Effect of sericin on diabetic hippocampal growth hormone/insulin-like growth factor 1 axis

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Abstract
Previous studies have shown that sericin extracted from silk cocoon significantly reduces blood glucose levels and protects the nervous system against diabetes mellitus. In this study, a rat type 2 diabetes mellitus model was established by intraperitoneal injection of 25 mg/kg streptozotocin for 3 successive days, following which the rats were treated with sericin for 35 days. After treatment, the blood glucose levels of the diabetic rats decreased significantly, the growth hormone level in serum and its expression in the hippocampus decreased significantly, while the insulin-like growth factor-1 level in serum and insulin-like growth factor-1 and growth hormone receptor expression in the hippocampus increased significantly. The experimental findings indicate that sericin improves disorders of the growth hormone/insulin-like growth factor 1 axis to alleviate hippocampal damage in diabetic rats.

Key Words
neural regeneration; traditional Chinese medicine; sericin; type 2 diabetes mellitus; hippocampus; growth hormone; insulin-like growth factor 1; growth hormone receptor; growth hormone/insulin-like growth factor 1 axis; streptozotocin; blood glucose; western blot assay; reverse transcription-PCR; grants-supported paper; neuroregeneration

Research Highlights
(1) The growth hormone/insulin-like growth factor 1 axis is an important anabolic-conditioning system that plays a critical role in the growth and development of the central nervous system.
(2) Enzyme-linked immunosorbent assay, western blot and reverse transcription-PCR were used to demonstrate the role of sericin in regulating the expression of hippocampal growth hormone/insulin-like growth factor 1 in diabetic rats.
(3) Sericin can alleviate diabetic hippocampal damage through improving disorders in the growth hormone/insulin-like growth factor 1.

INTRODUCTION
Diabetes mellitus is an endocrine and metabolic disease caused by absolute or relative insulin deficiency. Diabetes mellitus is characterized by hyperglycemia and highly prevalent complications caused by the low function of insulin secreting cells and inefficient insulin work capacity [1]. With the progress of society and the improvement of living standards, the incidence of diabetes mellitus is increasing yearly. Diabetes mellitus is regarded as equal to cardiovascular disease and malignant tumors as the most serious health risk, showing a high morbidity and mortality [2-3]. Type 2 diabetes mellitus is dominant and is characterized by elevated blood glucose, insulin resistance and impaired islet β-cell function [4-5]. In the...
treatment of type 2 diabetes mellitus, it is crucial to reduce blood glucose levels and promote insulin secretion from β-cells.

The existing treatments for diabetes mellitus include drugs, which constitute the main treatment, insulin injection, cell transplantation and gene therapy. Several drugs are available to regulate blood glucose, delay disease progression and reduce complications, including sulfonylureas, biguanides, α-glucosidase inhibitor, and insulin sensitizers. However, these drugs still produce side effects to varying degrees and fail to improve insulin resistance and protect islet cells, while long-term application leads to adverse reactions and drug resistance[6-8]. Although injection of exogenous insulin is a commonly used method to regulate blood glucose in the later stages of diabetes mellitus, the insulin injection is slowly absorbed and requires daily administration, leading to insufficient feedback regulation of blood glucose. Furthermore, the treatment effect is not satisfactory and is inconvenient to the patients[9-10]. Pancreas transplantation and islet transplantation have been applied for the treatment of diabetes mellitus (mainly type 1), but did not achieve satisfactory effects because of the shortage of transplant donors and transplant rejection[11]. The rapid development of recombinant DNA and transgenic technologies, particularly diabetes mellitus-related gene cloning and viral vector-based gene transfer technology, has enabled great progress to be made in gene therapy for diabetes mellitus, mainly focusing on type 1 diabetes mellitus[12]. Gene therapy is a commonly used treatment targeting type 1 diabetes mellitus; however, it is problematic because of the lack of an effective insulin gene transfer system, auto-immune destruction of the target cells, persistent gene expression, and the long-term impact on the body[13-14]. Therefore, increasing attention is being paid to the screening of Chinese herbal medicines for hypoglycemic natural substances with good absorption efficacy and an absence of toxic side effects.

