doi.org/10.36721/PJPS.2021.34.6.REG.2121-2129.1

Protective roles of liraglutide against brain injury of streptozotocin induced diabetic rats

Ying Jia[1], Pengtian Lu[1], Liu Qi[1], Jiayao Li[1], Mengdi Wang[1], Jiping Qi[1]* and Yuejia Song[2]*
[1]Departments of Pathology, The First Affiliated Hospital of Harbin Medical University, Harbin, PR China
[2]Endocrinology Departments, The First Affiliated Hospital of Harbin Medical University, Harbin, PR China

Abstract: Our study aimed to explore the impacts of liraglutide on brain dysfunction of type 2 diabetes mellitus. Rats in liraglutide treatment group were diabetic rats further received daily intraperitoneal administration of liraglutide for continuous 6 weeks. Body weight and blood glucose were measured weekly. Vascular structure changes in brain tissues were evaluated by Periodic acid-Schiff (PAS) staining. Angiopoietin-2 (ANG-2), high-mobility group box 1 (HMGB-1), CD105, NeuN, Oligo-2 in brain tissues were measured by immunohistochemistry staining and ANG-2, HMGB-1, and matrix metalloproteinase-9 (MMP-9) were detected by western blotting. Blood glucose levels of rats in diabetic model group were significantly elevated and blood glucose levels of rats in liraglutide treatment group were reduced to comparable levels with control group. PAS staining showed vascular basement membrane of rats in the diabetic model group was thicker than that of the control group. ANG-2, HMGB1 and MMP-9 were up-regulated in the diabetic model group comparing the control group, while down-regulated after treated with liraglutide ($p<0.05$). NeuN expressions were significantly higher in liraglutide treatment group. Liraglutide may have protective roles against brain injury of streptozotocin induced diabetic rats by inhibiting HMGB1, which further suppressing the MMP-9 and ANG-2.

Keywords: Type 2 diabetes mellitus, liraglutide, blood glucose.

INTRODUCTION

Diabetes mellitus is a complex and heterogeneous metabolic disease, with the characterization of hyperglycemia, which is resulted from dysfunctions of insulin secretion or insulin action (Petersmann *et al*., 2019). Around 463 million people worldwide had diabetes mellitus in 2019, and the number of people with diabetes mellitus is predicted to rise to 578 million by 2030, to 642 million by 2040 and to 700 million by 2045 (Saeedi *et al*., 2019 and Williams *et al*., 2020). The prevalence of diabetes mellitus is increasing worldwide and becoming one of the most important public health challenges of the twenty-first century (Glovaci *et al*., 2019).

Type 2 diabetes mellitus, which accounts for approximately 90-95% of patietns with diabetes mellitus, is characterized by reduced insulin sensitivity and relative insulin deficiency (Kaur *et al*., 2018). Chronic hyperglycemia of diabetes can lead to micro vascular and macro vascular complications, which are associated with long-term organ damages, defections and failures of the eyes, nerves, brain kidneys, heart and blood vessels (McCrimmon *et al*., 2012 and Xia *et al*., 2020). Adults with type 2 diabetes have increased risk of developing certain brain or mental disorders (such as stroke, dementia and depression) and diabetes-related cerebral micro vascular dysfunction are mainly caused by hyperglycemia, obesity and insulin resistance and hypertension, indicating diabetes-related cerebral micro vascular dysfunction is associated with a higher risk of stroke, cognitive dysfunction and depression (van Sloten *et al*., 2020). Under the condition of hyperglycemia, glucagon-like peptide-1 (GLP-1) promotes insulin secretion and suppresses glucagon secretion in the pancreatic islets (Gallwitz, 2014). For treatment of type 2 diabetes, therapies targeting GLP-1, including GLP-1 receptor agonists and dipeptidyl peptidase 4 (DPP-4) inhibitors (that delay the degradation of endogenous GLP-1) have become widely used in clinical (Scheen, 2013). Liraglutide is one of the GLP-1 receptor agonists and has been shown to improve glycemic control and β cell function with a low risk of hypoglycaemia in people with type 2 diabetes (Seufert and Gallwitz, 2014). After six-month treatment of liraglutide, greater improvements of arterial stiffness and left ventricular myocardial deformation were observed in subjects with newly diagnosed type 2 diabetes compared to the standard treatment with metformin (Lambadiari *et al*., 2018). Moreover, it has been demonstrated that liraglutide could function as a neuroprotective agent and attenuate the rat brain neuronal damage caused by cerebral ischemia through inhibiting apoptosis and reducing oxidative stress (Briyal *et al*., 2014, Wiciński *et al*., 2019). However, the impacts of liraglutide on type 2 diabetes mellitus with regarding to the brain dysfunction were seldomly studied.

Our current study aimed to explore whether liraglutide has protective roles against brain dysfunction of type 2 diabetes. Combination of high fat diet and multiple low dose of streptozotocin (STZ) could be used to develop the animal model mimicing the metabolic characteristics of

**Corresponding author:* e-mail: songyuejia_1@163.com

type 2 diabetes mellitus in humans, which has been usually applied as useful tool for anti-diabetic drug screening (Nath *et al*., 2017). In our study, rat model of type 2 diabetes mellitus was established based on the above method and vascular structure changes in brain tissues were evaluated by Periodic acid-Schiff staining. Moreover, diabetic rats were further received daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg body weight for continuous 6 weeks. The expression changes of angiopoietin-2 (ANG-2), high-mobility group box 1 (HMGB-1), CD105, NeuN, Oligo-2 in brain tissues were evaluated by immunohistochemistry staining and ANG-2, HMGB-1 and matrix metalloproteinase-9 (MMP-9) in brain tissues were detected by western blotting.

