Insulin and liraglutide attenuate brain pathology in diabetic mice by enhancing the Wnt/β-catenin signaling pathway

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Abstract. Insulin and liraglutide have been demonstrated to control blood glucose and exert neuroprotective effects. However, the impact of liraglutide or insulin alone or in combination on brain pathology in type 1 diabetes mellitus (T1DM) and their underlying mechanisms are unclear. In the present study, diabetes mellitus (DM) was induced via intraperitoneal injection of streptozotocin in mice and subsequently mice were treated with insulin, liraglutide, a combination of the two drugs or saline. Changes in body weight and blood glucose were assessed weekly. The pathological changes in the brain tissue and the apoptosis of neurons were assessed using H&E staining and TUNEL staining. The mRNA and protein expression levels of apoptosis-related proteins were detected using reverse transcription-quantitative PCR (RT-qPCR) and western blotting, respectively. Moreover, Ki67 protein expression was analyzed using immunohistochemistry and the mRNA and protein expression levels of Wnt/β-catenin signaling pathway-related proteins were examined using RT-qPCR and western blotting, respectively. The results of the present study suggested that DM mice developed hyperglycemia and weight loss and also exhibited significantly increased neural cell apoptosis and significantly reduced numbers of Ki67-positive cells. Liraglutide significantly decreased blood glucose levels in DM mice, whereas both insulin and the combination of the two drugs failed to control blood glucose well. Insulin, liraglutide and their combination also failed to control body weight well, but significantly attenuated brain pathological changes and activation of the pro-apoptotic proteins Caspase-3 and Bax, which may have resulted in the significant increase in the expression levels of Wnt/β-catenin signaling pathway-associated molecules such as Wnt3a and S9-pGSK-3β. Liraglutide also promoted the protein expression of the neurogenesis marker of Ki67 and the antiapoptotic factor Bcl-2. These results suggested that insulin and liraglutide may improve brain damage via upregulation of the Wnt/β-catenin signaling pathway and could be of therapeutic relevance for improvement of cognitive impairment in patients with DM.

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Introduction

Type 1 diabetes mellitus (T1DM) has been considered to be a risk factor for inducing stroke, Alzheimer's disease (AD), vascular dementia and other types of dementia (1-3). Moreover, it has been reported that in T1DM mice and rats impairments in cognitive function increase brain cell apoptosis, tau protein expression and oxidative stress (4,5). It has also been demonstrated that insulin, the effective drug in the treatment of T1DM, improves cognitive function in comorbid patients with diabetes and AD (6).

Glucagon-like peptide 1 (GLP-1) is a growth factor and endogenous incretin hormone and its analogs, such as liraglutide and exenatide, are currently used in the treatment of type 2 diabetes mellitus (DM) (7,8). Furthermore, in addition to improving glycemic control, liraglutide has been demonstrated to cross the blood-brain barrier and bind to the GLP-1 receptor in the brain, which exerts neuroprotective effects in several neurological disorders, such as stroke, AD and Parkinson's disease (9,10). Liraglutide administered peripherally attenuates impairments in cognition and synaptic plasticity, promotes neurogenesis and reduces cell apoptosis in the streptozotocin (STZ)-induced T1DM mouse model (11,12).

Furthermore, the Wnt/β-catenin signaling pathway is an essential pathway for regulating cell proliferation, migration and differentiation (13). In the brain, the Wnt/β-catenin signaling pathway regulates neuronal survival, differentiation and synaptogenesis and serves an important role in the pathogenesis of AD (14,15). Moreover, the Wnt/β-catenin signaling pathway also mediates post-stroke angiogenesis and neurogenesis (16,17). Therefore, the present study aimed to investigate the effects of liraglutide, insulin and their co-treatment on neuronal apoptosis in STZ-induced diabetic mice, and if the activation of the Wnt/β-catenin signaling pathway was associated with the underlying mechanism.

Materials and methods

Animals and study design. In total 40 female C57BL/6J mice (age, 10-11 weeks; weight, 19.1-21.5 g) were purchased from Beijing Huafukang Biotechnology Co., Ltd. and were allowed to acclimate for 1 week before the experiments. Subsequently, the T1DM model was established via a single intraperitoneal injection of STZ (150 mg/kg) and control mice, referred to as the normal glucose tolerance (NGT; n=8) group, were injected with citrate buffer (100 mM citrate; pH 4.2-4.5).

After 2 weeks, mice injected with STZ and confirmed to have diabetes (random blood glucose, ≥250 mg/dl), were randomly assigned to the following four treatment groups for 8 weeks (n=8/group): i) DM model group (STZ), treated with subcutaneous injection of normal saline; ii) insulin group (INS), treated with subcutaneous injection of insulin (10 units/kg body weight/day insulin determir; Levemir®; Novo Nordisk A/S); iii) liraglutide group (LRG), treated with subcutaneous injection of liraglutide (0.6 mg/kg/day; Novo Nordisk A/S); and iv) combined insulin and liraglutide group (LRG + INS), subcutaneous injection of insulin (10 units/kg/day) and liraglutide (0.6 mg/kg/day). Furthermore, although it was not expected, a rapid decrease in body weight of >15-20% was defined as a potential humane endpoint for the study.

