Original Article
JPQ downregulates the P38MAPK signal pathway in skeletal muscle of diabetic rats and increases the insulin sensitivity of Skeletal Muscle

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Abstract: This study aims to explore the acting mechanism of the Jianpi Qinghua Recipe (JPQ) in skeletal muscle of rats. A high fat diet combined with streptozotocin (STZ) was adopted to establish a type 2 diabetic rat model system. The rats were randomly divided into a normal control group (CON), a model group (IR group), a Jianpi Qinghua Receipt group (JPQ group) and a pioglitazone treatment group (PIO group), and were administered drugs intragastrically for 8 consecutive weeks. Compared with the model group, the mRNA and protein expression levels of P38MAPK and MKK6 in the JPQ group were significantly lower (P < 0.05), while those of MKK3 were significantly higher (P < 0.05). Western blot results showed that the expression of Glut-4 protein was higher in the JPQ group than in the model group (P < 0.05). Blood glucose was lower in the JPQ group than in the IR group (P < 0.01) The glucose infusion rate of diabetic rats after JPQ treatment was significantly higher than in the model group (P < 0.01), and was not significantly different to that of the PIO (Western medicine) group (P > 0.05). JPQ enhances the insulin sensitivity of skeletal muscle. The mechanism may involve downregulation of the p38MAPK signal pathway in skeletal muscleand increased expression of GLUT4.

Keywords: Jianpi Qinghua Recipe, P38MAPK, skeletal muscle, GLUT4

Introduction

In recent years, with the improvement of living standards and associated lifestyle changes, the prevalence of diabetes is increasing, making it the most common metabolic endocrine disease. Studies have demonstrated that the occurrence and development of type 2 diabetes are closely linked to states of chronic inflammation in the human body [1]. Among these, insulin resistance (IR), an important pathogenesis [2], may be induced by inflammatory factors, promoting the progression of diabetes through a variety of signaling pathways [3].

It has been found that skeletal muscle tissue plays a crucial role in peripheral insulin resistance [4]. As an insulin-sensitive tissue, it is an important site for the transport and absorption of glucose. Insulin may act on skeletal muscle tissues for glucose transportation and uptake through a series of complex enzymatic cascade reactions involving mitogen-activated protein kinases (MAPKs) [5]. Previous studies have found high expression levels of tumor necrosis factor-α and other inflammatory factors in tissues displaying insulin resistance in diabetic patients, especially in the liver and skeletal muscles [6]. At the same time, the MAPK signaling pathway was found to be abnormally activated. Regulation of abnormal levels of glucose metabolism in order to reduce insulin resistance may present a new avenue for the prevention and treatment of diabetes.

Jianpi Qinghua receipt (JPQ) is a cipher prescription in our hospital and functions to invigorate the spleen and kidney and lower blood sugar, and has effective hypoglycemic properties in clinical applications. However further study is required to determine whether it enables improved insulin resistance in skeletal muscle through an anti-inflammatory mechanism, thereby lowering blood sugar.

Table 1. Primer sequence

Protein	Forward primer sequence	Reverse primer sequence
P38MAPK	5'-TAGACGAATGGAAGAGCCTGAC-3'	5'-GGCACTTGAATGGTATTTGGAG-3'
MKK6	5'-CACATGAACTTGAACAGATGCAG-3'	5'-CAGTGCTTCTCGGCTGATCC-3'
MKK3	5'-TCCGCCATGAGAATGTTATAGGC-3'	5'-GGTGGTGTTGATAAGCAGATTGG-3'
Glut4	5'-TGGCTGGAAGTTTGGGACCTA-3'	5'-GTCCAACATCAGCAGGTTCAT-3'
β-actin	5'-ACTCCCATTCCTCCACCTTT-3'	5'-TTACTCCTTGGAGGCCATGT-3'

sheng Technology Co. Ltd; an Insulin Kit was purchased from Beyotime Biotechnology, Dalian.