MATERIALS AND METHODS

Animals and experimental groups

Male Sprague Dawley (SD) rats with the weight of 200-250g were purchased from the Animal Experimental Center in The Second Affiliated Hospital of Harbin Medical University (Harbin, China). Rats were housed in a well-ventilated animal unit under a 12h light/dark cycle (humidity: 50±10%; temperature: 22±2°C). Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Harbin Medical University (Harbin, China, No. 2018014).

The male SD rats were randomly divided into three groups: Control group (n=10), diabetic model group (n=10) and liraglutide treatment group (n=10). The diabetic model was established by streptozotocin induction. Briefly, diabetic rats were induced by intraperitoneal injection of streptozotocin with the dose of 35mg/kg body weight after 4 weeks of continuous feeding with high-fat and high-carbohydrate diet (Vatandoust *et al*., 2018). The rats in liraglutide treatment group were STZ-induced diabetic rats further received daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg body weight for continuous 6 weeks.

Body weight and blood glucose measurements

The body weight of each rat was measured weekly for 6 weeks. Meanwhile, blood glucose meter and test strips were used to measure blood glucose of rats. To be more specific, the rat tail and blood sampling needle were firstly sterilized with alcohol cotton. Blood samples were collected by puncturing sampling needle into the vein of the rat tail and dripped onto the blood glucose test strips (Sinocare Inc, Changsha, China). The blood glucose of rat was measured by the blood glucose meter (Sinocare Inc, Changsha, China) and results data were recorded.

Periodic acid-Schiff and immunohistochemistry staining

Brain tissues of rats were paraffin-embedded after fixation, dehydration and paraffin infiltration. Coronal sections with the thickness of 4μm were made using sliding microtome (Leica, Solms, Germany). Sections were stained with standard protocols of Periodic acid-Schiff (PAS) for evaluating the vascular changes. Moreover, immunohistochemistry staining was also performed after de-paraffinizing. For immunohistochemistry staining, after incubation in 0.03% hydrogen peroxide to block the activity of endogenous peroxidase and exposure the epitopes antigens by high pressure heating, the sections were incubated with primary antibodies against ANG-2 (mouse anti-ANG-2; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), HMGB-1 (rabbit anti-HMGB-1; 1:200; Abcam, Cambridge, UK), CD105 (rabbit anti-CD105; 1:200; Abcam, Cambridge, UK), NeuN (rabbit anti-NeuN; 1:500; Abcam, Cambridge, UK), or Oligo-2 (rabbit anti-Oligo-2; 1:200; Abcam, Cambridge, MA, USA) at 4ºC overnight. The slides were washed with phosphate buffer solution (PBS) and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, UK) for 1h at room temperature. After washing with PBS, the sections were visualized using 3, 3-diaminobenzidine (DAB) kit (ZLI-9017; Zhongshan Biotechnology Co., Ltd., Beijing, China). The slides were counterstained with hematoxylin. Images were taken by using Leica microscope (DMI6000B, Leica, Solms, Germany). Image J software was used to perform immunopositive area quantitation.

Western blotting

The protein levels in brain tissues were measured by Western blotting (Taylor and Posch, 2014). Rat brain tissues were lysed in radio immunoprecipitation assay (RIPA) lysis buffer with protease inhibitors, and lysate was separated by centrifugation with the speed of 12,000 × g at 4°C for 15 min. The protein was quantified using the bicinchoninic acid (BCA) assay kit (P0012, Beyotime Biotech, Shanghai, China), separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 300 mA for 1.5 h. The membrane was blocked with 0.1% bovine serum albumin (Beyotime Biotech, Shanghai, China) for 1 h at room temperature, and then incubated with primary antibodies against ANG-2 (mouse anti-ANG-2; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), HMGB-1 (rabbit anti-HMGB-1; 1:200; Abcam, Cambridge, MA, USA), MMP-9 (rabbit anti-MMP-9; 1:200; Abcam, Cambridge, MA, USA) and β-actin (mouse anti-β-actin; 1:1,000, Zhongshan Biotechnology Co., Ltd., Beijing, China) at 4ºC overnight. After the membrane was washed with Tris-Buffered Saline with Tween 20 (TBS-T), the membranes were incubated with goat anti-mouse or anti-rabbit secondary antibodies (1:500, Zhongshan Biotechnology Co., Ltd., Beijing, China) for 1h at 37°C. Then, the membranes were washed with T-BST again. Protein bands were visualized by Beyo ECL Plus kits (Beyotime Biotech,

Shanghai, China). The optical densities of the detected proteins were obtained using UVP Software iBox 500 Imaging System (Upland, CA, USA).

STATISTICAL ANALYSIS

The Shapiro-Wilk test was used to assess the normality of the quantitative variables. One way Analysis of variance (ANOVA) was used for comparisons of normally distributed variables, followed by least significant difference (LSD) test. Non-normally distributed variables were evaluated by using Kruskal-Wallis test, and Bonferroni procedure was applied to adjust for multiple testing. Data were assessed using the SPSS software (version 22.0, SPSS Inc, Chicago, IL, USA). The level of significance was $p<0.05$.

RESULTS

Effects of liraglutide on body weight and blood glucose

The body weight and blood glucose of diabetic rats in control group, diabetic model group and liraglutide treatment group were listed in table 1. According to the statistical analysis, no significant difference was observed in the body weight of rats in liraglutide treatment group at day 0, day 7, day 14, day 21, day 28, day 35, and day 42, when comparing with control group or diabetic model group ($p>0.05$) (table 1).