All mice were housed under standard laboratory conditions from the start of acclimatization in an air-conditioned atmosphere with a 12-h light/dark cycle, a humidity of 40-60% and a temperature of 22˚C. Mice were provided with *ad libitum* access to water and food for 11 weeks. Body weight and pedal dorsal vein blood glucose, which was assessed using the Accu-Chek compact glucometer (Roche Diagnostics), were recorded once a week. At the end of the experiment, mice were sacrificed using an overdose of isoflurane (5%) and were subsequently decapitated. Trunk blood (0.8-1 ml) was collected from the severed neck. One hemisphere of the brain was quickly dissected and stored at -80˚C until RNA and protein extraction (n=4/group) was performed. Meanwhile the other hemisphere was fixed with 4% paraformaldehyde overnight at room temperature for histopathological and immunohistochemical assays (n=4/group). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Laboratory Animals Science of the Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). Experiments were performed according to the Laboratory Animal Management Regulations in China (18) and also adhered to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (19). Moreover, all animal studies were performed in accordance with the Animal Research: Reporting of *In Vivo* Experiments guidelines (20). During the course of this study, none of the animals exhibited a weight loss of >20%, one mouse in each of the STZ, INS, and INS + LRG groups were sacrificed because of significantly elevated blood glucose, obvious signs of dehydration and weakness. In addition, two more mice in the INS + LRG group died at week 14 and 15, respectively, within a few hours after injection of the treatment drug, probably due to hypoglycemia (Fig. S1). Data from these mice were excluded from the analysis.

H&E staining. The cerebral hemispheres fixed in 4% paraformaldehyde overnight were embedded in paraffin, and sections 5-μm thick were prepared. The tissues were then stained with hematoxylin solution (cat. no. G1004-100ML; Wuhan Servicebio Technology Co., Ltd.) for 5 min at room temperature, followed by immersion in 1% acid alcohol differentiation solution for 5 min to be de-stained. Subsequently, the tissues were rinsed in distilled water, stained again with eosin dye (cat. no. G1001-100ML; Wuhan Servicebio Technology Co., Ltd.) for 5 min at room temperature, dehydrated with anhydrous ethanol and xylene and then mounted with neutral gum. Morphological changes in hippocampal and cortical neurons were observed using light microscopy. In total five fields were randomly selected from the hippocampus and cortex (magnification, x400) and the number of neurons were counted using image analysis software (ImageJ; version 1.46a; National Institutes of Health).

TUNEL staining. Dewaxed tissue sections were dewaxed with anhydrous ethanol and xylene for 45 min at room temperature, followed by fixation in proteinase K working solution (cat. no. G1205; Wuhan Servicebio Technology Co., Ltd.) at 37˚C for 30 min and then rinsed using phosphate-buffered saline (PBS) solution. Subsequently, sections were soaked in 0.2% Triton X-100 solution for 5 min at room temperature to enhance permeability and incubated with the TUNEL reaction mixture (cat. no. G1501; Wuhan Servicebio Technology Co., Ltd.) for 60 min at 37˚C. The samples were washed with PBS and then incubated with DAPI solution (cat. no. G1012; Wuhan Servicebio Technology Co., Ltd.) for 10 min at room temperature, followed by washing with PBS, and then mounted with anti-fade mounting medium. Samples were imaged using a fluorescence microscope. In total five fields of the cortex were randomly selected from each section and the number of apoptotic cells was quantified as apoptotic rate (%)=(number of apoptosis-positive cells/total cells) x100.

Immunohistochemistry for Ki67. First, 30-μm thick sections were deparaffinized in xylene for 2 min at room temperature and then rehydrated in descending grades of ethanol (100, 95 and 70% ethanol) for another 5 min at room temperature. The sections were washed with PBS at room temperature and then incubated in 3% H_2O_2 at 37˚C for 25 min to block endogenous peroxidase activity. After blocking in 3% bovine serum albumin (cat. no. GC305010-25G; Wuhan Servicebio Technology Co., Ltd.) for 30 min at room temperature, sections were incubated with primary antibody against Ki67 (1:500; cat. no. GB111141; Wuhan Servicebio Technology Co., Ltd.) overnight at 4˚C. Following the primary incubation cells were incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; 1:1,000; cat. no. 7074S; Cell Signaling Technology, Inc.) for 1 h at room temperature. The peroxidase was visualized using the DAB detection kit (cat. no. G1212-200T; Wuhan Servicebio Technology Co., Ltd.) and counterstained with hematoxylin solution (cat. no. G1004-100ML; Wuhan Servicebio Technology Co., Ltd.) for 10 min at room temperature. Brain sections were imaged using light microscopy.