Modeling, grouping and drug administration

In this study we successfully developed a rat model of type 2 diabetes and observed the effect of JPQ on the expression levels of p38MAPK in skeletal muscle tissue. This allowed us to explore its hypoglycemic effect on type 2 diabetes and its effect on the improvement of insulin resistance in skeletal muscle.

Materials and methods

The experimental animals

A total of 40 healthy male specific pathogen free (SPF) Sprague Dawley (SD) rats at 6 weeks of age and weighing (250 ± 50) g were used in this study. All rats were adaptively fed for 1 week and housed at a temperature of 24 ± 2°C, humidity of 40%-60%, and in a light-dark cycle (14:10). The rats were fed regularly and were free to drink, ensuring sufficiency of food and water.

Experimental reagents

Jianpi Qinghua decoctions (*Radix codonopsis*, *Astragalus mongholicus*, *Rhizoma dioscorea*, *Radix puerariae*, *Rhizoma coptidis*, *Scutellaria*, *Polygonatum sibiricum*, and *Ramulus euonymi alati* [5:5:5:5:1:3:5:5]) was provided by the Department of Pharmacy, ShangHai Shuguang Hospital. The received material was decocted and condensed into a TCM decoction containing 1.3 g/mL of raw medicine, which was stored in a refrigerator at 4°C until further use. Pioglitazone tablets (Huadong Medicine Co., Ltd.; approval No. H20060664; strength: 30 mg/tablet) were dissolved in saline solution to 1.5 mg/mL. Rabbit P38MAPK and P-P38MAPK, rabbit MKK3 and MKK6, goat polyclonal antibody IgG against glucose transporter 4 (GLUT4), and Rabbit anti-GAPDH antibody were all bought from Cell signaling Technology, Boston, MA, USA. Chemiluminescence (ECL) kits were purchased from Zhongshan Company in Beijing; TaKaRa RNA PCR kit was bought from Dalian biologic engineering Co., Ltd.; a blood glucose kit was bought from Shanghai Rong-

40 rats were randomly divided into a normal group (CON), a model group (IR group), a Jianpi Qinghua Receipt group (JPQ group), and a pioglitazone treatment group (PIO group). The rats in IR group, PIO group and JPQ group were fed a high-fat and high-sugar diet for 8 weeks, after which they were fasted for 12 h, weighed, and given a single STZ (35 mg/kg) intraperitoneal injection. After 7 days of injection, fasting blood glucose was measured by tail vein blood sampling. If the fasting blood glucose content was 16.7 mmol/L or more, the T2DM rat model was considered to have been successfully established [7]. Different drugs were administered to different groups, with the rats in PIO group given 15 mg/kg of pioglitazone while those in JPQ group were given 13 g/kg by intragastric administration once a day for consecutive 12 weeks. The control group and model group were given the same volume of normal saline by intragastric administration.

Sample collection

After 12 weeks of continuous treatment, the rats in each group were fasted and denied liquids for 12 h, weighed, and anaesthetized by intraperitoneal injection of 2% pentobarbital sodium (40 mg/kg). Blood was collected from abdominal aorta, and blood glucose was assayed. After drawing, the blood was stored for 1 h at room temperature and then centrifuged at 3500 r/min for 15 min. The serum was collected and stored at -80°C. Rat gastrocnemius tissue was recovered and preserved by liquid nitrogen flash-freezing after washing in ice-cold physiological saline.

Detection of the expression of P38MAPK, MKK6, and MKK3 protein in gastrocnemius muscle by protein immunoblotting

Cold PMSF, RIPA, and phosphatase inhibitors were added to 100 mg of gastrocnemius tissue to extract tissue protein. Samples were homogenized on ice and centrifuged at 12,000 rpm for 10 min at 4°C, after which the supernatant