Diabetes mellitus is regarded as wasting-thirst disease, and the treatment principles are based on the production of sperm, clearing heat, nourishing yin and moistening dryness. Chinese ancient medical masters have accumulated rich experience in the prevention and treatment of diabetes mellitus, which has been frequently documented in classic medical books including Synopsis of Golden Chamber, Medical Secrets of An Official, Thousand Golden Prescriptions and Internal Doctrine of the Yellow Emperor. Currently, treatment of diabetes mellitus with Chinese herbal medicine is based on the differentiation of symptoms and signs, and can be regulated according to the yin and yang in blood, cold-heat and deficiency-excess, and internal and external toxins. However, these herbal therapies do not meet clinical needs.

The cocoon shell of the silkworm, Bombyx mori, is sweet, warm in nature and non-toxic, and it can nourish yin, moisturize dryness, enhance sperm production, quench thirst and promote granulation. It can also be used to treat polydipsia, kidney consumption and cloudy urine, as well as polyphagia and emaciation[15-16]. A piece of floss is bonded by two monofilament fibers comprising central silk fibroin and peripheral sericin. The silk industry originated in China and has developed for thousands of years. The majority of silk applications involve silk fibroin, which is used for clothing, while sericin, which accounts for 30% floss, is discarded. Increasing attention has been paid to the effective use of a large number of high-quality proteins, and sericin possesses many advantages for beauty, skin care, nutrition, anti-oxidation, and anti-cancer treatment[17-21]. The silk cocoon soaked in water is a prescription for regulating blood glucose levels, and sericin can effectively protect islet cells, gonads and kidney[22-27]. Preliminary studies by our research group have shown that sericin might improve aberrant Akt signaling, decrease heme oxygenase-1 expression in the hippocampus.
and cerebral cortex, and reduce the apoptosis of hippocampal neurons in diabetic rats, thus protecting the nervous system.[28-29]

The growth hormone/insulin-like growth factor 1 axis is an important anabolic-conditioning system that plays a critical role in the growth and development of the central nervous system. The hippocampal expression of insulin-like growth factor 1 can promote synaptic regeneration, neuroprotection, myelin formation, neuroregeneration and dendritic branch formation after hippocampal injury.[30-31] The growth hormone/insulin-like growth factor 1 axis undergoes abnormal changes in diabetes mellitus, which aggravate the disease progression and trigger complications.[32]

To improve diabetes mellitus and its chronic complications, improvement of growth hormone/insulin-like growth factor 1 axis anomalies is one of the treatment strategies. The present study aimed to explore the protective effect of the hippocampal growth hormone/insulin-like growth factor 1 axis in type 2 diabetic rats.

RESULTS

Quantitative analysis of experimental animals

Thirty Sprague-Dawley rats were used, 10 of which were randomly assigned to a control group that received no treatment. The remaining 20 rats were used to establish a model of type 2 diabetes mellitus, and then randomly divided into a model group and a sericin group. The model group received no treatment and the sericin group was given sericin via intragastric administration. All rats entered the final analysis without any losses.

Sericin significantly reduced blood glucose levels in diabetic rats

A glucose oxidase test showed that blood glucose levels in the diabetic model rats were significantly increased compared with the control group (P < 0.01). After sericin administration for 35 days, the blood glucose levels in the diabetic rats decreased significantly (P < 0.01; Table 1).

Effect of sericin on serum growth hormone and insulin-like growth factor 1 levels in diabetic rats

Enzyme-linked immunosorbent assay showed that the levels of serum growth hormone were significantly increased in diabetic rats, while the levels of insulin-like growth factor 1 were significantly decreased compared with the control group (P < 0.01). After sericin administration for 35 days, the serum growth hormone levels were significantly lower, and the insulin-like growth factor-1 levels were significantly higher in the diabetic rats (P < 0.01; Table 1).