Western blotting. The isolated brain tissues were homogenized on ice in RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology) and phenylmethanesulfonyl fluoride in the presence of protease and phosphatase inhibitors. The homogenates were centrifuged at 12,000 x g for 15 min at 4˚C and the supernatants were extracted to quantify protein concentration using a BCA Protein Assay Kit (cat. no. P0012S; Beyotime Institute of Biotechnology). Equal amounts of protein (50 µg per lane) were separated using SDS-PAGE on a 12% gel, transferred to polyvinylidene difluoride membranes and blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated with the following primary antibodies: rabbit anti-Wnt3a (1:1,000; cat. no. 26744-1-AP; ProteinTech Group, Inc.), S33-phosphorylated (p)β-catenin (1:5,000; cat. no. 80067-1-RR; ProteinTech Group, Inc.), β-catenin (1:5,000; cat. no. 51067-2-AP; ProteinTech Group, Inc.), GSK-3β (1:1,000; cat. no. 22104-1-AP; ProteinTech Group, Inc.), Caspase-3 (1:1,000; cat. no. 9662S; Cell Signaling Technology, Inc.), Bcl-2 (1:1,000; cat. no. 12789-1-AP; ProteinTech Group, Inc.), Bax (1:5,000; cat. no. 50599-2-Ig; ProteinTech Group, Inc.), mouse anti-S9-pGSK-3β (1:1,000; cat. no. 67558-1-Ig; ProteinTech Group, Inc.), rabbit anti-β-actin (1:1,000; cat. no. 4970S; Cell Signaling Technology, Inc.) primary antibodies at 4˚C overnight. After washed with TBST, the membranes were incubated with goat anti-rabbit secondary antibody conjugated to HRP (1:1,000; cat. no. 7074S; Cell Signaling Technology, Inc.) or horse anti-mouse secondary antibody conjugated to HRP (1:1,000; cat. no. 7076S; Cell Signaling Technology, Inc.) at room temperature for 1 h. Proteins were visualized using an enhanced chemiluminescence (ECL) reagent (cat. no. P06M31M; Gene-Protein Link). The results were normalized to β-actin and the protein band densitometry was semi-quantified using ImageJ software (version 1.46a; National Institutes of Health) All protein bands in a given western blot image were derived from the same membrane.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using Tissue Total RNA Isolation Kit V2 (cat. no. RC112; Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. Complementary DNA was synthesized using the PrimeScript™ RT Reagent Kit with Genomic DNA (cat. no. RR047A; Takara Biotechnology Co., Ltd.), according to the manufacturer's instructions. qPCR primers (Table I) were synthesized by Beijing Nuosai Genome Research Center Co., Ltd. qPCR was performed using TB Green® Premix Ex Taq™ II (cat. no. RR82LR; Takara Biotechnology Co., Ltd.). The thermocycling conditions were as follows: After the initial denaturation for 30 sec at 95˚C, 40 PCR cycles were performed (95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 30 sec). The relative mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (21) with the housekeeping gene β-actin as an internal control.

Statistical analysis. All data analysis was performed using SPSS 25.0 software (IBM Corp.). All figures were created using GraphPad Prism 8.0 software (GraphPad Software, Inc.). Data from at least three independent experiments are presented as the mean ± SD. One-way ANOVA was used to make statistical comparisons among more than two groups followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')
Wnt3a	TGGAGGAATGGTCTCTCGGG
	GCACTTGAGGTGCATGTGAC
GSK-3β	GTAGCCCAGGGAGGTCACTA
	CAGCCTTCCTAAGCTGGCAT
β-catenin	CTGGGACTCTGCACAACCTT
	CAGTGTCGTGATGGCGTAGA
Bax	TCTCCGGCGAATTGGAGATG
	ACCCGGAAGAAGACCTCTCG
Bcl-2	GCAGCTTCTTTTCGGGGAAG
	CTCCAGCATCCCACTCGTAG
Caspase-3	TGGCTTGCCAGAAGATACCG
	ATGCTGCAAAGGGACTGGAT
β-actin	CACTGTCGAGTCGCGTCCA
	GTCATCCATGGCGAACTGGT

Results

Effect of insulin, liraglutide and combined drugs on metabolic parameters in DM mice. Compared with the NGT control, saline-treated DM mice exhibited significant hyperglycemia and weight loss, which indicated the successful establishment of the T1DM mouse model (Fig. 1; Tables SI and SII) (22). However, compared with saline-treated DM mice, once-daily insulin treatment failed to control blood glucose levels or lower body weight. Furthermore, liraglutide monotherapy had no effect on body weight compared with the saline-treated DM group and the INS group but exhibited significantly lower blood glucose levels after week 17 and approached those of the control group after week 20. However, the combined treatment (LRG + INS) group did not significantly improve glycemic control and led to further weight loss compared with the saline-treated DM group. These results suggested that liraglutide monotherapy exerted the greatest efficacy in reducing metabolic disturbances in DM mice.

Insulin and liraglutide attenuate diabetes-induced neuronal damage in mice. The pathological damage of brain tissue in different regions of the brain in each group of mice was assessed using H&E staining (Fig. 2A). Compared with the NGT group, neurons in the STZ group exhibited marked pathological changes in the cortex and hippocampal cornu ammonis-1 and dentate gyrus (DG) regions, as demonstrated by loose cortical interstitium and neuronal degeneration, including irregular neuronal arrangement, increased intercellular space, nucleus condensation and significantly decreased neuronal density (Fig. 2B). However, neurons in the INS, LRG and INS + LRG groups were neatly arranged with clearly visible nucleus and cytoplasm, displaying round vesicular nuclei and prominent nucleoli (Fig. 2A), and significantly increased neuronal density