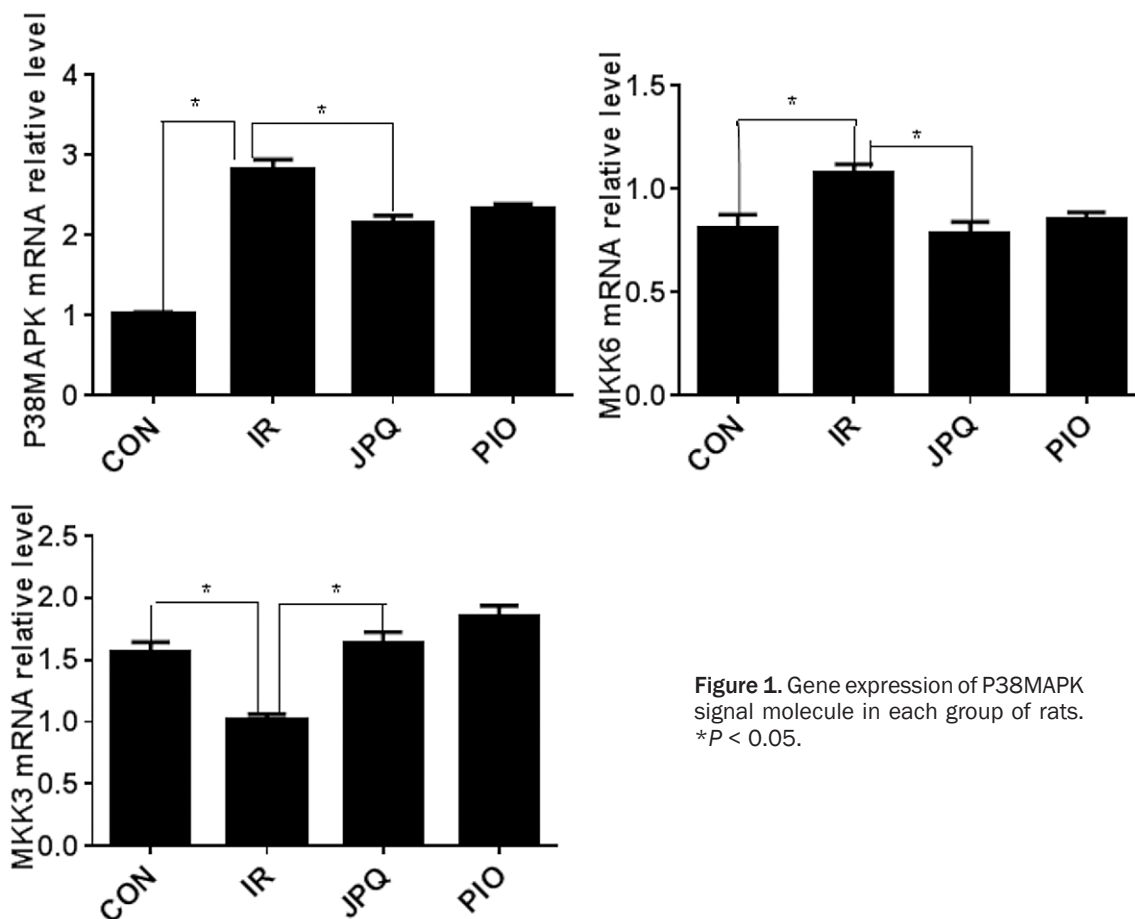Figure 1. Gene expression of P38MAPK signal molecule in each group of rats. *P < 0.05.

was extracted and used to quantify protein using the BCA method. Proteins were resolved by SDS-acrylamide gel electrophoresis, transferred to a membrane, and incubated with the appropriate primary antibody. The transfer membrane was washed with TBST and a secondary antibody added, followed by a second wash step with TBST. Blots were developed with ECL chemiluminescent agent and images captured using a gel imaging system. Gray scale values of imaged target strips were determined using Image J software.