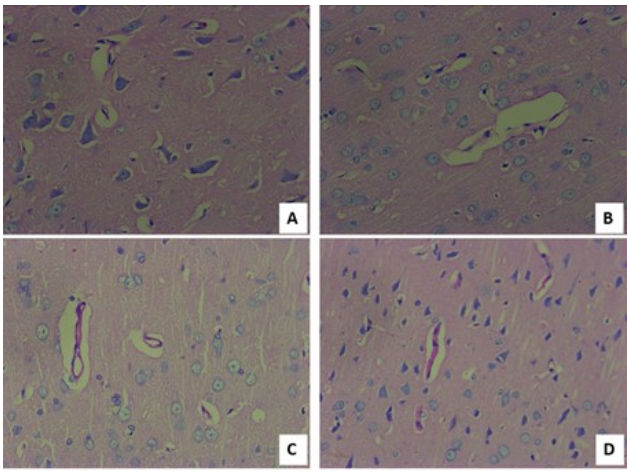

Fig. 1: Comparison of Periodic acid-Schiff (PAS) staining for vascular structures in brain tissues between rats of control group and rats of diabetic model group model. A, B: periodic acid-Schiff staining for vascular structures in brain tissues of rats in the control group; C, D: periodic acid-Schiff staining for vascular structures in brain tissues of rats in the diabetic model group. Representative images are shown at 40 ×10 magnifications.

The blood glucose levels of rats in diabetic model group were significantly higher than those of the control group at day 0 ($p<0.001$), day 7 ($p<0.001$), day 14 ($p=0.001$), day 21 ($p<0.001$), day 28 ($p<0.001$), day 35 ($p<0.001$) and day 42 ($p<0.001$). After daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg for 14 days, the blood glucose levels of rats in liraglutide treatment group were comparable with those in control group ($p>0.05$).

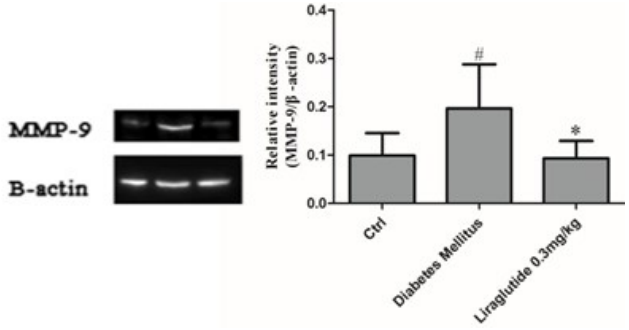

Fig. 2: Protein expression levels of matrix metalloproteinase-9 (MMP-9) in brain tissues of rats in the control group, diabetic model group, and liraglutide treatment group by western blotting. #$p<0.05$ compared with control group; *$p<0.05$ compared with diabetic model group.

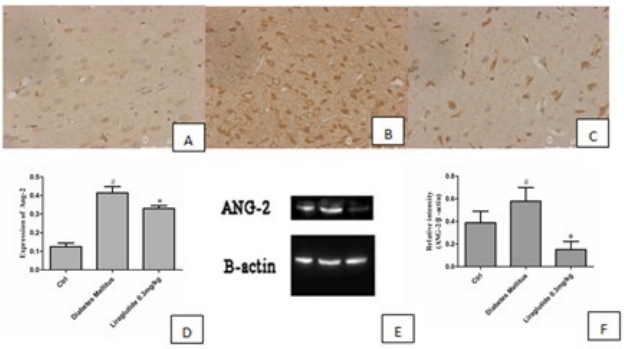

Fig. 3: Protein expression levels of angiopoietin-2 (ANG-2) in brain tissues by immunohistochemistry staining and western blotting. A, immunohistochemistry staining of ANG-2 in brain tissues of rats in control group; B, immunohistochemistry staining of ANG-2 in brain tissues of rats in diabetic model group; C, immunohistochemistry staining of ANG-2 in brain tissues of rats in liraglutide treatment group; D, comparison of ANG-2 expression levels detected by immunohistochemistry staining; E, western blotting image; F, comparison of ANG-2 expression levels measured by western blotting. #$p<0.05$ compared with control group; *$p<0.05$ compared with diabetic model group. Representative images of immunohistochemistry staining are shown at 40×10 magnifications.

Histopathological examination

Periodic acid-Schiff staining for vascular structures in brain tissues of rats in the control group (fig. 1A and 1B) and diabetic model group (fig. 1C and 1D) were conducted. The images showed vascular basement membrane of rats in the diabetic model group was thicken than that of the control group.

Protein expression levels of MMP-9 in brain tissues

According to the western blotting analysis, the protein expression levels of MMP-9 were significantly elevated in brain tissues of diabetic rats in the diabetic model group when comparing with the control group ($p<0.05$) (fig. 2). After daily intraperitoneal administration of liraglutide with the dose of 0.3 mg/kg for 6 weeks, the protein expression levels of MMP-9 in brain tissues of rats in the liraglutide treatment group were significantly lower than those in the diabetic model group ($p<0.05$) (fig. 2).

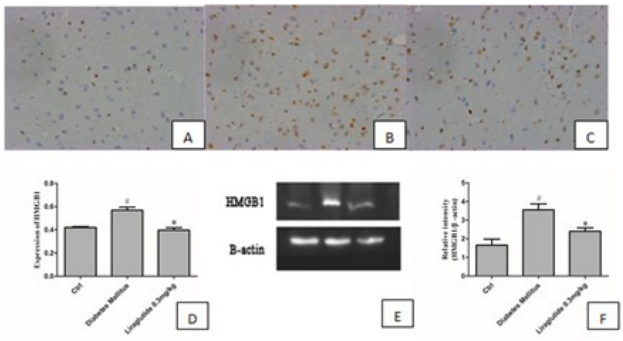

Fig. 4: Protein expression levels of high-mobility group box 1 (HMGB-1) in brain tissues by immunohistochemistry staining and western blotting. A, immunohistochemistry staining of HMGB-1 in brain tissues of rats in control group; B, immunohistochemistry staining of HMGB-1 in brain tissues of rats in diabetic model group; C, immunohistochemistry staining of HMGB-1 in brain tissues of rats in liraglutide treatment group; D, comparison of HMGB-1 expression levels detected by immunohistochemistry staining; E, western blotting image; F, comparison of HMGB-1 expression levels measured by western blotting. #$p<0.05$ compared with control group; *$p<0.05$ compared with diabetic model group. Representative images of immunohistochemistry staining are shown at 40 ×10 magnifications.

