**Giti Kalantarian[1] / Nasrin Ziamajidi[1] / Roghayeh Abbasalipourkabir[1] / Reza Mahjub[2] / Mohammad Taghi Goodarzi[3] / Massoud Saidijam[4] / Sara Soleimani Asl[5] / Mohammad Jamshidi[6]**

# Effect of insulin-loaded trimethyl chitosan nanoparticles on genes expression in the hippocampus of diabetic rats

[1] Department of Biochemistry, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran, E-mail: rpourkabir@hotmail.com. https://orcid.org/0000-0002-6345-1410.
[2] Department of Pharmaceutics, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran
[3] Department of Biochemistry, Shahrood Branch, Islamic Azad University, Shahrood, Iran
[4] Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
[5] Endometrium and Endometriosis Research Centre, Hamadan University of Medical Sciences, Hamadan, Iran
[6] Department of Laboratory Sciences, School of Allied Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

**Abstract:**
**Background:** Diabetes mellitus is a chronic metabolic disorder that undesirably affects both central and peripheral nervous systems through the apoptosis of neurons. Insulin and insulin-like growth factors (IGFs) inhibit apoptosis of oligodendrocytes. The objective of this study was to determine whether oral insulin in the form of nanoparticles may have similar effects to injectable insulin in increasing the gene expression of *IGF1* and *IGF2*.
**Methods:** Insulin-loaded trimethyl chitosan nanoparticles were prepared using the polyelectrolyte complex method and characterized for size, polydispersity index, zeta potential, drug loading, and entrapment efficiency. An *in vivo* study was performed in different groups of male Wistar rats with diabetes mellitus type 1 treated with insulin-loaded trimethyl chitosan nanoparticles and subcutaneous injection of trade insulin (neutral protamine Hagedorn). The hippocampus of rats were studied for the expression of *IGF1* and *IGF2* genes by using real-time PCR, and the fold changes in gene expression were evaluated using the $2^{-\Delta\Delta Ct}$ method.
**Results:** The expression of *IGF1* and *IGF2* genes in the groups treated with nano-insulin and injected insulin were significantly higher than that in the diabetic control group ($p<0.001$) and meaningfully lower than that in the healthy control group. However, there was no significant difference to the treated groups.
**Conclusion:** Our findings suggest that future research might provide a new formulation of drugs for treating type 1 diabetes, in the form of oral insulin.

**Keywords:** diabetes mellitus, IGF1, IGF2, insulin, nanoparticles, real-time PCR
**DOI:** 10.1515/jbcpp-2019-0147
**Received:** June 13, 2019; **Accepted:** September 6, 2019

## Introduction

Various pathological processes play a role in the development of diabetes, from the autoimmune destruction of β-pancreatic cells, and consequently insulin deficiency, to processes that lead to insulin resistance, which is the basis of disorders in the metabolism of carbohydrates, lipids, and proteins. Disorders of insulin secretion and insufficiency of insulin function are commonly found in diabetic patients, and it is often unclear which of these disorders are the main cause of hyperglycemia [1]. Currently, researchers are trying to achieve a non-invasive method for using insulin orally, which has fewer side effects for patients. There are many barriers to oral insulin administration, including physical agents (e.g. mucosal layers, mucin, electrolytes, and tight joints); enzymatic barriers, including pepsin enzymes, trypsin, and chymotrypins; and insulin instability in the gastrointestinal tract due to high pH changes. These agents cause insulin to be absorbed orally, and about 2% of the amount of insulin consumed enters the bloodstream. However, over the past decades, a nanoparticle program has been developed to treat diabetes with the aim of delivering insulin through nanoparticles [2], [3], [4], [5]. In nanotechnology, the material is loaded onto polymer nanoparticles that direct it for a purpose into a region of the gastrointestinal tract. As mentioned, cognitive impairment due to damage to hippocampal neurons is also a complication of diabetes, and deficits in memory, learning, and recognition are more common in diabetics