The detection of the expression of P38MAPK, MKK6, MKK3 and GLUT4 genes in the gastrocnemius muscle of rats by real-time fluorescence quantitative PCR

Total RNA was extracted from 100 mg of gastrocnemius muscle tissue using an RNAiso Plus kit and used to synthesize cDNA by reverse transcription. PCR was conducted using an AccuPower® 2X Green StarqPCR Master Mix containing SYBR Green. The primers were designed by Primer Premier 5 software and synthesized by Shanghai Sangon Biotechnology Co., Ltd. The conditions of PCR amplification were: The thermal cycling for the PCR reaction began with a 95°C incubation for 5 min. This was followed by 45 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 5 s. The ramping temperature rate to the annealing step was set at 1.0°C/s. The specificity of primers (**Table 1**) was determined from the melting curve and the relative expression of relevant genes in gastrocnemius muscle was determined by means of the $2^{-\Delta\Delta CT}$ method. (http://www.biotechniques.com/softlib/qgene.html) quantitative software was used to analyze gene expression based on cycle threshold values normalized to β-actin expression.

Glucose infusion rate detection by hyperinsulinemic-euglycemic clamp

Three rats were selected and denied food, but allowed water, for 12 hours, after which they were anaesthetized by injection of 2% pentobarbital sodium into the abdominal cavity. The rats were fixed to isolate the left carotid artery
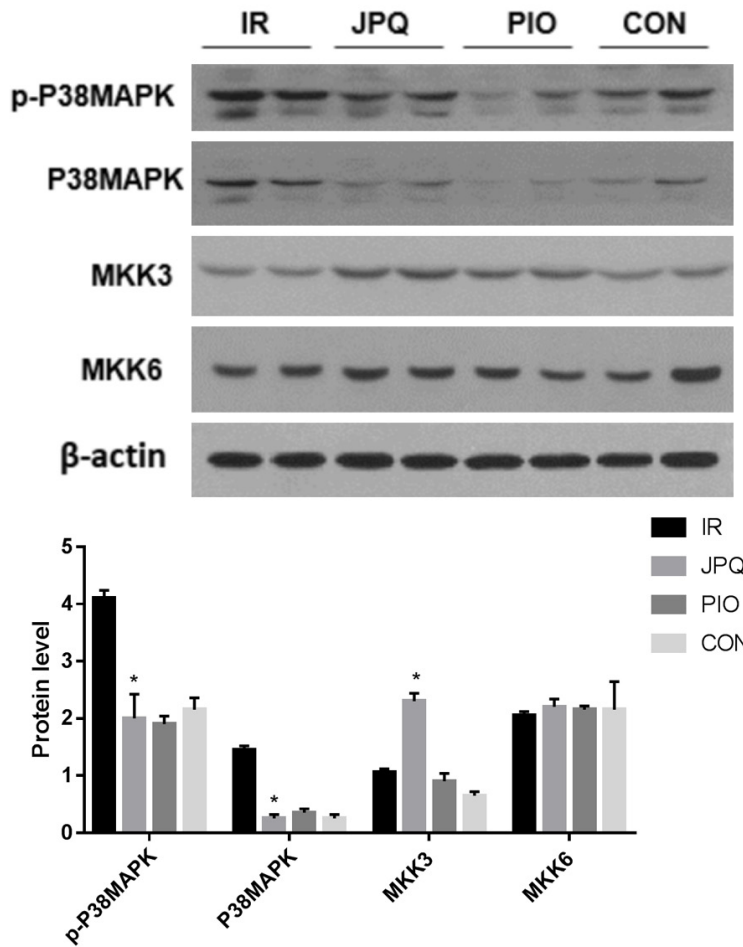
Figure 2. Wester-blot expression of P38MAPK signal molecule in each group of rats. *P < 0.05.

and the area under the insulin response curve (AUC) was calculated.

Statistical method

SPSS17.0 (Tianjin Soft Kewang Science and Technology Co., Ltd.) was used for the statistical analysis. Mean ± the standard deviation ($x ± SD$) was used to express the measurement data, and the two-sample t test was adopted. Multiple groups were analyzed by application of ANOVA. *P* value of < 0.05 indicated statistical difference.

Results

The effect of JPQ on the mRNA expression of P38MAPK signaling pathway

The mRNA expression levels of P38MAPK and MKK6 in rats from the model group were significantly higher than of those in the control group (P < 0.05) while the mRNA expression level of MKK3 were significantly lower (P < 0.05). Compared with the model group, the mRNA expression levels of P38MAPK and MKK6 in JPQ group were significantly decreased (P < 0.05), and the mRNA expression levels of MKK3 significantly increased (P < 0.05) (**Figure 1**).