Protein expression levels of ANG-2 in brain tissues

The immunohistochemical staining revealed that the ANG-2 was highly expressed in the brain tissues of diabetic rats in the diabetic model group in comparison with the control group ($p<0.05$) (fig. 3). An obvious decrease was observed in the positive expression area of ANG-2 in brain tissues of rats in the liraglutide treatment group after daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg for 6 weeks, comparing with the diabetic model group ($p<0.05$) (fig. 3). Moreover, the results of western blotting analysis were consistent with those of the immunohistochemical staining analysis (fig. 3).

Protein expression levels of HMGB-1 in brain tissues

The expressions of HMGB-1 were markedly higher in the brain tissues of diabetic rats in the diabetic model group in comparison with the control group based on the results of immunohistochemical staining ($p<0.05$) (fig. 4). In the brain tissues of rats in the liraglutide treatment group, the positive expression areas of HMGB-1 were obviously reduced by comparing with the diabetic model group ($p < 0.05$) (fig. 4).

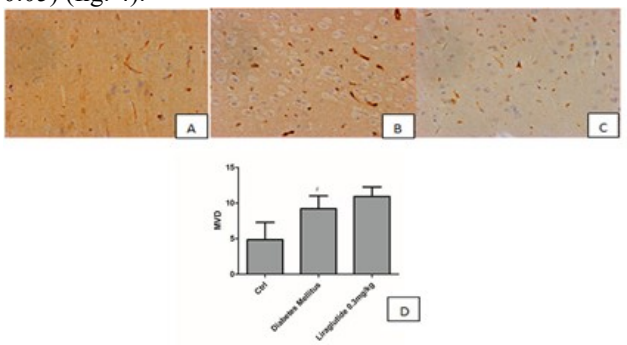

Fig. 5: Assessment of microvessel density (MVD) in brain tissues by immunohistochemistry staining of CD105. A, immunohistochemistry staining of CD105 in brain tissues of rats in control group; B, immunohistochemistry staining of CD105 in brain tissues of rats in diabetic model group; C, immunohistochemistry staining of CD105 in brain tissues of rats in liraglutide treatment group; D, comparison of CD105 expression levels detected by immunohistochemistry staining. #$p< 0.05$ compared with control group. Representative images of immunohistochemistry staining are shown at 40×10 magnifications.

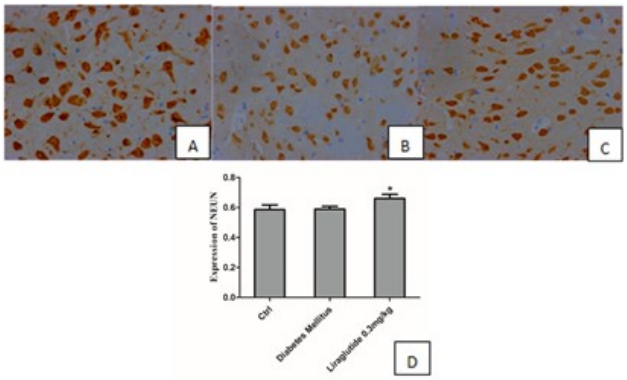

Fig. 6: Immunohistochemistry staining of NeuN for labeling neurons in brain tissues by marking. A, immunohistochemistry staining of NeuN in brain tissues of rats in control group; B, immunohistochemistry staining of NeuN in brain tissues of rats in diabetic model group; C, immunohistochemistry staining of NeuN in brain tissues of rats in liraglutide treatment group; D, comparison of NeuN expression levels detected by immunohistochemistry staining. *$p<0.05$ compared with diabetic model group. Representative images of immunohistochemistry staining are shown at 40 ×10 magnifications.

Similarly, western blotting analysis showed the expressions of HMGB-1 were significantly higher in diabetic model group, while decreased tendency was

Table 1. Body weight and blood glucose of each rat in the control group, diabetic model group, and liraglutide treatment group at different time points