**Roghayeh Abbasalipourkabir** is the corresponding author.

than in non-diabetics [6]. The hippocampus is sensitive to high blood glucose, as an important learning and memory center, and its neurons are extremely vulnerable to type 1 diabetes [7], [8]. Although the mechanism of destruction of diabetes-induced nerve cells in the hippocampus is not well known, dendritic atrophy, decrease in glucocorticoid receptors, changes in the expression of insulin-like growth factor (IGF) receptors, insulin secretion, and induction of apoptosis have been proposed [9]. Research has also shown that IGF1 has a role of regulating brain hexokinase activity, which may contribute to reducing glucose consumption. This information demonstrates the role of anabolic IGF1 signaling in brain metabolism [10]. Another purpose of insulin IGF1 signaling is via AKT in the brain with respect to glycogen β3 synthase kinase (GSK3β) [11]. Research has shown that IGF1 inhibits the activity of β3GSK and promotes neuronal growth, and indeed GSK3β acts as a pro-apoptotic factor in neurons [2], [12], [13]. Changes in the level of IGF1 related to diabetes mellitus occur practically due to reduced free-living physical activity and reduced bioavailability of the growth factor. By considering the positive potential and supportive effects of IGF1 in the processes of tissue repair and regeneration of damaged cells, reducing the free surface of this substance causes or intensifies various disorders and complications. Early and progressive reductions in serum IGF1 levels that occur in parallel with aging in diabetic patients cause the IGF2-protective and other protective effects on the kidneys, eyes, and neurons to be inadequate, and provide a basis for the onset of complications in these organs [13]. Nanoparticles, including nanospheres and nanocapsules, can absorb and encapsulate drugs, thereby protecting against enzymatic and chemical degradation. Nanocapsules are vesicular systems in which the drug is placed in a hole surrounded by a polymeric membrane, while nanospheres are matrix systems in which the drug is dispersed physically and uniformly in the carrier. In recent years, biodegradable polymer nanoparticles have received considerable attention as potential systems for drug delivery [2]. Polymers of nanoparticles, including biodegradable polymers of chitosan, were reported by Hu and Luo for oral delivery of insulin nanoparticles prepared from polysaccharides, including chitosan, alginate, dextran, and glucan [14]. These polymers are considered for their biocompatibility and usability in natural pathways. Chitin is a mucopolysaccharide derived naturally and found to be produced abundantly (second to cellulose) through biosynthesis. Chitins are characterized as white, non-elastic, hard, nitrogenous polysaccharides that have been estimated to be synthesized at approximately 1 billion tons annually [15], [16]. Chitosan, a polysaccharide derived from chitins, which are extracted from the shells of arthropods such as crab, shrimp, and lobster, is becoming the most wanted biopolymer for use toward therapeutic interventions [17]. The aim of this study was to investigate the effect of insulin-loaded trimethyl chitosan nanoparticles on the gene expression of IGF1 and IGF2 in the hippocampus of male Wistar rats with diabetes mellitus type 1.

## Materials and methods

### Preparation of insulin-loaded nanoparticles

Insulin-loaded trimethyl chitosan nanoparticles were prepared using the modified complex polyelectrolyte method [18]. The basis of this method is the reaction between the positively charged polymer and the negatively charged insulin. Briefly, 15 mL of polymer solution, trimethyl chitosan, was added to equal volume of insulin at pH 3 for 20 min and centrifuged at 500 *g* (4 °C) for 20 min. The sediment was used to prepare the lyophilized powder. For this purpose, the sediment was dissolved with alginate powder (90 mg) and mannitol (450 mg) in distilled water (5 mL). Thereafter, it was sonicated for 3 min at 24 °C and was subsequently kept frozen at −20 °C and lyophilized in a freeze dryer at −53 °C temperature and 38 mm Hg pressure for 5 days. The lyophilizing process was done until the lyophilized powder was prepared.

### Physicochemical characterization

Zetasizer (Malvern Instruments, Malvern, UK) was used to determine the particle size in disposable polystyrene cuvettes at 25 °C. A transmission electron microscope (Zeiss S500, Germany) with acceleration voltage of 0.8 kV was used for morphological examination. High-pressure liquid chromatography was used to determine the entrapment efficiency (EE) and drug loading (DL) of the prepared insulin-loaded nanoparticles. Insulin loading content and loading efficiency were calculated [19], [20]. Insulin-loaded trimethyl chitosan was characterized *in vitro* for particle size, particle size distribution, and zeta potential.

## Inductionof diabetes mellitus type 1

Diabetes mellitus type 1 was induced by intraperitoneal injection of 60 mg/kg freshly prepared streptozotocin (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Fasting blood sugar (FBS) level >250 mg/dL was considered as diabetes. Measurements of FBS were also repeated at 8 and 10 weeks after diabetes induction.