The effect of JPQ on protein expression of P38MAPK signaling pathway

The protein expression levels of P38MAPK in rats from the model group were significantly higher than those from the control group (P < 0.05). Compared with the model group, the protein expression of P38MAPK was decreased significantly (P < 0.05), and the protein expression of MKK3 was increased significantly (P < 0.05) (**Figure 2**).

The effect of JPQ on Glu-4 gene expression and protein levels

The PCR results suggest that Glu-4 in the model group decreased significantly (P < 0.05).

and the right internal jugular vein, and a PE-50 catheter was inserted into the blood vessel, to which insulin infusion and glucose infusion pumps were connected. Basal insulin and basal blood glucose (BBG) were measured, after which insulin was continuously infused at a rate of 0.12 U/kg/h. Blood glucose was measured every 5 min. Glucose (10%) was infused and the glucose infusion rate adjusted in order to maintain blood sugar in the range of 0.5 mmol/L. Blood glucose was measured a total of 24 times per experiment.

The detection of intraperitoneal insulin tolerance test (IPITT) by injection in the fasting state

After 4 weeks of drug administration, the rats were denied food, except water, for 12 hours. Rats from each group were injected with insulin intraperitoneally and assayed the insulin level at four time points (0, 30, 60 and 120 mins)
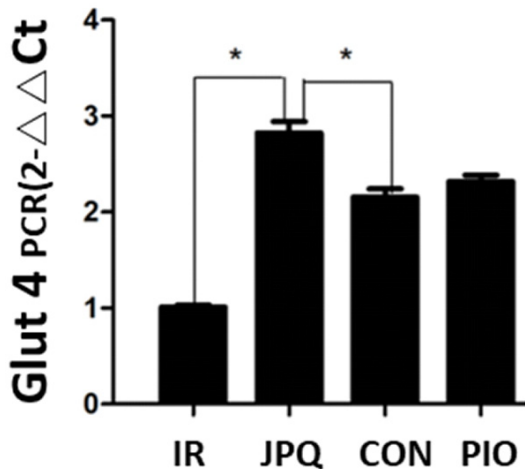
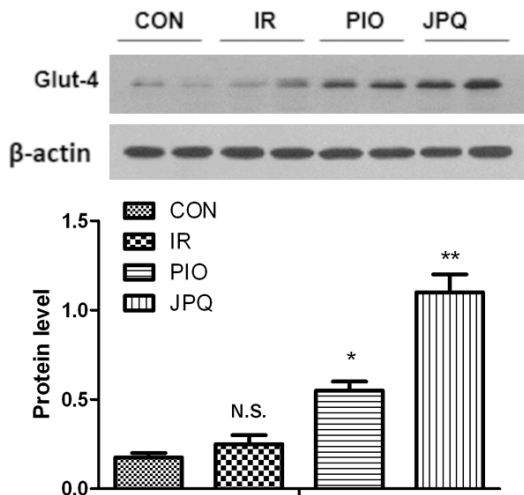Figure 3. Gene expression of Glut-4 in each group of rats. *$P < 0.05$.

Figure 4. Western-blot expression of Glut-4 signal molecule in each group of rats. Note: compared with CON, NS $P > 0.05$, *$P < 0.05$, **$P < 0.01$.

The expression of Glut-4 in JPQ group was higher than that of the model group ($P < 0.05$) (**Figure 3**). The Western blot results demonstrate that the expression of Glut-4 protein in the JPQ group was higher than that in the model group ($P < 0.05$) (**Figure 4**).

Effect of JPQ on insulin resistance

The experiment results showed that the blood glucose levels at different time points were lower in the JPQ group than in the IR group ($P < 0.01$) and that the AUC decreased significantly ($P < 0.01$), suggesting that the insulin tolerance in the JPQ group was improved (**Table 2**).