Group	Rat	Body weight (g)							Blood glucose (mmol/L)						
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Control	No.1	437	428	431	443	457	473	500	4.6	5.8	4.4	4.8	5.1	5.2	4.7
	No.2	375	345	340	363	390	391	391	3.2	5.1	4.1	4.2	4.4	3.9	5.1
	No.3	377	380	371	377	393	399	399	4.8	5.9	6.6	6.1	5.3	5.4	6.2
	No.4	394	352	392	365	395	393	399	4.9	5.2	5.7	5.9	6.1	6.0	5.8
	No.5	403	405	410	407	414	413	429	4.4	5.8	5.4	4.9	4.8	5.2	5.7
	No.6	390	380	390	400	402	419	430	5.2	5.5	5.4	5.3	5.4	5.8	5.1
	No.7	380	362	365	380	394	402	405	5.1	6.2	5.1	5.7	5.8	6.0	6.2
	No.8	392	378	394	400	420	411	425	5.0	5.9	6.4	6.2	5.8	6.4	6.2
	No.9	360	362	367	375	395	394	393	4.2	3.7	3.4	4.2	3.9	4.3	4.1
	No.10	343	362	362	360	365	374	370	4.4	4.1	4.5	4.7	5.0	4.2	4.8
Diabetic model	No.1	383	374	380	392	416	424	430	7.4	8.0	8.2	8.4	7.9	8.7	9.0
	No.2	420	404	413	425	441	450	457	7.4	18.2	18.4	16.5	18.0	>27.8	20.2
	No.3	405	392	397	399	394	401	399	7.1	16.1	16.5	15.5	19.5	19.6	23.0
	No.4	454	453	453	465	473	479	492	9.6	14.9	15.2	16.4	15.8	15.1	16.9
	No.5	373	370	367	392	399	403	417	9.1	9.3	15.2	18.2	17.3	15.9	22.0
	No.6	397	398	401	390	390	394	390	23.6	20.2	20.3	>27.8	27.1	>27.8	26.5
	No.7	357	360	365	363	374	390	395	7.2	10.8	12.5	11.0	14.5	13.9	17.1
	No.8	319	317	322	322	324	313	291	9.9	12.4	14.2	11.9	15.6	19.2	19.7
	No.9	335	327	325	314	307	297	295	13.1	14.2	15.2	15.9	>27.8	19.4	18.4
	No.10	325	295	234	264	255	248	254	14.9	13.4	15.4	17.5	18.4	19.5	22.3
Liraglutide treatment	No.1	392	392	373	394	396	400	414	5.1	6.5	8.7	6.1	7.2	7.6	7.9
	No.2	427	407	431	420	442	463	465	7.1	3.3	5.6	5.4	6.9	9.2	9.6
	No.3	362	303	320	294	298	295	290	23.0	24.4	22.8	26.4	>27.8	23.8	22.7
	No.4	361	332	322	319	343	345	346	15.3	6.6	4.0	4.1	2.8	5.0	6.1
	No.5	325	319	324	303	320	315	260	5.2	7.3	6.1	4.9	6.7	6.7	3.6
	No.6	394	390	365	367	365	371	302	7.0	5.6	5.4	6.1	2.8	8.1	11.8
	No.7	379	395	395	373	393	321	395	12.6	9.3	3.9	4.9	6.2	27.4	5.2
	No.8	418	420	424	432	440	395	452	13.0	17.4	5.8	8.0	8.2	7.4	7.8
	No.9	424	403	404	410	415	415	425	7.8	6.8	5.5	7.1	6.8	6.0	7.3
	No.10	430	399	401	407	414	401	390	21.5	25.9	18.8	20.0	24.9	>27.8	27.8

observed in rats of the liraglutide treatment group, which received daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg for 6 weeks (fig. 4).

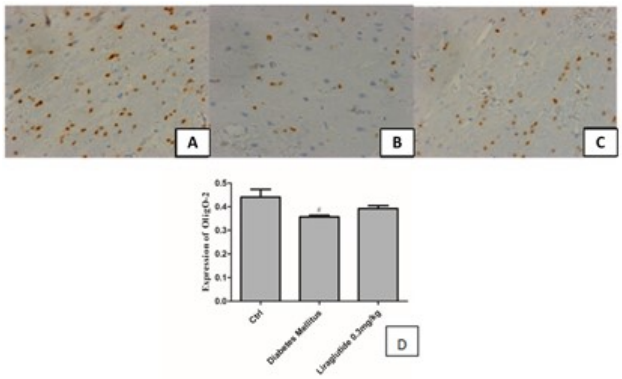

Fig. 7: Immunohistochemistry staining of Oligo-2 for labeling oligodendrocytes in brain tissues. A, immunohistochemistry staining of Oligo-2 in brain tissues of rats in control group; B, immunohistochemistry staining of Oligo-2 in brain tissues of rats in diabetic model group; C, immunohistochemistry staining of Oligo-2 in brain tissues of rats in liraglutide treatment group; D, comparison of Oligo-2 expression levels detected by immunohistochemistry staining. *#p<0.05 compared with control group. Representative images of immunohisto chemistry staining are shown at 40 ×10 magnifications.

Neovascularization evaluated by CD105

The micro vessel density (MVD) of brain tissues were assessed by immunohistochemistry staining of CD105. The MVD of brain tissues of rats in the diabetic model group were obviously higher than those of the control group ($p<0.05$) (fig. 5). After received daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg for 6 weeks, the MVD of brain tissues were further elevated (fig. 5).

Impacts of liraglutide on neurons and oligodendrocytes

Neurons and oligodendrocytes in brain tissues were labeled by NeuN and Oligo-2, respectively. Comparative expressions of NeuN were observed between control group and diabetic model group ($p>0.05$), while significantly higher expressions of NeuN were shown in brain tissues of rats in the liraglutide treatment group, which received daily intraperitoneal administration of

liraglutide with the dose of 0.3mg/kg for 6 weeks ($p< 0.05$) (fig. 6). On the other hand, by comparing with the control group, brain tissues of rats in the diabetic model group had obviously lower expressions of Oligo-2 ($p<0.05$) (fig. 7). No significant difference was found in the expression of Oligo-2 between diabetic model group and liraglutide treatment group ($p>0.05$) (fig. 6).

DISCUSSION

In our study, rats in diabetic model group had significantly higher blood glucose levels than control group at day 0, day 7, day 14, day 21, day 28, day 35 and day 42, suggesting type 2 diabetes mellitus model was successfully developed by combination of high fat diet and injection of STZ. Moreover, the vascular basement membrane in brain tissues rats of the diabetic model group was obviously thicker than that of the control group according to the PAS staining. After daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg for 14 days, the blood glucose levels of rats in liraglutide treatment group were comparable with those in control group, indicating liraglutide is useful for the treatment of induced type 2 diabetes mellitus.