Effect of sericin on blood glucose (mmol/L), serum growth hormone (ng/mL) and insulin-like growth factor 1 (ng/mL) levels in diabetic rats

Table 1 Effects of sericin on blood glucose (mmol/L), serum growth hormone (ng/mL) and insulin-like growth factor 1 (ng/mL) levels in diabetic rats

| Group     | Blood glucose | Growth hormone | Insulin-like growth factor 1 |
|-----------|---------------|----------------|------------------------------|
| Control   | 11.12±2.22    | 1.36±0.53      | 1.124.75±186.37              |
| Model     | 29.00±5.39*   | 2.56±1.13*     | 520.20±121.81*               |
| Sericin   | 14.03±3.98b   | 1.36±0.57b     | 981.10±180.88b               |
| β-actin   | 43 kDa        | 43 kDa         | 43 kDa                       |
| F         | 55.42         | 7.65           | 36.37                        |
| P         | <0.01         | <0.01          | <0.01                        |

Data are expressed as mean ± SD for 10 rats in each group. Comparisons between groups were tested by one-way analysis of variance and pairwise comparison was performed using the q test. *P < 0.01, vs. control group; **P < 0.01, vs. model group.

Western blot analysis demonstrated that growth hormone protein expression levels in the hippocampus of diabetic rats were significantly increased, while the growth hormone receptor protein expression levels were significantly decreased compared with the control group (P < 0.01). After sericin administration for 35 days, the hippocampal growth hormone expression levels in diabetic rats were significantly decreased, and the growth hormone receptor protein expression levels were significantly increased (P < 0.01; Figure 1, Table 2).

Effect of sericin on growth hormone and growth hormone receptor protein expression in the hippocampus of diabetic rats

Figure 1 Growth hormone (GH; A) and growth hormone receptor (GHR; B) expression in rat hippocampus (western blot assay).

The growth hormone expression levels were higher and the growth hormone receptor expression levels were lower in the model group than in the control group. In the sericin group, the growth hormone expression levels decreased, while the growth hormone receptor expression levels increased.

1: Control group; 2: model group; 3: sericin group.
Effect of sericin on growth hormone, growth hormone receptor and insulin-like growth factor 1 mRNA expression in the hippocampus of diabetic rats

Reverse transcription-PCR analysis showed that, compared with the control group, hippocampal growth hormone mRNA expression levels in the model group were significantly increased, while the growth hormone receptor and insulin-like growth factor 1 mRNA expression levels were significantly decreased ($P < 0.01$). After sericin administration for 35 days, the growth hormone mRNA expression levels in the rat hippocampus were significantly reduced, while the growth hormone receptor and insulin-like growth factor 1 mRNA expression levels were significantly increased ($P < 0.01$; Figure 2, Table 3).

**DISCUSSION**

The growth hormone/insulin-like growth factor 1 axis is a critical anabolic-conditioning system that plays an important role in tissue differentiation, proliferation and metabolism\[33-34\]. Growth hormone, insulin-like growth factor 1 and its receptor are present in brain tissue, and insulin-like growth factor 1 in the blood may bind to insulin-like growth factor 1 receptor in brain tissue after passing through the blood-brain barrier\[35\]. Brain tissue also secretes a small amount of growth hormone and insulin-like growth factor 1\[36-37\]. In addition to the effect of growth hormone/insulin-like growth factor 1 axis on growth and development, increasing attention has been paid to the regulatory effect of the growth hormone/insulin-like growth factor 1 axis on the central nervous system.

Both type 1 and type 2 diabetes mellitus are characterized by growth hormone/insulin-like growth factor 1 axis abnormalities, which are closely associated with hippocampal damage\[35, 38\]. Sustained hyperglycemia may decrease the hypothalamic regulation of glucose and weaken the hypothalamic inhibition of growth hormone, thus leading to an increase in growth hormone levels\[39\]. The elevated growth hormone levels are also the result of growth hormone resistance, reduced insulin-like growth factor 1 levels and suppressed negative feedback in the pituitary gland\[40\].