## Experimental design and sampling

Twenty-five male Wistar rats with an average weight of 200 g were obtained from Hamadan University of Medical Sciences animal laboratory (Hamadan, Iran). They were handled under conditions that were approved by the local Ethics Committee of the university and the National Medical Board, in accordance with the ethics standards of the Principles of Laboratory Animal Care. The animals were housed throughout the acclimatization and experimental periods in standard cages and appropriate conditions, including 12-h light/dark cycles, normal temperature (25±2 °C), and free access to commercial rat chow diet and tap water. After 1 week of acclimation, all rats except the normal group with no treatment (G1) were subjected to diabetes induction. Six weeks after diabetes induction, the animals were randomly divided into five groups, including the diabetes mellitus group with no treatment (G2), diabetes mellitus group treated with nanoparticles (G3: 1 mL/day by gavage), diabetes mellitus group treated with insulin-loaded nanoparticles (G4: 1 mL/day by gavage), and diabetes mellitus group treated with insulin (G5: 2 U/day by subcutaneous injection). Each group was equally assigned five rats, which were treated daily for 2 weeks. The body weight of all groups was measured before and after diabetes induction and every 2 weeks until the day the animals were sacrificed. The sera were prepared from the blood samples by centrifugation (3000×*g*, 10 min). The animals were sacrificed after anesthesia, and the hippocampus was removed from the brain. Thereafter, the hippocampus sample was kept at −80 °C in a freezer for the real-time test. All procedures for the treatment of animals were approved by the Research Committee of Hamadan University of Medical Sciences, Iran (IR. UMSHA.REC.1394.104).

## Real-time PCR analysis

RNA extraction from the hippocampus tissue was performed using RNX-Plus solution (Sinaclon, Tehran, Iran) according to the manufacturer's protocol. The RNA concentrations were evaluated by Nanodrop measurements, and the RNA quality was examined using gel electrophoresis. The cDNA was synthesized using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Vilnius, Lithuania), following the protocols of the manufacturer. The gene expression of IGF1 and IGF2 was measured using quantitative real-time PCR using the SYBR Premix ExTaq real-time PCR kit (Takara Bio Inc., Tokyo, Japan), according to the manufacturer's instructions. The primer sequences used in this study are shown in Table 1. The β-actin gene was used as the reference gene. The fold changes in gene expression were evaluated using the $2^{-\Delta\Delta Ct}$ method [21].

**Table 1:** Characteristics of primers of IGF1, IGF2, and β-actin genes.

| Genes | Primers | Primers | Primer length | GC% | Tm, °C | Product length |
|---|---|---|---|---|---|---|
| IGF1 | Forward | CTGGTGGACGCTCTTCAGTTC | 21 | 57.14 | 60.94 | 112 |
| | Reverse | ACTCATCCACAATGCCCGT | 19 | 52.63 | 59.31 | |
| IGF2 | Forward | GCTTGTTGACACGCTTCAGT | 20 | 50.00 | 59.34 | 153 |
| | Reverse | GGGGTGGCACAGTATGTCTC | 20 | 60.00 | 60.11 | |
| β-Actin | Forward | GATCAAGATCATTGCTCCTCCTGA | 24 | 45.83 | 60.20 | 170 |
| | Reverse | CAGCTCAGTAACAGTCCGCC | 20 | 60.00 | 60.74 | |

## Statistical analysis

All data were expressed as mean±standard deviation. One-way analysis of variance was used for statistical comparison of data, followed by a post-hoc Tukey test. Analyses were performed using Statistical Package for Social Sciences version 21 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered statistically significant at $p<0.05$. Non-normal variables were assessed using the Mann-Whitney U-test.

## Results

Insulin-loaded trimethyl chitosan was characterized *in vitro* for particle size, particle size distribution, zeta potential, and DL% and EE% (Table 2). The effect of treatment with insulin and nano-insulin on body weights, serum glucose, proteins of IGF1 and IGF2, and apoptosis levels in all rats with diabetes mellitus type 1 are shown in Table 3. The ΔΔCt values of genes in the studied groups were obtained from the difference of ΔCt in the treatment groups compared with the control group. Moreover, the formula $2^{-\Delta\Delta Ct}$ was used for the expression of genes, which provides comparative values in the statistical standpoint. Figure 1 presents the expression of IGF1 and IGF2 genes in all groups. In accordance with our previous study [22], body weights, serum glucose levels, proteins of IGF1 and IGF2, and apoptosis levels were obtained (as seen in the Table 3). In the present study, the correlation between these factors and the percentage expression of IGF1 and IGF2 genes showed a negative significant correlation with glucose and apoptosis, although this relationship for weight, protein of IGF1, and protein of IGF2 revealed a positive significant correlation (p<0.0) (Table 4).

