and then PBS douching 5min×3times. Thirdly, dropped reagent C to react at 37℃ for 10min, and then PBS douching 5min×3times. At last, colored by DAB chromogenic reagent and re-stain by haematoxylin. Under microscope, which cytoplasm or membrane appeared brown granules was considered as positive cells, while negative control sections stained with 0.1mol/L PBS instead of without primary antibody and no color appeared. Under optical microscope with magnified 400 times, five non-overlapping visual fields in each section were randomly selected to observe and analysis the absorbance value (*A*) of InsR expression by Image-Pro Plus software. The InsR expression intensity was presented by the positive cells *A* subtracting the background *A*.

4.4. Western blotting: According to total protein 50μg, InsR protein was separated by 8%SDS-PAGE electrophoresis, transferred to a PVDF membrane and sealed for 1h by 5% evaporated skimmed milk. Then added rabbit anti-mouse primary antibody (InsR 1:50; GAPDH, TA-08, 1:10000), 4℃concentrating table to stay overnight, TBST washing 10min×3times. Added peroxidase labeling goat anti-rabbit secondary antibodies (Abcam, Ab6721, 1:5000) and horseradish enzyme marker goat anti-mouse lgG (ZB-2305，1:10000) to incubate at 37℃ for 1h. Took out the membrane and washed with TBST for 10min×3times, TBS for 5min×2times. Then plus plus A, B liquid developer (1:1) and imaged by Vilber Fusion FX7 imaging system. Using Quantity One software to analysis the absorbance value (*A*) of the interest protein InsR (156kD) and internal reference (36kD), and calculate the relative content of InsR (InsR *A*/GAPDH *A* value). The experiment was repeated 3 times and result was presented as mean ± standard deviation ($\bar{x}$ ±s).

4.6. RT-PCR: Primers were synthesized by Shanghai Yingjiekai & Co. Ltd. InsR forward primer: 5′-ATG GGC TTC GGG AGA GGA-3′, reverse primer: 5′-GGA TGT CCA TAC CAG GGC AC-3′, product length 120bp; GAPDH forward primer: 5′-ACC ACA GTC CAT GCC ATC AC -3′, reverse primer: 5′-TCC ACC ACC CTG TTG CTG TA-3′， product length 452bp. RT-PCR: semi-quantitative PCR was conducted according to Takara DRR014A PrimeScript™ RT-PCR kit. The reactions were pre-degeneraed at 95℃ for 5min, then degenerated at 94℃ for 30s, amplified at 65℃ (for InsR) or 60℃ (for GAPDH) for 30s and extension at 72℃ for 40s, 35 cycles, and then followed by 72℃ for 10min. Electrophoresis: 50μl PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The absorbance (*A*) of interest gene InsR and internal reference GAPDH was imaged by Vilber Lourmat gel imaging system and analysed by Quantity One software. The InsR mRNA expression level was presented as a ratio of InsR *A*/GAPDH *A*. The experiment was repeated 3 times and result was presented as mean ± standard deviation ($\bar{x}$ ±s).

5. Statistical analysis

According to SPSS 17.0 software analysis, Data were expressed as mean±standard deviation ($\bar{x}$ ±s) that multi-groups were compared using analysis of variance (ANOVA), two groups were compared by *t*-test.

RESULTS

1. FBG levels

Before modeling, there was no significant difference of animal FBG level among two groups and control group (P >0.05). After modeling, these groups of animals FBG level had significant difference by analysis of variance (F=14.32, q=0.01-1.57, P<0.05), There was significantly higher compared model group or control group(t=2.64， P <0.05). each treatment group animals FBG levels were significantly lower than the model group (F=4.02, q=0.01-2.94, P <0.05). Table 1.

Table 1 FBG levels before and after treatment of mice ($\bar{x}$ ±s, n=10, mmol/L)

Groups	Before modeling	After modeling	After treatment
Control group	7.40±1.23	6.70±1.05	6.19±1.27
Model group	7.40±1.23	10.18±0.97[a b]	8.47±0.91[b]
Treatment group	7.40±1.23	9.95±1.03[a]	6.86±1.46[c]

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[a] Compared with before modeling, $P<0.05$; [b] Compared with control group, $P<0.05$; [c] Compared with model group, $P<0.05$

2. Tissue pathology

There was no significant difference among these groups of brainstem nerve cells with edge sharpness, structures normal. In control groups, hepatic lobule arranged in order, but in model groups appeared focal hepatocytes vacuolar degeneration. In control group, islets was demarcation clear and more cells, while reduced significantly in model groups of islet cells with vacuoles degeneration. After treated with LJPS, the morphology and structure of hepatocytes and islets improved significantly than those in model group. Figure 1.