### Table 2  Effect of sericin on growth hormone and growth hormone receptor protein expression in the hippocampus of diabetic rats (western blot assay)

| Group   | Growth hormone | Growth hormone receptor |
|---------|----------------|-------------------------|
| Control | 0.157±0.016    | 0.942±0.013             |
| Model   | 0.925±0.029a   | 0.355±0.017a            |
| (Q = 101.620) | (Q = 123.438)    |
| Sericin | 0.565±0.025b   | 0.539±0.015b            |
| (Q = 47.666) | (Q = 38.779)    |
| $F$     | 2.584.90       | 3.984.64                |
| $P$     | <0.01          | <0.01                   |

Expression of the target proteins is represented as the ratio of target band absorbance to β-actin absorbance. Data are expressed as mean ± SD for 10 rats in each group. Comparisons between groups were tested by one-way analysis of variance and pairwise comparison was performed using the q test. $^aP < 0.01$, vs. control group; $^bP < 0.01$, vs. model group.

### Figure 2  Effect of sericin on the mRNA expression of growth hormone (GH; A), growth hormone receptor (GHR; B) and insulin-like growth factor 1 (IGF-1; C) in the hippocampus of diabetic rats.

1: Control group, 2: model group, 3: sericin group. The bands for growth hormone, growth hormone receptor, insulin-like growth factor 1 and β-actin were 368, 394, 280 and 445 bp, respectively.
accompanied by hippocampal neuronal apoptosis, markedly decreased in the brains of diabetic mice and was resistance and reduce growth hormone-mediated insulin-like growth factor 1 levels lead to growth hormone receptor levels and reduced growth hormone function. Decreased function deficits [47] and the anti-apoptotic effects of insulin-like growth factor 1 are reduced in the central nervous system, mRNA expression [43]. Excessive growth hormone inhibits mone binding capacity and growth hormone receptor human growth hormone can upregulate the growth hor- mone and growth hormone receptor [44]. Therefore, gests a regulatory feedback mechanism between growth hormone receptor, which sug- gests the downregulation of growth hormone receptor and growth hormone binding protein; growth hormone receptor is the key functional factor in the growth hormone/insulin-like growth factor 1 axis, and growth hormone receptor gene expression may be influenced by diabetes mellitus [41-42]. Recombinant human growth hormone can upregulate the growth hormone binding capacity and growth hormone receptor mRNA expression [43]. Excessive growth hormone inhibits the expression of growth hormone receptor, which sugg- ests a regulatory feedback mechanism between growth hormone and growth hormone receptor [44]. Therefore, diabetes mellitus is accompanied by elevated growth hormone levels, decreased growth hormone receptor expression, reduced tissue sensitivity to growth hormone, and reduced growth hormone function. Decreased growth hormone receptor levels lead to growth hormone resistance and reduce growth hormone-mediated insulin-like growth factor 1 levels [45-46]. Insulin-like growth factor 1 levels are also decreased in diabetes mellitus, and accordingly growth hormone function is attenuated [47] and the anti-apoptotic effects of insulin-like growth factor 1 are reduced in the central nervous system, leading to hippocampal neuronal apoptosis and cognitive function deficits [48-51]. Insulin-like growth hormone system abnormalities are the key factors contributing to hippocampal neuronal apoptosis, and may be involved in the development of cognitive dysfunction [52]. Li et al. [53] found that insulin-like growth factor 1 expression was significantly decreased in the brains of diabetic mice and was accompanied by hippocampal neuronal apoptosis, re-

gardless of whether the disease duration was 2 or 8 months.

Sericin was shown to significantly reduce serum growth hormone levels, downregulate hippocampal growth hormone expression, increase serum insulin-like growth factor 1 levels, and upregulate expression of insulin-like growth factor 1 and growth hormone receptor in the hippocampus of diabetic rats. These results indicate that sericin can improve disorders of the hippocampal growth hormone/insulin-like growth factor 1 axis and may alleviate hippocampal damage.