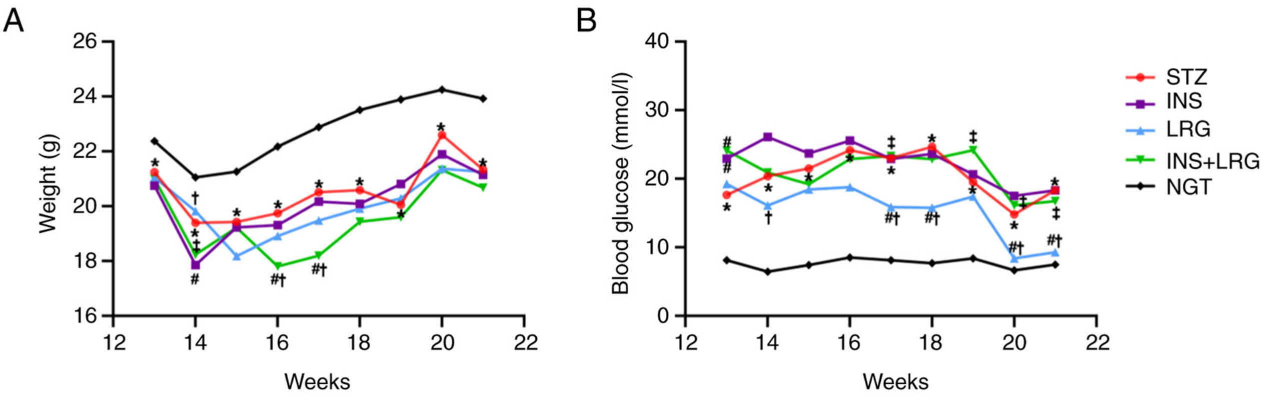

Figure 1. Changes in body weight and blood glucose of diabetic mice during treatments. (A) Weight per week (g). (B) Glucose level after feeding per week. Data are presented as the mean ± SD (n=4/group). *P<0.05 vs. NGT; #P<0.05 vs. STZ; †P<0.05 vs. INS; ‡P<0.05 vs. LRG. STZ, saline treated type 1 diabetes group; INS, insulin treatment group; LRG, liraglutide treatment group; INS + LRG, insulin and liraglutide treatment group; NGT, normal glucose tolerance group.

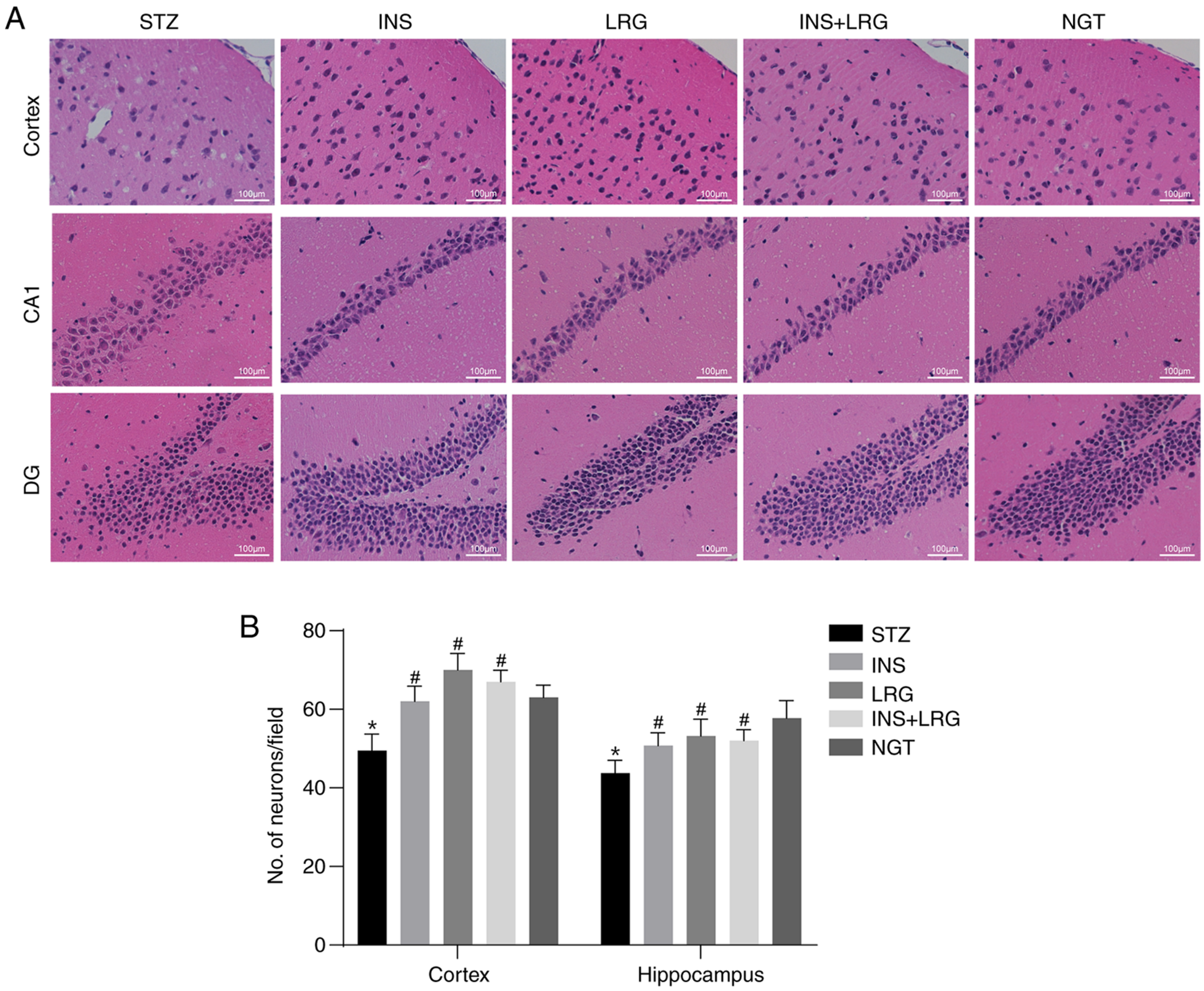

Figure 2. Effect of insulin, liraglutide and combined drugs on pathological changes in the cortex, hippocampal CA1 and DG regions in diabetic mice. (A) H&E staining of neurons in the cortex, hippocampal CA1 and DG regions. Scale bar, 100 μm. (B) Neuronal density in the cortex, hippocampus including CA1 and DG regions. Data are presented as the mean ± SD (n=4/group). *P<0.05 vs. NGT; #P<0.05 vs. STZ. CA1, cornu ammonis-1; DG, dentate gyrus; STZ, saline treated type 1 diabetes group; INS, insulin treatment group; LRG, liraglutide treatment group; INS + LRG, insulin and liraglutide treatment group; NGT, normal glucose tolerance group.