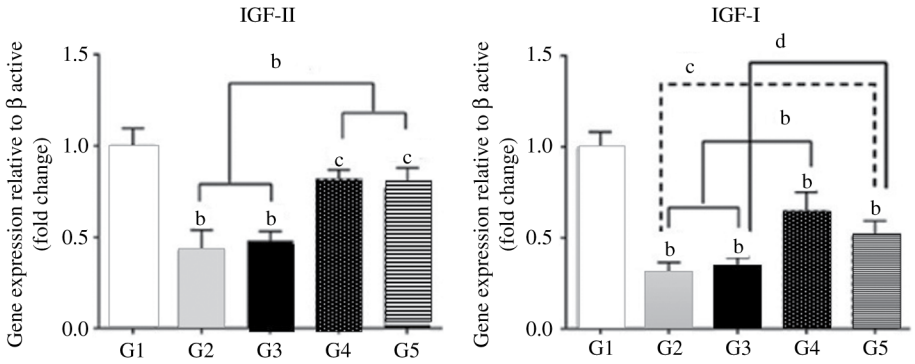


**Figure 1:** Graphs of expression of *IGF1/IGF2* genes in different groups.
G1, control normal group; G2, rats with diabetes mellitus with no treatment; G3, rats with diabetes mellitus treated with nanoparticles; G4, rats with diabetes mellitus treated with insulin-loaded nanoparticles; G5, rats with diabetes mellitus treated with insulin. Data are shown as mean±standard deviation. [a]Significant difference between group G1 and all other groups, p<0.01. [b]p<0.05, [c]p<0.01, [d]p<0.001.

**Table 2:** Particle sizes, polydispersity index, and zeta potential of insulin-loaded trimethyl chitosan nanoparticle formulation.

| Formulation | Particle size, nm | PI | Zeta potential, mV | Drug loading, % | Entrapment efficiency, % |
|---|---|---|---|---|---|
| Insulin-loaded trimethyl chitosan nanoparticles | 533.0±33.02 | 0.533±0.11 | 22.2±0.22 | 48.83±10.1 | 97.67±0.42 |

PI, polydispersity index.

**Table 3:** Effect of treatment with insulin and nano-insulin on body weights, serum glucose, proteins of IGF1 and IGF2, and apoptosis levels in male Wistar rats with diabetes mellitus type 1.

| Group | Glucose, mg/dL | Weight, g | IGF1, IHC% | IGF2, IHC% | Apoptosis, % |
|---|---|---|---|---|---|
| G1 | 84.60±7.45 | 307.6±17.3 | 16.2±3.96 | 16.7±5.96 | 14.1±3.6 |
| G2 | 507.5±129.2 | 177.6±28.4 | 12.6±4.14 | 21.8±4.66 | 26.0±4.18 |
| G3 | 488.4±65.19 | 180.5±18.6 | 11.1±2.72 | 12.9±2.68 | 24.1±5.6 |
| G4 | 294.1±51.54 | 217.0±8.16 | 13.1±2.64 | 12.5±1.71 | 20.1±7.20 |
| G5 | 268.3±75.5 | 239.4±10.2 | 26.6±10.8 | 18.5±7.9 | 21.2±1.8 |

G1, control normal group; G2, rats with diabetes mellitus with no treatment; G3, rats with diabetes mellitus treated with nanoparticle; G4, rats with diabetes mellitus treated with insulin-loaded nanoparticles; G5, rats with diabetes mellitus treated with insulin. Data are shown as mean±standard deviation.

**Table 4:** Correlation coefficients of IGF1 and IGF2 gene expression with weight, glucose, proteins of IGF1/IGF2, and apoptosis in male Wistar rats with diabetes mellitus type 1.

| Variable | Weight | Glucose | Protein IGF1 | Protein IGF2 | Apoptosis |
|---|---|---|---|---|---|
| IGF1 | 0.653[b] | −0.732[b] | 0.251[a] | 0.22[a] | −0.442[b] |
| IGF2 | 0.813[b] | −0.893[b] | 0.284[a] | 0.31[a] | −0.594[b] |

[a]$p<0.05$. [b]$p<0.01$.