### MATERIALS AND METHODS

#### Design

A randomized, controlled animal experiment.

#### Time and setting

The experiments were performed from October 2009 to December 2010 at the Institute of Basic Medical Sciences, Chengde Medical College, China.

#### Materials

**Animals**

Thirty healthy, clean, male Sprague-Dawley rats, aged 3 months and weighing 200-250 g, were provided by the Experimental Animal Center of Hebei Medical University (license No. 712024). All animals were reared in a clean animal laboratory at constant temperature (20 ± 2°C) and constant humidity (40-70%). Experimental disposals were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of China [54].

#### Drugs

Silk cocoon was soaked in water, decocted, filtered and concentrated to prepare sericin [26]. Sericin was provided by the Sericultural Institute of Chengde Medical College, and extracted at the Institute of Basic Medical Sciences, Chengde Medical College, China.

#### Methods

**Establishment of type 2 diabetes mellitus models**

Rats in the model group and the sericin group were injected with 2% streptozotocin (Sigma, St. Louis, MO, USA) via intraperitoneal injection (25 mg/kg) for 3 successive days to induce type 2 diabetes mellitus. The success of model induction was defined by fasting plasma glucose ≥ 16.7 mmol/L [55-56]. Rats in the control group were regularly fed with no treatment.

| Group   | Growth hormone | Growth hormone receptor | Insulin-like growth factor 1 |
|---------|----------------|-------------------------|------------------------------|
| Control | 0.179±0.016    | 0.618±0.020             | 1.502±0.028                  |
| Model   | 0.505±0.014    | 0.322±0.012             | 0.544±0.027                  |
| Sericin | 0.314±0.026    | 0.498±0.013             | 1.032±0.022                  |

The mRNA expression levels are represented as the ratio of the target band absorbance to β-actin absorbance. Data are expressed as mean ± SD for 10 rats in each group. Comparisons between groups were tested by one-way analysis of variance and pairwise comparison was performed using the q test. *P < 0.01, vs. control group; *P < 0.01, vs. model group.

The elevated growth hormone levels make blood glucose levels difficult to control and influence the occurrence and development of chronic diabetic complications. Tissue sensitivity to growth hormone is associated with growth hormone receptor and growth hormone binding protein; growth hormone receptor is the key functional factor in the growth hormone/insulin-like growth factor 1 axis, and growth hormone receptor gene expression may be influenced by diabetes mellitus [41-42]. Recombinant human growth hormone can upregulate the growth hormone binding capacity and growth hormone receptor mRNA expression [43]. Excessive growth hormone inhibits the expression of growth hormone receptor, which suggests a regulatory feedback mechanism between growth hormone and growth hormone receptor [44]. Therefore, diabetes mellitus is accompanied by elevated growth hormone levels, decreased growth hormone receptor expression, reduced tissue sensitivity to growth hormone, and reduced growth hormone function. Decreased growth hormone receptor levels lead to growth hormone resistance and reduce growth hormone-mediated insulin-like growth factor 1 levels [45-46]. Insulin-like growth factor 1 levels are also decreased in diabetes mellitus, and accordingly growth hormone function is attenuated [47] and the anti-apoptotic effects of insulin-like growth factor 1 are reduced in the central nervous system, leading to hippocampal neuronal apoptosis and cognitive function deficits [48-51]. Insulin-like growth hormone system abnormalities are the key factors contributing to hippocampal neuronal apoptosis, and may be involved in the development of cognitive dysfunction [52]. Li et al. [53] found that insulin-like growth factor 1 expression was significantly decreased in the brains of diabetic mice and was accompanied by hippocampal neuronal apoptosis, re-
**Sericin intervention**
Immediately after model establishment, rats in the sericin group received intragastric administration of sericin (2.4 g/kg) once a day for 35 days[27].

**Blood samples**
Rats were fasted for 12 hours after drug administration and then anesthetized by intraperitoneal injection of 4% chloral hydrate. A 3 mL volume of inner canthus blood was collected and centrifuged for 20 minutes at 3 000 r/min, and the serum was collected and stored at -20°C.