The effect of JPQ on glucose infusion rate (GIR)

The results of the hyperinsulinemic-euglycemic clamp test indicate that the glucose infusion rate in diabetic rats following the JPQ treatment was significantly higher than in rats from the model group ($P < 0.01$), but that no significant difference was observed compared to the PIO group ($P > 0.05$) (**Figure 5**).

Discussion

JPQ is a cipher prescription of our hospital and functions to invigorate the spleen and kidney and lower blood sugar. It has a powerful hypoglycemic effect, but further studies are necessary to explore whether JPQ improves insulin resistance in skeletal muscle, thereby lowering blood sugar through an anti-inflammatory mechanism. This study successfully established a model of type 2 diabetes in rats to observe the effect of JPQ on the expression level of p38MAPK in skeletal muscle tissue. The results illustrated that administration of the JPQ significantly improved the insulin resistance of skeletal muscle in diabetic rats and enhanced the insulin sensitivity of skeletal muscle. Insulin is a strong attachment inducer of Myocyte Enhancer Factor2 (MEF2) transcription factor to Glut4 gene promoter. Insulin enhances MEF2 binding to DNA by the means of p38 Mitogen Activated Protein Kinase (P38MAPK) [8]. The underlying mechanism may rely on the downregulation of the P38MAPK signaling pathway in skeletal muscle and increasing the expression of GLUT4.

Insulin resistance is an important pathophysiological mechanism in type 2 diabetes [9]. Skeletal muscle, as the main target organ for insulin-associated stimulation of glucose intake [10], we suppose that it may be a key component in the occurrence of peripheral insulin resistance in diabetes. The MAPK cascade signaling pathway is an important signaling pathway in diabetes, and includes MAP kinase, MAPK kinase (MEK) and MEK kinase (MEKK) [11-14]. Oxidative stress and inflammation are important mechanisms underlying the occurrence and development of diabetes and associated tissue damage [14, 15], which promote the activation of the p38MAPK signaling pathway. This leads to the expression of upstream inflammatory factors and fibrotic cytokines,

Table 2. Intraperitoneal insulin tolerance test (IPITT) ($\overline{X} \pm s$)

Group	0 min	30 min	60 min	120 min	AUC
CON	5.12±0.45	3.30±0.24	2.67±0.28	3.07±0.26	10.06±0.60
IR	14.11±3.86[**]	12.80±4.50[**]	8.29±3.02[**]	8.53±2.69[**]	32.41±10.53[*]
PIO	5.31±1.25[##]	3.33±0.62[##]	3.15±0.66[##]	3.94±0.59[##]	11.10±1.67[##]
JPQ	7.05±2.85[*,##]	5.15±1.84[*,##]	3.65±0.83[*,##]	3.84±0.96[*,##]	14.24±4.26[*,##]

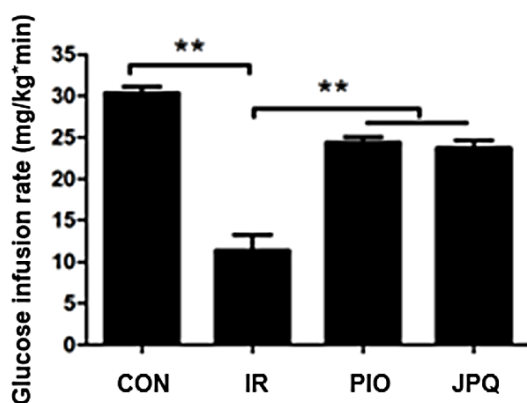Note: compared with CON, [*]$P < 0.05$, [**]$P < 0.01$; Note: compared with IR, [##]$P < 0.01$.

Figure 5. Change of glucose infusion rate. **P < 0.01.

and raises the levels of various inflammatory factors, which may promote inflammation, damaging tissues. Previous studies have shown that by inhibiting the activity of the p38MAPK signaling pathway, the expression of pro-fibrotic cytokines and inflammatory factors can be inhibited and renal fibrosis improved [16]. In this study, we found that the expression levels of p38MAPK and MKK6 mRNA in the model group were significantly higher than in the control group (P < 0.05), while the expression level of MKK3 mRNA was significantly lower than that of the control group (P < 0.05). This suggested that the p38MAPK signal pathway was activated, under the pathological condition of diabetes.