## Discussion

In this study, using the real-time PCR method, we examined the effect of injectable insulin and insulin nanoparticles on the expression of IGF1 and IGF2 genes in diabetic rats. According to the results in diabetic rats treated with nano-insulin, the level of IGF1 gene expression was significantly higher than that in the non-treated diabetic control group, and there was no significant difference in the IGF2 gene expression in the groups treated with injectable insulin and nano-insulin. Moreover, in the group of insulin-treated diabetic rats, the level of IGF2 gene expression was significantly higher than that in the diabetic control group without treatment, and the rate of increase of IGF2 gene expression in the groups treated with insulin and nano-insulin did not have a significant difference. These IGFs are neurotrophic factors that can support the growth and survival of central and peripheral neurons. IGF enhances the re-establishment of sympathetic nerves and also increases the survival of motor neurons. The occupancy of IGF receptors depends heavily on IGFs, in which IGF binding to its receptor increases neuronal growth and the expression of genes encoding actinic cytoskeleton proteins, such as tubulin and neurofilaments. Reducing age-related IGF and disease reduces the neurotrophic protection of IGF, which can be a pathogenic cause of progressive neurodegenerative disorders in diabetes [23], [24]. According to Jamali et al. and Huang et al., suppression of IGF1 signaling can induce apoptosis both *in vivo* and *in vitro*. The level of IGF1 in rats with streptozotocin-induced diabetes decreases [25], [26]. In the study of Zhuang et al., the mRNA of the IGF gene decreased in the neurons, liver, and nerve cord [23]. According to their results, the level of IGF2 gene in cerebrospinal and spinal cord tissue and neural tissue of diabetic rats had a significant reduction in non-diabetic control mice, and also the level of mRNA of the IGF1 gene in the spinal cord and nerves of diabetic rats vs. non-diabetic rats. Treatment with IGF can partly maintain the level of mRNA related to IGF1 and IGF2 in the nerve cord of diabetic rats that is dependent on insulin. The amount of mRNA related to IGF1 in the liver of diabetic rats that is dependent or independent on insulin has decreased. It is estimated that daily production of IGF1 in the liver provides sufficient blood levels of IGF1. The blood level of IGF1 decreases in insulin-dependent diabetic rats. Treatment with IGF can partly maintain the amount of mRNA related to IGF1 as well as its blood levels. The level of IGF2 is naturally low in adult rats, and this may be due down-regulation of IGF2 gene expression in the liver during evolution [23]. IGF1 enhances the phosphorylation of the pro-apoptosis factor and increases the level of Bcl-2 anti-apoptotic protein via the phosphatidylinositol 3 (PI3) kinase pathway in brain cells [27]. In previous studies, the authors examined the mechanisms involved in type 1 diabetes and its effects on apoptosis of hippocampal neurons. Their results indicated that cognitive impairment in diabetic rats was not detectable early in the first 2 months of life and hippocampal lesions could be detected within 8 months [28], [29]. These lesions of the hippocampus had cellular DNA fragmentation, TUNEL staining positivity, increased Bax/Bcl-x ratio, increased activity of caspase-3, and decreased neuronal density in the hippocampus of diabetic rats. Insulin and IGF1 have anti-apoptotic effects. Insulin produces its anti-apoptotic effects through the Raf-I messaging mechanism, which activates NF-κB via the PI3 kinase pathway [30]. In addition, Stern et al., in their recent work, reported that IGF2 promotes memory enhancement [31]. They tested the effect of intracerebral injections of IGF1, IGF2, or insulin on memory consolidation and persistence in rats. They found that a bilateral injection of insulin into the dorsal hippocampus transiently enhances hippocampal-dependent memory, whereas an injection of IGF1 has no effect. Stern et al. concluded that insulin can improve memory via a mechanism that involves IGF2. They used a pH-responsive nanoparticle system for the oral delivery of insulin, which includes chitosan and poly-γ-glutamic acid [14], [32]. Polysaccharide chitosan is a non-toxic soft-tissue-compatible cationic polymer that is able to attach to the mucosal surface and open the tight junctions between contiguous epithelial cells.