(Fig. 2B), similar to those exhibited by the NGT group. These results suggested that either insulin, liraglutide, or combined drugs prevented neuronal damage in DM condition.

Insulin and liraglutide reduce the apoptotic rate of neurons and regulate the expression levels of related proteins in DM mice. The mRNA and protein expression levels of Bax, Bcl-2

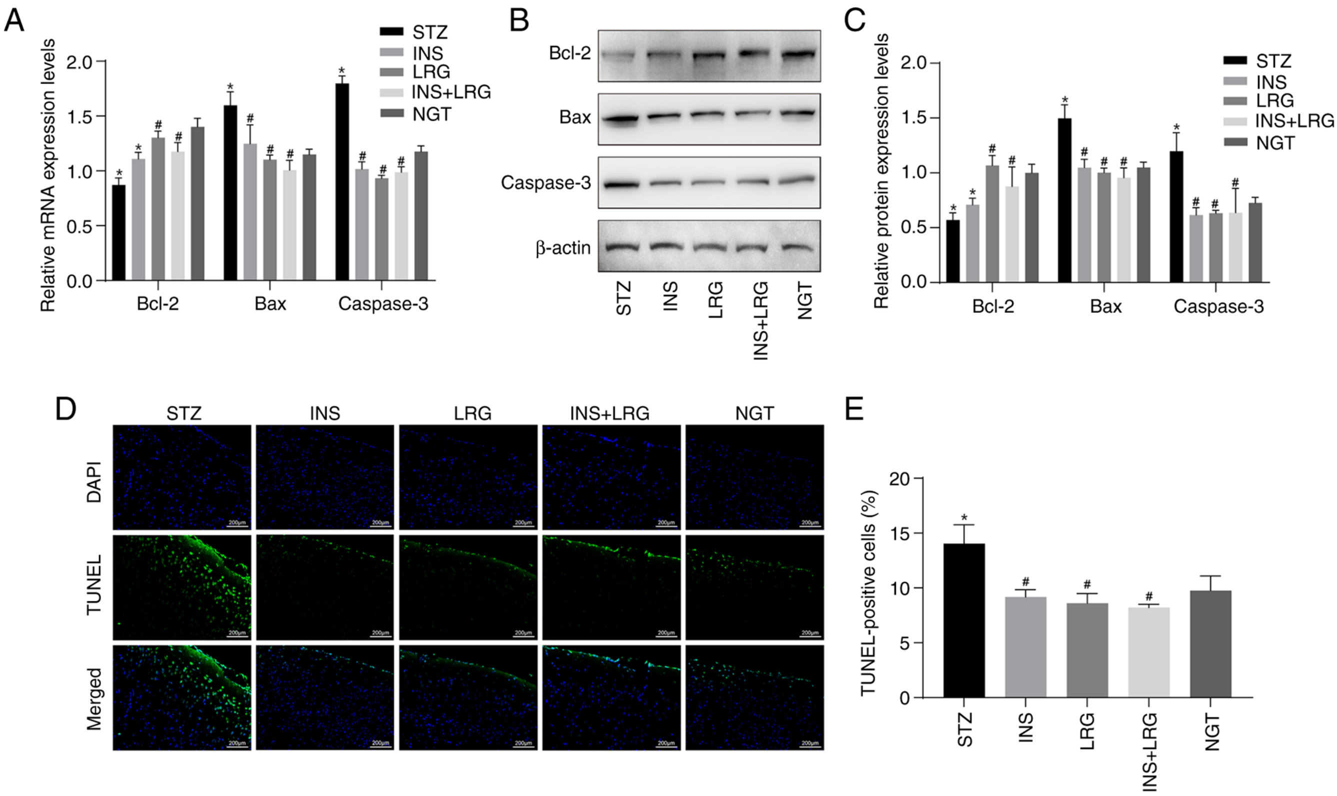

Figure 3. Effect of insulin, liraglutide and combined drugs on neuronal apoptosis in diabetic mice. (A) Relative mRNA expression levels of apoptosis-related genes were detected via reverse transcription-quantitative PCR. (B and C) Relative protein expression levels of apoptosis-related proteins were detected via western blotting. (D and E) Apoptosis of neurons in the cortex was detected using the TUNEL assay. Scale bar, 200 μm. β-actin was included as a reference for normalization. Data are presented as the mean ± SD (n=4/group). *P<0.05 vs. NGT; #P<0.05 vs. STZ. STZ, saline treated type 1 diabetes group; INS, insulin treatment group; LRG, liraglutide treatment group; INS + LRG, insulin and liraglutide treatment group; NGT, normal glucose tolerance group.