Glucose transporter 4 (GLUT4) plays an important role in the transportation of glucose and changes in its activity are closely associated with insulin resistance in skeletal muscle. Insulin resistance of skeletal muscle can be improved by increasing the expression of GLU4. Phosphorylated p38MAPK reduces the transmembrane transport of glucose by decreasing GLUT4 expression [17, 18]. Hyperglycemia, inflammatory responses and insulin resistance in type 2 diabetes can aggravate the activation of p38MAPK and promote the progression of diabetes and its complications [19-21].

In our study, GLUT4 in the rats of the model group decreased significantly. Glut-4 expression in the JPQ group increased compared with the model group. Western blot results demonstrated that the expression of Glut-4 protein in the JPQ group was higher than in the model group, prompting the conclusion that JPQ downregulated the over-activation of p38MAPK to improve the expression of GLUT4, which increased the transport and uptake of glucose in skeletal muscle, and reduced the level of blood glucose.

Insulin infusion rate (GIR) is a sensitive indicator for the evaluation of insulin activity and its value is positively correlated with insulin sensitivity [17]. The results of this study showed that the GIR in the Jianpi Qinghua Group was significantly higher than that in the model group, suggesting that the sensitivity of the peripheral tissue of rats to insulin was higher in JPQ group. This reflected the improvement of the insulin resistance in the human body and consequently resulted in the decrease of blood glucose at each time point compared with IR group.

In conclusion, JPQ improves the insulin resistance in diabetic rats and promotes the insulin sensitivity. The underlying mechanism may involve downregulation of the p38MAPK signal pathway in skeletal muscle and increased expression of GLUT4. However, the study did not analyze the correlation between the p38MAPK signaling pathway and inflammatory factors; this will be the subject of a follow-up study.

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Disclosure of conflict of interest

None.

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References

[1] Stringhini S, Zaninotto P, Kumari M, Kivimäki M and Batty GD. Lifecourse socioeconomic status and type 2 diabetes: the role of chronic inflammation in the english longitudinal study of ageing. Sci Rep 2016; 6: 24780.

[2] Pourfarzam M, Zadhoush F and Sadeghi M. The difference in correlation between insulin resistance index and chronic inflammation in type 2 diabetes with and without metabolic syndrome. Adv Biomed Res 2016; 5: 153.

[3] Hossain IA, Akter S, Bhuiyan FR, Shah MR, Rahman MK and Ali L. Subclinical inflammation in relation to insulin resistance in prediabetic subjects with nonalcoholic fatty liver disease. BMC Res Notes 2016; 9: 266.

[4] Di Meo S, Iossa S and Venditti P. Skeletal muscle insulin resistance: role of mitochondria and other ROS sources. J Endocrinol 2017; 233: R15-R42.

[5] Diamond-Stanic MK, Marchionne EM, Teachey MK, Durazo DE, Kim JS and Henriksen EJ. Critical role of the transient activation of p38 MAPK in the etiology of skeletal muscle insulin resistance induced by low-level in vitro oxidant stress. Biochem Biophys Res Commun 2011; 405: 439-444.

[6] Miyamoto Y, Miyazaki T, Honda A, Shimohata H, Hirayama K and Kobayashi M. Retention of acetylcarnitine in chronic kidney disease causes insulin resistance in skeletal muscle. J Clin Biochem Nutr 2016; 59: 199-206.

[7] Zhao Y, Zhang L, Qiao Y, Zhou X, Wu G, Wang L, Peng Y, Dong X, Huang H and Si L. Heme oxygenase-1 prevents cardiac dysfunction in streptozotocin-diabetic mice by reducing inflammation, oxidative stress, apoptosis and enhancing autophagy. PLoS One 2013; 8: e75927.