Due to these features, chitosan nanoparticles are a suitable candidate for drug delivery compared with conventional tablets and powder formulations. After permeating through the opened paracellular pathway, insulin is carried into the systemic circulation [32].

## Conclusions

Taken together, the results of the present study showed the ability of nano-insulin to increase the expression of IGF1 and IGF2 in rats with streptozotocin-induced diabetes. In addition, according to our previous study, we found significant correlations between IGF1/IGF2 and weight, glucose, protein of IGF1, protein of IGF2, and apoptosis percentage. This result focuses on the premise that chitosan nanoparticles can adhere to and infiltrate the mucus layer of the small intestine. Subsequently, the infiltrated chitosan nanoparticles transiently open the tight junctions between epithelial cells. Because they are pH sensitive, the nanoparticles become less stable and disintegrate, releasing the loaded insulin, which enters the bloodstream. Our results also indicate that it could be a suitable and effective alternative to injectable insulin for the treatment of diabetes mellitus type 1. However, further research is still required to achieve this outcome.

### Acknowledgments

The authors would like to thank the animal house of Hamadan University of Medical Sciences for supporting this project.

**Research funding:** The present study was funded by Vice Chancellor for Research and Technology, Hamadan University of Medical Sciences.

**Competing Interests:** The authors declare that there are no conflicts of interest.

**Author contributions:** RA and NZ designed the project. GK and MJ conducted the experiments. MS and SSA analyzed the data. RM and MTG assisted analyzing of experimental data. RA and GK wrote the manuscript. All authors read and accepted the manuscript.

**Ethical approval:** Research involving animals complied with all relevant national regulations and institutional policies (Hamadan University of Medical Sciences, Iran) for the care and use of animals. (IR.UMSHA.REC.1394.104).

## References

[1] Bastaki A. Review diabetes mellitus and its treatment. Int J Diabetes Metab 2005;13:111–34.
[2] Kaparissides C, Alexandridou S, Kotti K, Chaitidou S. Recent advances in novel drug delivery systems. J Mater Online 2006;2:1–11.
[3] Alai MS, Lin WJ, Pingale SS. Application of polymeric nanoparticles and micelles in insulin oral delivery. J Food Drug Anal 2015;23:351–8.
[4] Wong CY, Al-Salami H, Dass CR. Potential of insulin nanoparticle formulations for oral delivery and diabetes treatment. J Control Release 2017;264:247–75.
[5] Calzoni E, Cesaretti A, Polchi A, Di Michele A, Tancini B, Emiliani C. Biocompatible polymer nanoparticles for drug delivery applications in cancer and neurodegenerative disorder therapies. J Funct Biomater Rev 2019;10:4.
[6] Reagan LP, Magarinos AM, McEwen BS. Neurological changes induced by stress in streptozotocin diabetic rats. Ann N Y Acad Sci 1999;893:126–37.
[7] Sima AAF, Li Z-g, Zhang W. The insulin-like growth factor system and neurological complications in diabetes. Exp Diabetes Res 2003;4:235–56.
[8] Cheng CM, Tseng V, Wang J, Wang D, Matyakhina L, Bondy CA. Tau is hyperphosphorylated in the insulin-like growth factor-I null brain. Endocrinology 2005;146:5086–91.
[9] Crowder RJ, Freeman RS. Glycogen synthase kinase-3β activity is critical for neuronal death caused by inhibiting phosphatidylinositol 3-kinase or Akt but not for death caused by nerve growth factor withdrawal. J Biol Chem 2000;275:34266–71.
[10] Hetman M, Cavanaugh JE, Kimelman D, Xia Z. Role of glycogen synthase kinase-3β in neuronal apoptosis induced by trophic withdrawal. J Neurosci 2000;20:256–74.
[11] Li M, Wang X, Meintzer MK, Laessig T, Birnbaum MJ, Heidenreich KA. Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3β. Mol Cell Biol 2000;20:9356–63.
[12] Tong N, Sanchez JF, Maggirwar SB, Ramirez SH, Guo H, Dewhurst S, et al. Activation of glycogen synthase kinase 3β (GSK-3β) by platelet activating factor mediates migration and cell death in cerebellar granule neurons. Eur J Neurosci 2001;13:1913–22.
[13] Janssen JA, Lamberts SW. Circulating IGF-I and its protective role in the pathogenesis of diabetic angiopathy. Clin Endocrinol (Oxf) 2000;52:1–9.
[14] Hu Q, Luo YC. Recent advances of polysaccharide-based nanoparticles for oral insulin delivery. Int J Biol Macromol 2018;120:775–82.
[15] Muzzarelli RA. Chitin. Oxford, UK: Pergamon Press, 1977.
[16] Muzzarelli RA. Chitosan chemistry: relevance to the biomedical sciences. Polysaccharides 2005;186:151–209.