and Caspase-3 in brain tissue were determined using RT-qPCR and western blotting. The mRNA and protein expression levels of Bax and Caspase-3 were significantly higher in neurons of the STZ group compared with the NGT group, along with significantly lower expression levels of Bcl-2 (Fig. 3A-C). Compared with the STZ group, the mRNA and protein expression levels of Bax and Caspase-3 were significantly decreased in the brain tissue of the INS, LRG and LRG + INS groups, whereas Bcl-2 mRNA and protein expression levels were significantly increased in the LRG and LRG + INS groups. Furthermore, Bcl-2 mRNA and protein expression levels in the INS group was not significantly different compared with the STZ group. Apoptosis of neurons in the brain was detected using the TUNEL assay. The results demonstrated that the mean percentage of TUNEL-positive cells was significantly increased in the STZ group compared with the NGT group (Fig. 3D and E). However, among the INS, LRG and LRG + INS groups, the mean percentage of apoptotic cells was significantly lower compared with the STZ group and no significant difference was observed when compared with the NGT group. These results suggested that either liraglutide, insulin, or the combination of these drugs may potentially inhibit neuronal apoptosis in DM mice.

Insulin and liraglutide activate the Wnt/β-catenin signaling pathway in DM mice. mRNA and protein expression levels of Wnt/β-catenin signaling pathway-associated proteins in the brains of mice were determined (Fig. 4). Compared with the STZ group, the expression levels of Wnt3a and S9-pGSK-3β were significantly increased, whereas the expression levels of GSK-3β and S33-pβ-catenin were significantly decreased in the INS, LRG and LRG + INS groups. In the LRG and LRG + INS groups, β-catenin mRNA and protein expression levels were significantly upregulated compared with the STZ and INS groups, while there was no significant difference among the STZ, INS and NGT groups. These results suggested that either liraglutide, insulin or the combination drug therapy activated the Wnt/β-catenin signaling pathway in the brain of DM mice.

Liraglutide promotes neurogenesis in DM mice. Neuronal proliferation was investigated using Ki67 immunostaining of the hippocampus. The results demonstrated that compared with the NGT group the number of Ki67-positive neurons in the DG region was significantly reduced in the STZ group, whereas it was significantly increased in the LRG and LRG + INS groups (Fig. 5). No significant differences were observed between the STZ and INS groups.

Discussion

Multiple epidemiological studies have demonstrated that numerous patients with T1DM are at an increased risk for stroke, cognitive impairment, dementia and neurodegenerative diseases (23-25). However, there is a lack of effective clinical drug therapy due to incomplete knowledge of the underlying