[8] Nikzamir A, Palangi A, Kheirollaha A, Tabar H, Malakaskar A, Shahbazian H and Fathi M. Expression of glucose transporter 4 (GLUT4) is increased by cinnamaldehyde in C2C12 mouse muscle cells. Iran Red Crescent Med J 2014; 16: e13426.

[9] Jia G, DeMarco VG and Sowers JR. Insulin resistance and hyperinsulinaemia in diabetic cardiomyopathy. Nat Rev Endocrinol 2016; 12: 144.

[10] McCormack SE, McCarthy MA, Harrington SG, Farilla L, Hrovat MI, Systrom DM, Thomas BJ, Torriani M, McInnis K and Grinspoon SK. Effects of exercise and lifestyle modification on fitness, insulin resistance, skeletal muscle oxidative phosphorylation and intramyocellular lipid content in obese children and adolescents. Pediatr Obes 2014; 9: 281-291.

[11] Li LL, Ginet V, Liu X, Vergun O, Tuittila M, Mathieu M, Bonny C, Puyal J, Truttmann AC and Courtney MJ. The nNOS-p38MAPK pathway is mediated by NOS1AP during neuronal death. J Neurosci 2013; 33: 8185-8201.

[12] Luo B, Li B, Wang W, Liu X, Liu X, Xia Y, Zhang C, Zhang Y, Zhang M and An F. Rosuvastatin alleviates diabetic cardiomyopathy by inhibiting NLRP3 inflammasome and MAPK pathways in a type 2 diabetes rat model. Cardiovasc Drugs Ther 2014; 28: 33-43.

[13] Nair AR, Elks CM, Vila J, Del Piero F, Paulsen DB and Francis J. A blueberry-enriched diet improves renal function and reduces oxidative stress in metabolic syndrome animals: potential mechanism of TLR4-MAPK signaling pathway. PLoS One 2014; 9: e111976.

[14] Rains JL and Jain SK. Oxidative stress, insulin signaling, and diabetes. Free Radic Biol Med 2011; 50: 567-575.

[15] Wellen KE and Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005; 115: 1111-1119.

[16] Mao ZM, Shen SM, Wan YG, Sun W, Chen HL, Huang MM, Yang JJ, Wu W, Tang HT and Tang RM. Huangkui capsule attenuates renal fibrosis in diabetic nephropathy rats through regulating oxidative stress and p38MAPK/Akt pathways, compared to α-lipoic acid. J Ethnopharmacol 2015; 173: 256-265.

[17] Liu J, Wen D, Fang X, Wang X, Liu T and Zhu J. p38MAPK signaling enhances glycolysis through the up-regulation of the glucose transporter GLUT-4 in gastric cancer cells. Cell Physiol Biochem 2015; 36: 155-165.

[18] Lawan A, Min K, Zhang L, Canfran-Duque A, Jurczak MJ, Camporez JPG, Nie Y, Gavin TP, Shulman GI and Hernandez-Fernando C. Skeletal muscle-specific deletion of MKP-1 reveals a p38 MAPK/JNK/Akt signaling node that regulates obesity-induced insulin resistance. Diabetes 2018; db170826.

[19] Chen HL, Wan YG, Zhao Q, Huang YR, Shi XM, Meng XJ and Yao J. Regulative mechanism of renal inflammatory-related p38MAPK signaling pathway in diabetic nephropathy and interventional effects of Chinese herbal medicine. Zhongguo Zhong Yao Za Zhi 2013; 38: 2268-2272.

[20] Mao Z, Wan Y, Sun W, Chen H, Huang Y, Shi X and Yao J. Effects and mechanisms of huang-kui capsule ameliorating renal fibrosis in diabetic nephropathy rats via inhibiting oxidative stress and p38MAPK signaling pathway activity in kidney. Zhongguo Zhong Yao Za Zhi 2014; 39: 4110-4117.

[21] Price DM, Wloka MT, Chik CL and Ho AK. Mitogen-activated protein kinase phosphatase-1 (MKP-1) preferentially dephosphorylates p42/44MAPK but not p38MAPK in rat pinealocytes. J Neurochem 2007; 101: 1685-1693.