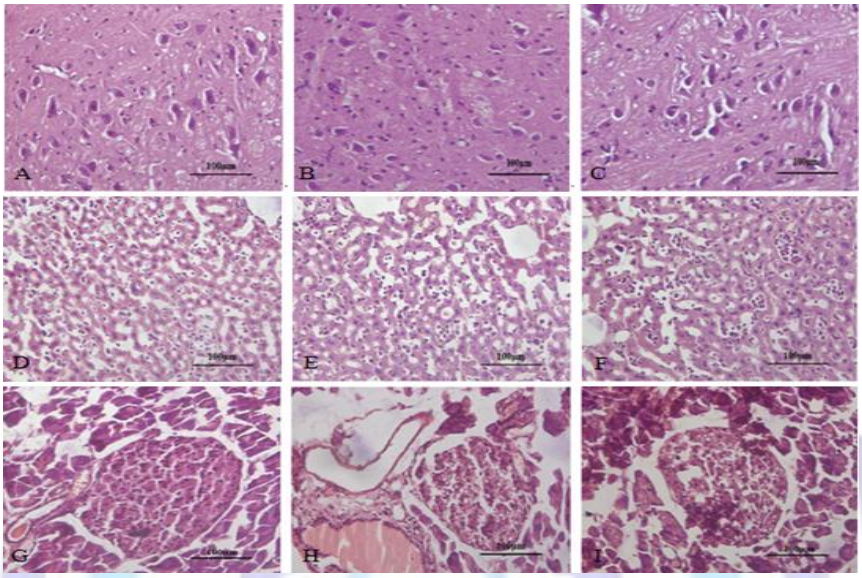

Figure 1 The with pathology structures in brainstem, liver and pancreatic tissue of mice, HE×400

A-C: Brainstem (control, model and treatment groups); D-F: Liver (control, model and treatment groups); G-I: Pancreas (control, model and treatment groups)

3. Immunohistochemical staining

There was no significant differences of InsR expression level in brainstem tissue between control, model and treatment groups ($P>0.05$). In liver and pancreas tissue, InsR expression levels in model group decreased significantly than those in control groups ($P<0.05$), while in treatment group increased significantly than those in model groups ($P<0.05$) . Figure 2 and Table 2.

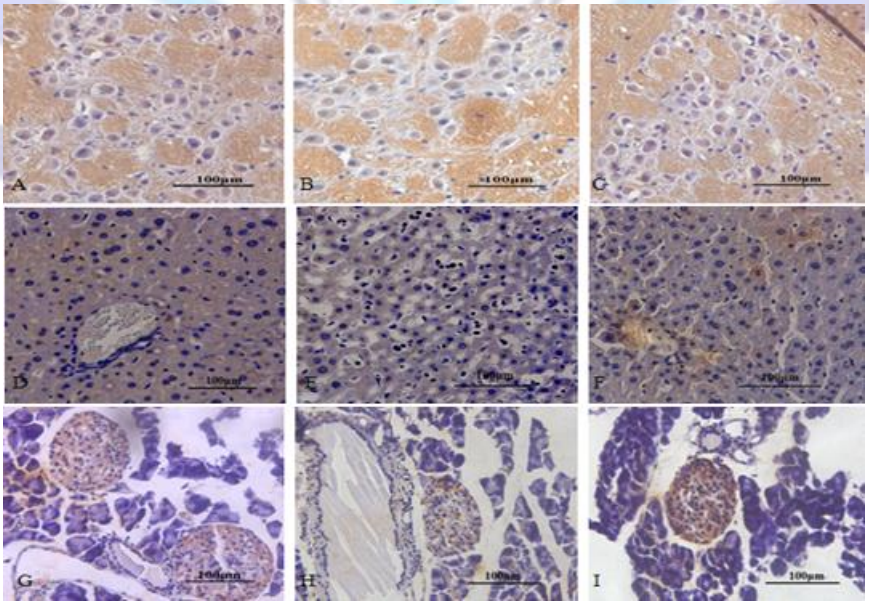

Figure 2 The expressions of InsR in brainstem, liver and pancreatic tissue of mice, IC×400

A-C: Brainstem (control, model and treatment groups); D-F: Liver (control, model and treatment groups); G-I: Pancreas (control, model and treatment groups)