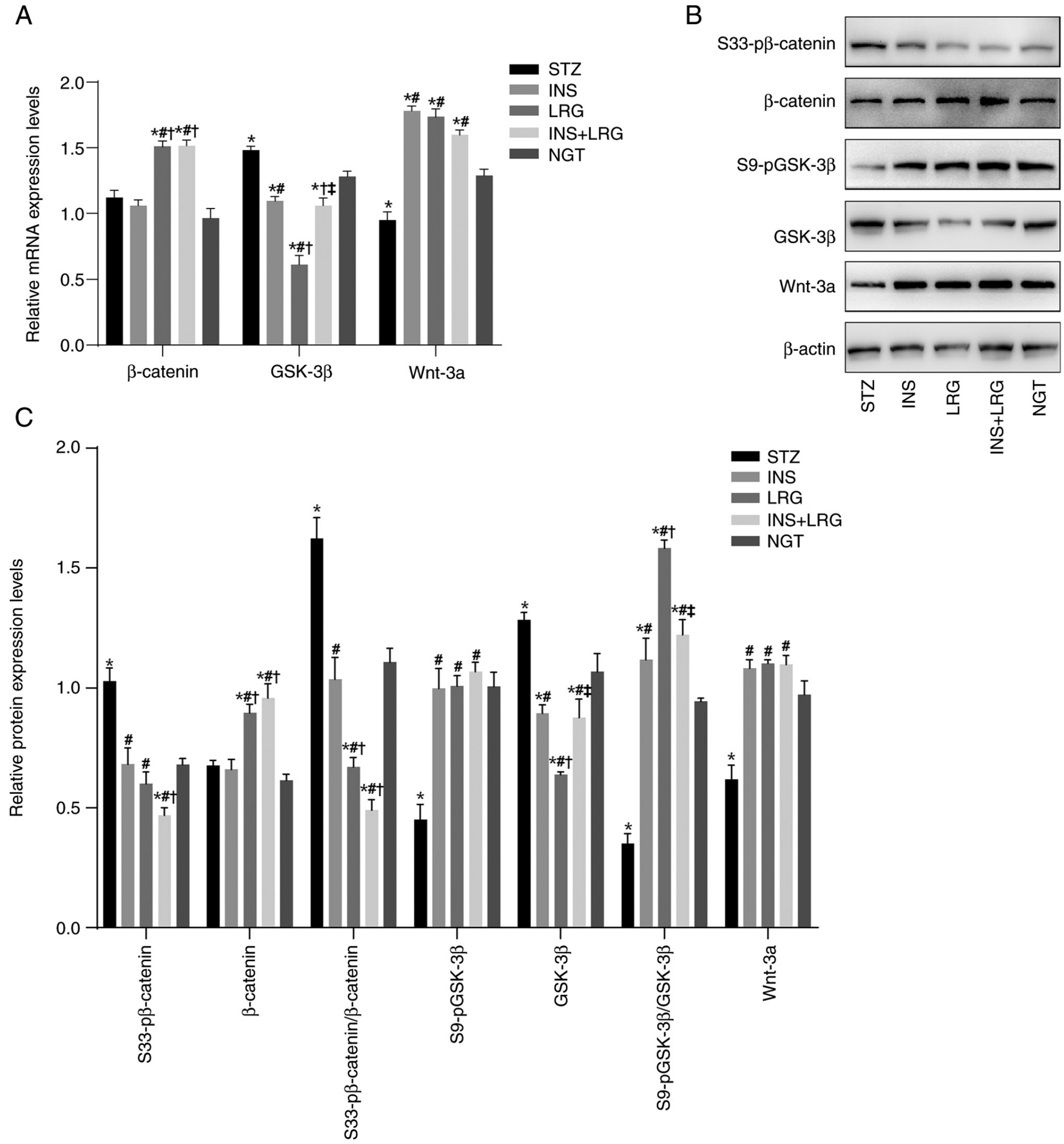

Figure 4. Effect of insulin, liraglutide and combined drugs on the expression of Wnt/β-catenin signaling pathway-related mRNAs and proteins in diabetic mice. (A) Relative mRNA expression levels of Wnt/β-catenin signaling pathway-related proteins were determined using reverse transcription-quantitative PCR. (B and C) Relative protein expression levels of Wnt/β-catenin signaling pathway-related proteins were detected via western blotting. β-actin was included as a reference for normalization. Data are presented as the mean ± SD (n=4/group). *P<0.05 vs. NGT; #P<0.05 vs. STZ; †P<0.05 vs. INS; ‡P<0.05 vs. LRG. STZ, saline treated type 1 diabetes group; INS, insulin treatment group; LRG, liraglutide treatment group; INS + LRG, insulin and liraglutide treatment group; NGT, normal glucose tolerance group; p, phosphorylated.

disease process. Over the last few years the roles of insulin and GLP-1 analogs in the central nervous system of animal models with DM have been increasingly investigated (6,10). To the best of our knowledge, this is the first study to have compared the effects of peripherally-administered insulin, liraglutide and their combination, on brain pathological changes in an STZ-induced mouse model of T1DM and to explore the underlying mechanisms. The present study demonstrated that insulin, liraglutide and the drugs combined equally significantly alleviated DM-induced hippocampal and cortical neuronal injuries and loss. Furthermore, treatment with liraglutide alone or in combination with insulin administration significantly increased the proliferation of newborn neurons (Ki67-positive neurons) in the hippocampal DG region of DM mice. These protective effects may involve the activation of the Wnt/β-catenin signaling pathway.

In the present study, the mortality rate in diabetic mice was lower compared with previous studies in which the same model was established but the mean blood glucose was higher (26,27), and two mice in the combined treatment group may have died due to hypoglycemia. Furthermore, the results demonstrated that liraglutide, insulin and the combination of both drugs had no significant effect on improving body weight, but liraglutide significantly decreased blood glucose levels in DM mice. Moreover, insulin monotherapy and the combination of the two drugs failed to control blood glucose well. However, the mean blood glucose level in the liraglutide treated group (16.41±6.36 mmol/l) was much higher than the normal standard, which is consistent with the results of previous studies (12,28). It has also previously been reported that GLP-1 and its analogs exert neuroprotective effects without significant improvement in blood glucose levels in

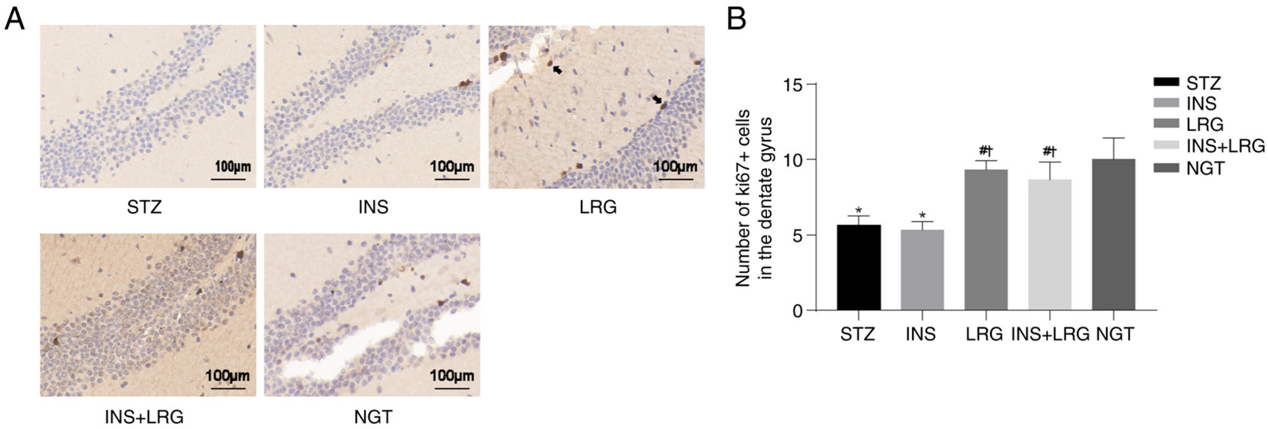

Figure 5. Effect of insulin, liraglutide and combined drugs neurodegeneration in the hippocampal DG of diabetic mice. (A) Representative Ki67 immunostaining in the DG region of the hippocampus. Arrows indicate Ki67-positive cells. Scale bar, 100 μm. (B) Number of Ki67-positive cells in each group. Data are presented as the mean ± SD (n=4/group). *P<0.05 vs. NGT; #P<0.05 vs. STZ; †P<0.05 vs. INS. DG, dentate gyrus; STZ, saline treated type 1 diabetes group; INS, insulin treatment group; LRG, liraglutide treatment group; INS + LRG, insulin and liraglutide treatment group; NGT, normal glucose tolerance group.