Predominant structural changes in the brain have been reported in type 1 and type 2 diabetes mellitus, such as changes in cerebral perfusion, neural slowing, microstructural abnormalities in white matter tracts and increased cortical atrophy (de Bresser, Tiehuis *et al*., 2010, Manschot, Biessels *et al*., 2007, Roy, Ehlert *et al*., 2020). Hippocampal atrophy is a more pronounced feature of type 2 diabetes than of type 1 diabetes (Convit, Wolf *et al*., 2003). It seems to be that insulin resistance, hypertension, dyslipidaemia, and cerebro vascular disease are of great importance to the cognitive dysfunction in type 2 diabetes (Lyu, Wu *et al*., 2020, van Sloten, Sedaghat *et al*., 2020). ANG-1 and ANG-2 are secreted growth factors that exert downstream signaling through the Tie2 receptor tyrosine kinase, which is predominantly expressed in endothelial cells and participates in vessel remodeling and maturation, and Tie2 signaling in activated endothelium is mainly regulated via the expression of ANG-2 (Reiss, Scholz *et al*., 2015, Scholz, Harter *et al*., 2016). Moreover, highest levels of ANG-2 are found in myeloid-dominated inflamed brain tissues, and ANG-2 mediates the blood-brain barrier permeability, maintaining homeostasis of the central nervous system (Gurnik, Devraj *et al*., 2016, Scholz, Lang *et al*., 2011). Previous studies have showed that significantly higher level of ANG-2 is closely related to vascular complications of type 2 diabetes mellitus (Li, Qian *et al*., 2015, Li, Y *et al*., 2016). In our study, immunohistochemical staining and western blotting revealed that ANG-2 was highly expressed in the brain tissues of diabetic rats in the diabetic model group in comparison with the control group, while down-regulated after receiving daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg for 6 weeks. Thus, liraglutide may have protective roles against brain injury of streptozotocin induced diabetic rats by reducing expressions of ANG-2.

What's more, up-regulated intravitreal concentrations of ANG-2 have been found significantly correlated with high MMP-9 levels in diabetic eyes, which may promote retinal angiogenesis synergistically (Loukovaara, Robciuc *et al*., 2013). We found protein expression levels of MMP-9 were significantly elevated in brain tissues of diabetic rats and reduced levels of MMP-9 were observed after daily intraperitoneal administration of liraglutide with the dose of 0.3mg/kg for 6 weeks. It is well known that MMP-9 is implicated in cerebral ischemic injury, and higher levels of MMP-9 may cause blood-brain barrier degradation, vasogenic edema and hemorrhage (Leonardo and Pennypacker, 2009, Rempe, Hartz *et al*., 2016). Qiu *et al*. have uncovered that HMGB1 promotes MMP-9 expression in neurons and astrocytes after cerebral ischemia, which primarily mediated via the Toll-like Receptor 4 (TLR4), suggesting HMGB1/TLR4 signaling pathway might be potential therapy target for cerebral ischemia to reduce acute inflammatory response and tissue damage (Qiu, Xu *et al*., 2010). HMGB1, a family member of molecules called alarmins, is rapidly released from injured cells after ischemia, and neuroprotective effects have been reported by suppression of HMGB1 via siRNA or neutralizing antibodies (Kim, Sig Choi *et al*., 2006, Liu, Mori *et al*., 2007). Higher circulating levels of HMGB1 have been found in type 2 diabetic patients, and expression of HMGB1 in the retinas of diabetic patients and rat models with retinopathy were elevated (Skrha, Kalousová *et al*., 2012, Yu, Yang *et al*., 2015). In our study, expressions of HMGB-1 were significantly higher in diabetic model group, while decreased tendency was observed in rats of the liraglutide treatment group based on the immunohistochemical staining and western blotting. Therefore, reduced expressions of HMGB1 caused by of liraglutide may suppress the MMP-9 expression to protect brain from injuries.

Neurons in brain tissues were labeled by NeuN and significantly higher expressions of NeuN were shown in brain tissues of rats in the liraglutide treatment group. Studies have shown neuroprotective actions of liraglutide in degenerative neurological disease models for Parkinson's disease, Alzheimer's disease, and neurovascular complications (such as stroke) (Femminella, Frangou *et al*., 2019, Yan, Pang *et al*., 2019). What's more, immunohistochemical staining of Oligo-2 showed number of oligodendrocytes was obviously lower. It has been reported that streptozotocin-induced diabetic rats nhibits the activation of astrocytes, exacerbates the demyelination and delays the remyelination processes, which promoting the detrimental effects of hyperglycemia on ischemic brain damage (Baig and Panchal, 2019, Jing, He *et al*.,

2013). Our results were similar to previous studies. However, no significant difference was found in the expression of Oligo-2 between diabetic model group and liraglutide treatment group, which might be due to the dose and duration of liraglutide injection. Thus, further studies with regarding to the different doses and durations of liraglutide injection are still needed.

CONCLUSION

In conclusion, diabetic rats induced by combination of high fat diet and injection of streptozotocin (STZ), which were further received intraperitoneal administration of liraglutide to study the impacts of liraglutide on type 2 diabetes mellitus with regarding to the brain injury. Blood glucose levels of rats were reduced to comparable levels with control group after treated with liraglutide for 14 days. Expressions of ANG-2, HMGB1 and MMP-9 were highly expressed in the diabetic model group comparing the control group, while down-regulated after treated with liraglutide. NeuN expressions were significantly higher in liraglutide treatment group. Our results suggested that liraglutide may have protective roles against brain injury of streptozotocin induced diabetic rats by reducing expressions of HMGB1, which further suppressing the MMP-9 and ANG-2.

ACKNOWLEDGEMENTS

This study was funded by National Natural Science (Grant No.81900743) and Heilongjiang Youth Science Foundation (Grant No. QC2017100).

REFERENCES

Baig MA and Panchal SS (2019). Streptozotocin-Induced diabetes mellitus in neonatal rats: An insight into its applications to induce diabetic complications. *Curr Diabetes Rev.,* **16**(1): 26-39.

Briyal S, Shah S and Gulati A (2014). Neuroprotective and anti-apoptotic effects of liraglutide in the rat brain following focal cerebral ischemia. *Neuroscience,* **281**: 269-281.