[17] Gan Q, Wang T. Chitosan nanoparticle as protein delivery carrier-systematic examination of fabrication conditions for efficient loading and release. Colloids Surf B Biointerfaces 2007;59:24–34.
[18] Mahjub R, Dorkoosh FA, Amini M, Khoshayand MR, Rafiee-Tehrani M. Preparation, statistical optimization, and in vitro characterization of insulin nanoparticles composed of quaternized aromatic derivatives of chitosan. AAPS PharmSciTech 2011;12:1407–19.
[19] Ma Z, Lim TM, Lim LY. Pharmacological activity of peroral chitosan-insulin nanoparticles in diabetic rats. Int J Pharm 2005;293:271–80.
[20] Sonaje K, Lin YH, Juang JH, Wey SP, Chen CT, Sung HW. In vivo evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery. Biomaterials 2009;30:2329–39.
[21] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔC(T)) method. Methods 2001;25:402–8.
[22] Kalantarian G, Ziamajidi N, Mahjub R, Goodarzi MT, Saidijam M, Soleimani Asl S, et al. Effect of insulin-loaded trimethyl chitosan nanoparticles on IGF-1, IGF-2, and apoptosis in the hippocampus of diabetic male rats. Restor Neurol Neurosci 2018;36:571–81.
[23] Zhuang HX, Wuarin L, Fei ZJ, Ishii DN. Insulin-like growth factor (IGF) gene expression is reduced in neural tissues and liver from rats with non-insulin-dependent diabetes mellitus, and IGF treatment ameliorates diabetic neuropathy. J Pharmacol Exp Ther 1997;283:366–74.
[24] Song W, Jiang W, Wang C, Xie J, Liang X, Sun Y, et al. Jinmaitong, a traditional Chinese compound prescription, ameliorates the streptozotocin-induced diabetic peripheral neuropathy rats by increasing sciatic nerve IGF-1 and IGF-1R expression. Front Pharmacol 2019;10:255.
[25] Jamali R, Bao M, Arnqvist H. IGF-I but not insulin inhibits apoptosis at a low concentration in vascular smooth muscle cells. J Endocrinol 2003;179:267–74.
[26] Kangmao H, Yanyan C, Rui Z, Yizheng W, Yan M, Xiangqian F, et al. Honokiol induces apoptosis and autophagy via the ROS/ERK1/2 signaling pathway in human osteosarcoma cells in vitro and in vivo. Cell Death Dis 2018;9:157.
[27] Fernandez M, Sanchez-Franco F, Palacios N, Sanchez I, Fernandez C, Cacicedo L. IGF-I inhibits apoptosis through the activation of the phosphatidylinositol 3-kinase/Akt pathway in pituitary cells. J Mol Endocrinol 2004;33:155–60.
[28] Li ZG, Zhang W, Grunberger G, Sima AA. Hippocampal neuronal apoptosis in type 1 diabetes. Brain Res. 2002;946:221–31.
[29] Gómez RL, Edgin JO. The extended trajectory of hippocampal development: implications for early memory development and disorder. Dev Cognit Neurosci 2016;18:57–69.
[30] Hui L, Pei DS, Zhang QG, Guan QH, Zhang GY. The neuroprotection of insulin on ischemic brain injury in rat hippocampus through negative regulation of JNK signaling pathway by PI3K/Akt activation. Brain Res 2005;1052:1–9.
[31] Stern SA, Chen DY, Alberini CM. The effect of insulin and insulin-like growth factors on hippocampus- and amygdala-dependent long-term memory formation. Learn Memory 2014;21:556–63.
[32] Sung HW, Sonaje K, Liao Z-X, Hsu LW, Chuang E-Y. pH-responsive nanoparticles shelled with chitosan for oral delivery of insulin: from mechanism to therapeutic applications. Acc Chem Res 2012;45:619–29.