T1DM models (11,29). It can therefore be hypothesized that liraglutide potentially exerts direct neuroprotective effects independently from its hypoglycemic effects.

Cell apoptosis is dependent on caspases, of which Caspase-3 is central to the apoptotic signaling pathway (30). The proapoptotic factor Bax and the antiapoptotic factor Bcl-2, members of the Bcl-2 family, control the release of cytochrome *c*, which is involved in the activation of Caspase-3 (31). Previous studies have reported that liraglutide alleviates neuronal apoptosis in STZ-induced T1DM mouse models via modulating the mTOR or PI3K/Akt signaling pathways (12,28). Furthermore, insulin may prevent brain cell apoptosis by reducing brain mitochondrial dysfunction and brain oxidative stress via its antioxidant effects (32,33). In the present study it was demonstrated that STZ-induced DM mice exhibited significantly decreased mRNA and protein expression levels of Bcl-2 and significantly increased mRNA and protein expression levels of Bax and Caspase-3 compared with the NGT group. Moreover, the STZ group exhibited increased apoptosis of cortical neurons. However, insulin, liraglutide and the drugs combined equally significantly inhibited the mRNA and protein expression of Bax and Caspase-3 and had a significant inhibitory effect on apoptosis in cortical neurons, and Bcl-2 expression was significantly upregulated in either the liraglutide monotherapy and combined drug groups, without significant changes after insulin treatment. These results suggested that insulin and liraglutide potentially inhibited Caspase-dependent apoptosis in STZ-induced DM mice.

Ki67 is a commonly used marker for assessing cell proliferation (34). It has been indicated that Ki67 immunoexpression is significantly reduced in the DG of STZ-induced DM rats (35,36). The results of the present study demonstrated that the number of Ki67-positive cells was significantly decreased in the hippocampal DG of mice in the STZ group and significantly increased after liraglutide treatment, which suggested that liraglutide may have alleviated diabetes-induced neurogenesis defects. A previous study demonstrated that insulin-mediated protection of the hippocampus did not involve neurogenesis (37), which is consistent with the results of the present study. However, a limitation of the present study is that Ki67 is simply a marker for proliferating cells, hence the use of 5-bromo-2'-deoxyuridine as a marker for neurogenesis would be more effective (38).

The Wnt/β-catenin signaling pathway serves an important role in the regulation of numerous cellular events, including the prevention of apoptosis as well as the enhancement of cell proliferation (39). In the brain, the Wnt/β-catenin signaling pathway has been shown to alleviate the cognitive decline associated with AD by increasing neurogenesis in DM rats (40). Moreover, 10-O-(N N-dimethylaminoethyl)-ginkgolide B methane-sulfonate alleviates cerebral ischemic injury induced by middle cerebral artery occlusion/reperfusion surgery in mice via activation of the Wnt/β-catenin signaling pathway to exert antiapoptotic and neurogenetic activity (41). It has also previously been reported that insulin and GLP-1 are direct activators of the Wnt/β-catenin signaling pathway in multiple tissues and organs and that insulin promotes the phosphorylation and inhibition of GSK-3β (42-45). He *et al* (46) demonstrated that liraglutide restores the viability, inhibits apoptosis and protects the neuronal growth of cortical neurons under oxidative stress possibly via activation of the Wnt/β-catenin signaling pathway. Insulin contributes to the healing of diabetic corneal epithelial wounds and recovery from nerve damage via Wnt/β-catenin signaling (44). In the present study, to the best of our knowledge, it was investigated for the first time whether the application of insulin and liraglutide could inhibit apoptosis in neurons of DM mice via the activation of the Wnt/β-catenin signaling pathway. The results demonstrated that either insulin, liraglutide or the combined drugs led to the significantly decreased apoptosis of brain cells. This was accompanied by a significant increase in the expression levels of Wnt3a and S9-pGSK-3β and a significant decrease in GSK-3β and S33-pβ-catenin protein expression levels in brain tissues.

In conclusion, the results of the present study suggested that insulin, liraglutide and the combination of the two drugs exerted similar neuroprotective effects on neuronal loss and apoptosis in the hippocampus and cortex in an STZ-induced T1DM mouse model. Moreover, these effects appeared to be associated with the activation of the Wnt/β-catenin signaling pathway. These results provide a theoretical basis for the potential of insulin and liraglutide as a new drug with the capacity against diabetes-induced cognitive impairments. A limitation of our present study is the lack of Wnt3a-overexpression or knockdown experiments, and more experimental data are required to explore the underlying mechanisms in the future.

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Availability of data and material

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

Authors' contributions

YZ was responsible for data acquisition and drafting of the manuscript. JY performed the animal experiments and data acquisition. FP, LX and WL performed data acquisition and analyzed and interpreted the data. HZ and YL were responsible for the study concept and design, critical revision of the manuscript for important intellectual content and study supervision. All authors read and approved the manuscript for publication. HZ and YL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Laboratory Animals Science, Chinese Academy of Medical Sciences and Peking Union Medical College (approval no. XHDW-2018-00; Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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