Convit A, Wolf OT, Tarshish C and de Leon MJ (2003). Reduced glucose tolerance is associated with poor memory performance and hippocampal atrophy among normal elderly. *Proc. Natl. Acad Sci. USA,* **100**(4): 2019-2022.

de Bresser J, Tiehuis AM, van den Berg E, Reijmer YD, Jongen C, Kappelle LJ, Mali WP, Viergever MA and Biessels GJ (2010). Progression of cerebral atrophy and white matter hyperintensities in patients with type 2 diabetes. *Diabetes Care,* **33**(6): 1309-1314.

Femminella GD, Frangou E, Love SB, Busza G, Holmes C, Ritchie C, Lawrence R, McFarlane B, Tadros G, Ridha BH, Bannister C, Walker Z, Archer H, Coulthard E, Underwood BR, Prasanna A, Koranteng P, Karim S, Junaid K, McGuinness B, Nilforooshan R, Macharouthu A, Donaldson A, Thacker S, Russell G, Malik N, Mate V, Knight L, Kshemendran S, Harrison J, Hölscher C, Brooks DJ, Passmore AP, Ballard C and Edison P (2019). Evaluating the effects of the novel GLP-1 analogue liraglutide in Alzheimer's disease: Study protocol for a randomised controlled trial (ELAD study). *Trials,* **20**(1): 019-3259.

Gallwitz B (2014). Extra-pancreatic effects of incretin-based therapies. *Endocrine,* **47**(2): 360-371.

Glovaci D, Fan W and Wong ND (2019). Epidemiology of diabetes mellitus and cardiovascular disease. *Curr. Cardiol. Rep.,* **21**(4): 019-1107.

Gurnik S, Devraj K, Macas J, Yamaji M, Starke J, Scholz A, Sommer K, Di Tacchio M, Vutukuri R, Beck H, Mittelbronn M, Foerch C, Pfeilschifter W, Liebner S, Peters KG, Plate KH and Reiss Y (2016). Angiopoietin-2-induced blood-brain barrier compromise and increased stroke size are rescued by VE-PTP-dependent restoration of Tie2 signaling. *Acta Neuropathol.,* **131**(5): 753-773.

Jing L, He Q, Zhang JZ and Li PA (2013). Temporal profile of astrocytes and changes of oligodendrocyte-based myelin following middle cerebral artery occlusion in diabetic and non-diabetic rats. *Int. J. Biol. Sci.,* **9**(2): 190-199.

Kaur R, Kaur M and Singh J (2018). Endothelial dysfunction and platelet hyperactivity in type 2 diabetes mellitus: molecular insights and therapeutic strategies. *Cardiovasc Diabetol.,* **17**(1): 018-0763.

Kim JB, Sig Choi J, Yu YM, Nam K, Piao CS, Kim SW, Lee MH, Han PL, Park JS and Lee JK (2006). HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. *J. Neurosci.,* **26**(24): 6413-6421.

Lambadiari V, Pavlidis G, Kousathana F, Varoudi M, Vlastos D, Maratou E, Georgiou D, Andreadou I, Parissis J, Triantafyllidi H, Lekakis J, Iliodromitis E, Dimitriadis G and Ikonomidis I (2018). Effects of 6-month treatment with the glucagon like peptide-1 analogue liraglutide on arterial stiffness, left ventricular myocardial deformation and oxidative stress in subjects with newly diagnosed type 2 diabetes. *Cardiovasc Diabetol.,* **17**(1): 017-0646.

Leonardo CC and Pennypacker KR (2009). Neuroinflammation and MMPs: Potential therapeutic targets in neonatal hypoxic-ischemic injury. *J. Neuroinflammation,* **6**(13): 1742-2094.

Li L, Qian L and Yu ZQ (2015). Serum angiopoietin-2 is associated with angiopathy in type 2 diabetes mellitus. *J. Diabetes Complications,* **29**(4): 568-571.

Li L, Y ZHQ, Juan Yu H, Jian Yong X, Fan L, Guang Chun Z, Lei Z, Hui Ming G, Si Jing Z and Meng J (2016). Association between interleukin-19 and angiopoietin-2 with vascular complications in type 2 diabetes. *J. Diabetes Investig.,* **7**(6): 895-900.

Liu K, Mori S, Takahashi HK, Tomono Y, Wake H, Kanke

T, Sato Y, Hiraga N, Adachi N, Yoshino T and Nishibori M (2007). Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *Faseb J.,* **21**(14): 3904-3916.

Loukovaara S, Robciuc A, Holopainen JM, Lehti K, Pessi T, Liinamaa J, Kukkonen KT, Jauhiainen M, Koli K, Keski-Oja J and Immonen I (2013). Ang-2 upregulation correlates with increased levels of MMP-9, VEGF, EPO and TGFβ1 in diabetic eyes undergoing vitrectomy. *Acta Ophthalmol.,* **91**(6): 531-539.

Lyu F, Wu D, Wei C and Wu A (2020). Vascular cognitive impairment and dementia in type 2 diabetes mellitus: An overview. *Life Sci.,* **254**(117771): 11.

Manschot SM, Biessels GJ, de Valk H, Algra A, Rutten GE, van der Grond J and Kappelle LJ (2007). Metabolic and vascular determinants of impaired cognitive performance and abnormalities on brain magnetic resonance imaging in patients with type 2 diabetes. *Diabetologia.,* **50**(11): 2388-2397.

McCrimmon RJ, Ryan CM and Frier BM (2012). Diabetes and cognitive dysfunction. *Lancet.,* **379**(9833): 2291-2299.

Nath S, Ghosh SK. Choudhury Y (2017). A murine model of type 2 diabetes mellitus developed using a combination of high fat diet and multiple low doses of streptozotocin treatment mimics the metabolic characteristics of type 2 diabetes mellitus in humans. *J. Pharmacol. Toxicol. Methods,* **84**: 20-30.