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Table 2 The expressions of InsR in different tissues of mice ($\bar{x}$ ±s, n=5)

Groups	InsR-B	InsR-L	InsR-P
Control group	0.245±0.023	0.171±0.004	0.298±0.007
Model group	0.237±0.002	0.124±0.017$^{\triangle a}$	0.195±0.009$^{\triangle b}$
Treatment group	0.242±0.014	0.159±0.015$^{\blacktriangle a}$	0.259±0.003$^{\blacktriangle b}$

Compared to control group, $^{\triangle a}t$=23.72, $^{\triangle b}t$ =20.25, P<0.05；Compared to model group

5. Western bloting

No significant differences of InsR protein expression level in brainstem tissue existed between control, model and treatment groups (t=1.39, t=-0.89, P＞0.05). In liver and pancreas tissue, InsR protein expression levels in model group were significantly lower than those in control group (t=17.44, t=21.10, P＜0.05), but in treatment group higher than those in model groups (t=15.75, t= 9.73, P＜0.05) . Figure 3 and Table 3.

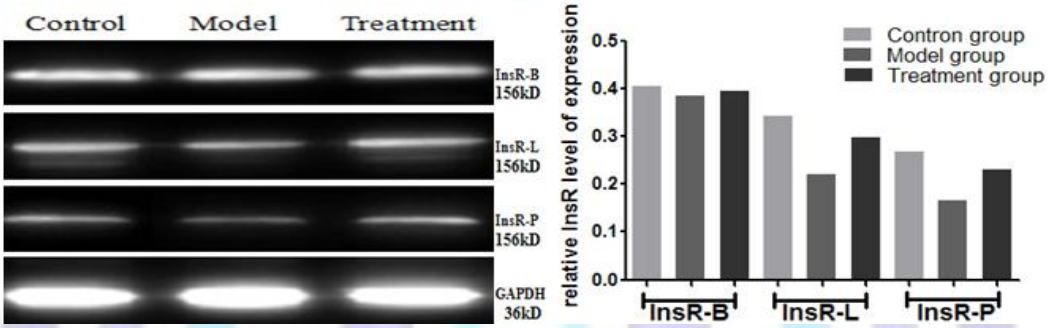

Figure 3 The expressions of InsR protein in different tissues of mice

InsR-B: Brainstem；InsR-L: Liver；InsR-P: Pancreas

Table 3 The expressions of InsR protein in different tissues of mice ($\bar{x}$ ±s, n=5)

Groups	InsR-B	InsR-L	InsR-P
Control group	0.403±0.021	0.343±0.014	0.268±0.009
Model group	0.384±0.021	0.221±0.007$^{\triangle a}$	0.165±0.006$^{\triangle b}$
Treatment group	0.395±0.018	0.298±0.008$^{\blacktriangle a}$	0.229±0.013$^{\blacktriangle b}$

Compared to control group, $^{\triangle a}t$=17.44, $^{\triangle b}t$=21.10, P<0.05；Compared to model group, $^{\blacktriangle}t$=17.44, $^{\blacktriangle b}t$=21.10, P<0.05

6. RT-PCR

In liver and pancreas tissue, InsR mRNA expression in model groups were significantly decreased than those in control groups(t=13.41, t=30.51, P＜0.05), while increased significantly than those in model groups (t=-9.60, t=-18.25, P＜0.05). There was no significant differences of InsR mRNA expression in rainstem tissue between the control , model and treatment groups (t=1.55, t=1.53, P＞0.05). Figure 4 and Table 4.

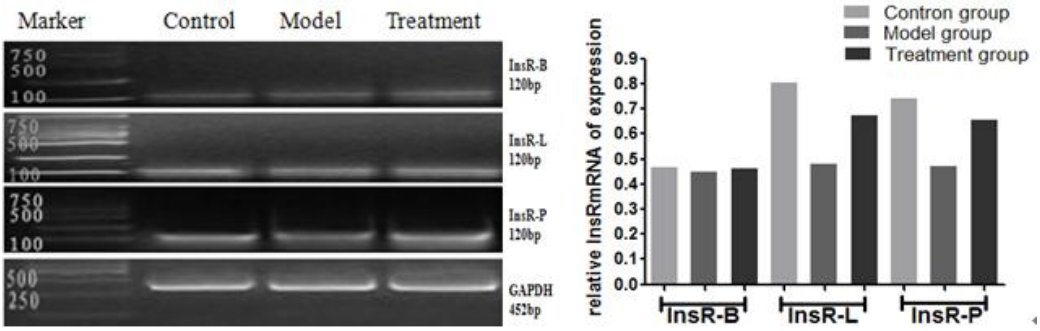

Figure 4 The expression of InsR mRNA in different tissues of mice InsR-B: Brainstem；InsR-L: Liver；InsR-P: Pancreas

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Table 4 The expression of InsR mRNA in different tissues of mice ($\bar{x}$ ±s, n=5)

Groups	InsR mRNA-B	InsR mRNA-L	InsR mRNA-P
Control group	0.465±0.021	0.805±0.038	0.742±0.014
Model group	0.449±0.011	0.480±0.039$^{\triangle a}$	0.469±0.039$^{\triangle b}$
Treatment group	0.462±0.016	0.673±0.022$^{\blacktriangle a}$	0.655±0.018$^{\blacktriangle b}$

Compared with control group, $^{\triangle a}$ t=13.41, $^{\triangle b}$ t =30.51, P<0.05; Compared with model group, $^{\blacktriangle a}$ t= 9.60, $^{\blacktriangle b}$ t =18.25, P<0.05

DISCUSSION

Type 2 DM is a group of metabolic diseases, characterized by high blood glucose in the context of insulin resistance, and relative insulin insufficiency [22] Almost all the metabolisms in the body are regulated by insulin. IR is the common pathway of the metabolism disorder. In 1998, Stern [23] proposed the "common soil" hypothesis considering that IR and its secondary metabolic disorder are the common soil of coronary heart disease, diabetes and hypertension. A chronic physiology increment in plasma glucose concentration could lead to "glucose toxicity", inhibit the expression of insulin gene and generate oxidative stress[24]. Only under the condition of high blood glucose, high fatty acid impairs the function of beta cells, which is regarded as the consequence of "glucose toxicity". Combined with InsR on the target cell membrance, insulin caused the receptor tyrosine residuses phosphorylation itself and β subunits tyrosine protein kinase activation, and the tyrosine protein kinase induced the target cell substrates such as insulin receptor substrate1 (IRSI) or Src homology collagen protein phosphoryzation of tyrosine residues, which played an important role in the InsR signaling. The main activity of activation of the InsR is inducing glucose uptake. For the reason "insulin insensitivity" or a decreasing in insulin receptor signaling, it leads to Type 2 DM. Metabolically, the insulin receptor plays a key role in the regulation of glucose homeostasis [25-26]. Zhang [27] reports indicated that traditional Chinese medicine berberine could promote the expression of InsR gene in liver and muscle cells by protein kinase C (PKC) dependent InsR enhancing the sensitivity to the InsR in the peripheral tissue. Takikawa et al. [28] found that blueberry (main composition is anthocyanin) could improve blood glucose and increase the sensitivity to InsR by AMP activitied protein kinase (AMPK). Werner et al [29] reported that a new type of powerful selective pancreatic glucogen peptide 1 (Glp1) agonists, Lixisenatifde, could strengthen bio-synthesis of InsR and stimulate the β cell multiplication. It has been previously reported that kelp polysaccharides could play a function of partial pancreatic islet cells, improve the level of serum insulin which is benefit to the blood glucose control in type 2 DM. Any of the expression disorder or dysfunction with Insulin and InsR will lead to insulin resistance. Signal pathway conduction dysfunction is one of the main reasons for the pathogenesis of insulin resistance [30]. So, it will be helpful to clarify the molecular mechanism of how LJPS or other interference factors improve the insulin resistance.

This experiment confirmed that FBG of animal had significantly higher level, focal cell vacuolar degeneration in the liver and pancreatic tissues in model groups, pancreatic β cell be damaged and insulin secretion function synchronized declined on type 2 diabetic mouse. Immunohistochemistry, Western blot and RT-PCR experiments indicated that no significant difference of InsR expression in brainstem tissue between groups of mouse. InsR has no affected by blood glucose and maintain its stability, importance to maintaining stability of the physiological functions of the brain. In liver and pancreatic tissues, InsR mRNA and protein expression levels decreased significantly in model groups, so induced IR due to InsR number reducing and sensibilities descend in type 2 DM. After treated with LJPS, InsR expression levels increased significantly, while tissues morphology has improved and FBG levels were decline in liver and pancreatic. The further confirmation of LJPS could improve InsR expression levels; reduce IR and glucose metabolism processes in type 2 diabetes, to play hypoglycemic activity in liver and pancreatic tissues.

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