Petersmann A, Müller-Wieland D, Müller UA, Landgraf R, Nauck M, Freckmann G, Heinemann L and Schleicher E (2019). Definition, classification and diagnosis of diabetes mellitus. *Exp. Clin. Endocrinol.***,** **127**(S 01): S1-S7.

Qiu J, Xu J, Zheng Y, Wei Y, Zhu X, Lo EH, Moskowitz MA and Sims JR (2010). High-mobility group box 1 promotes metalloproteinase-9 upregulation through Toll-like receptor 4 after cerebral ischemia. *Stroke* **41**(9): 2077-2082.

Reiss Y, Scholz A and Plate KH (2015). The angiopoietin-tie system: Common signaling pathways for angiogenesis, cancer and inflammation. *In*: Schmidt MHH and Liebner S (editors). Endothelial signaling in development and disease. Springer New York, USA. pp.313-328.

Rempe RG, Hartz AMS and Bauer B (2016). Matrix metalloproteinases in the brain and blood-brain barrier: Versatile breakers and makers. *J. Cereb. Blood Flow Metab.,* **36**(9): 1481-1507.

Roy B, Ehlert L, Mullur R, Freeby MJ, Woo MA, Kumar R and Choi S (2020). Regional brain gray matter changes in patients with type 2 diabetes mellitus. *Sci. Rep.,* **10**(1): 020-67022.

Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, Colagiuri S, Guariguata L, Motala AA, Ogurtsova K, Shaw JE, Bright D and Williams R (2019). Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition. *Diabetes Res. Clin. Pract.,* **157**(107843): 10.

Scheen AJ (2013). GLP-1 receptor agonists or DPP-4 inhibitors: How to guide the clinician? *Ann Endocrinol.,* **74**(5-6): 515-522.

Scholz A, Harter PN, Cremer S, Yalcin BH, Gurnik S, Yamaji M, Di Tacchio M, Sommer K, Baumgarten P, Bähr O, Steinbach JP, Trojan J, Glas M, Herrlinger U, Krex D, Meinhardt M, Weyerbrock A, Timmer M, Goldbrunner R, Deckert M, Braun C, Schittenhelm J, Frueh JT, Ullrich E, Mittelbronn M, Plate KH and Reiss Y (2016). Endothelial cell-derived angiopoietin-2 is a therapeutic target in treatment-naive and bevacizumab-resistant glioblastoma. *EMBO. Mol. Med.,* **8**(1): 39-57.

Scholz A, Lang V, Henschler R, Czabanka M, Vajkoczy P, Chavakis E, Drynski J, Harter PN, Mittelbronn M, Dumont DJ, Plate KH and Reiss Y (2011). Angiopoietin-2 promotes myeloid cell infiltration in a β_2-integrin-dependent manner. *Blood,* **118**(18): 5050-5059.

Seufert J and Gallwitz B (2014). The extra-pancreatic effects of GLP-1 receptor agonists: A focus on the cardiovascular, gastrointestinal and central nervous systems. *Diabetes Obes Metab.,* **16**(8): 673-688.

Skrha J, Jr., Kalousova M, Svarcová J, Muravska A, Kvasnička J, Landová L, Zima T and Skrha J (2012). Relationship of soluble RAGE and RAGE ligands HMGB1 and EN-RAGE to endothelial dysfunction in type 1 and type 2 diabetes mellitus. *Exp. Clin. Endocrinol. Diabetes*, **120**(5): 277-281.

Taylor SC and Posch A (2014). The design of a quantitative western blot experiment. *Biomed Res. Int.* 361590.

van Sloten TT, Sedaghat S, Carnethon MR, Launer LJ and Stehouwer CDA (2020). Cerebral microvascular complications of type 2 diabetes: Stroke, cognitive dysfunction, and depression. *Lancet Diabetes Endocrinol.,* **8**(4): 325-336.

Vatandoust N, Rami F, Salehi A, Khosravi S, Dashti G, Eslami G, Momenzadeh S and Salehi R (2018). Novel high-fat diet formulation and streptozotocin treatment for induction of prediabetes and type 2 diabetes in rats. *Adv. Biomed. Res.,* **7**: 107.

Wiciński M, Socha M, Malinowski B, Wódkiewicz E, Walczak M, Górski K, Słupski M and Pawlak-Osińska K (2019). Liraglutide and its neuroprotective properties-focus on possible biochemical mechanisms in alzheimer's disease and cerebral ischemic events. *Int. J. Mol. Sci.,* **20**(5): 1050.

Williams R, Karuranga S, Malanda B, Saeedi P, Basit A, Besançon S, Bommer C, Esteghamati A, Ogurtsova K, Zhang P and Colagiuri S (2020). Global and regional estimates and projections of diabetes-related health expenditure: Results from the International Diabetes

Federation Diabetes Atlas, 9th edition. *Diabetes Res. Clin. Pract.,* **162**(108072): 13.

Xia SS, Xia WL, Huang JJ, Zou HJ, Tao J and Yang Y (2020). The factors contributing to cognitive dysfunction in type 2 diabetic patients. *Ann. Transl Med.,* **8**(4): 113.

Yan W, Pang M, Yu Y, Gou X, Si P, Zhawatibai A, Zhang Y, Zhang M, Guo T, Yi X and Chen L (2019). The neuroprotection of liraglutide on diabetic cognitive deficits is associated with improved hippocampal synapses and inhibited neuronal apoptosis. *Life Sci.,* **231**(116566): 13.

Yu Y, Yang L, Lv J, Huang X, Yi J, Pei C and Shao Y (2015). The role of high mobility group box 1 (HMGB-1) in the diabetic retinopathy inflammation and apoptosis. *Int. J. Clin Exp. Pathol.,* **8**(6): 6807-6813.