**Determination of blood glucose levels using the glucose oxidase test**
Blood glucose levels were determined using a Boehringer Mannheim in/Hitachi 717 automatic clinical biochemistry analyzer (Hitachi, Tokyo, Japan) and a glucose detection kit (batch No. 20071030, Baoding Great Wall Clinical Reagent Co., Ltd., Baoding, Hebei Province, China)[58-59].

**Analysis of serum growth hormone and insulin-like growth factor 1 levels by enzyme-linked immunosorbent assay**
The serum growth hormone and insulin-like growth factor 1 levels were determined, according to the instructions of assay kits for growth hormone (Rb, West Hills, CA, USA) and insulin-like growth factor 1 (Wuhan Boster Biotechnology Co., Ltd., Wuhan, Hubei Province, China), and the plates were read on a Multiskan MK3 Micro-plate reader (Thermo, Waltham, MA, USA)[60-61]. Samples (10 μL) were diluted with sample diluent (40 μL), and blank control wells without reagent or samples were also included. The plates were incubated at 37°C for 30 minutes, rinsed five times for 30 seconds each, and further incubated with 50 μL of reagent (except for blank control wells) and washed. The samples were developed with 50 μL of chromogenic agents A and B at 37°C in the dark for 15 minutes, and the reactions were terminated with termination solution. The absorbance value was read at a wavelength of 450 nm.

**Hippocampal tissue**
Rats were decapitated and the brains were rapidly harvested, from which the hippocampi were isolated and stored in liquid nitrogen[27, 62].

**Analysis of hippocampal growth hormone and growth hormone receptor protein expression by western blot assay**
Total hippocampal protein was extracted and the protein concentration was determined using the BCA protein assay kit (Beijing Taigemei Science and Technology Co., Ltd., Beijing, China). The proteins were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with mouse anti-rat growth hormone monoclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-rat growth hormone receptor polyclonal antibody (1:200; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) and mouse anti-rat β-actin monoclonal antibody (1:1 000; Santa Cruz Biotechnology) at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:5 000; KPL Corp., Gaithersburg, MD, USA) at room temperature for 1 hour, and bound antibodies were detected with Super ECL Plus ultra-sensitive luminescent solution (Beijing Taigemei). The films were scanned with an EPSON scanner (Epson China Co., Ltd., Beijing, China) and the developing strip was analyzed using Quantity One-4.6.2 software (BIO-RAD, Hercules, CA, USA). The relative expression levels of target protein were represented as the ratio of target band absorbance to β-actin absorbance[63-64].

**Analysis of hippocampal growth hormone, growth hormone receptor and insulin-like growth factor-1 mRNA expression by reverse transcription-PCR**
Total hippocampal RNA was extracted with Trizol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA. PCR primers were synthesized by Sangonitech (Shanghai) Co., Ltd., Shanghai, China.

The PCR primer sequences are as follows:

| Gene                          | Primer sequence                  | Tm (°C) | Cycle | Product length (bp) |
|------------------------------|----------------------------------|---------|-------|---------------------|
| Growth hormone               | F: 5′-TGA CAC CTA CAAGAAGT CGA AGC-3′   | 65      | 33    | 368                 |
|                             | R: 5′-TGT TGG CGT CAA ACT TGT CAT AGG-3′   |         |       |                     |
| Growth hormone receptor      | F: 5′-CTG GGT GTA GTT CAT TGA GCT | 62      | 31    | 394                 |
|                             | R: 5′-TGT AGG GAG GAG TTG GTG GGT TGA C-3′ |         |       |                     |
| Insulin-like growth factor 1 | F: 5′-CTG GTG GAC GCT CTT CAG TTC | 59      | 31    | 280                 |
|                             | G-3′                              |         |       |                     |
| β-actin                      | F: 5′-GAG GGA AAC CGT GCG TGA C-3′   | 55      | 29    | 445                 |
|                             | R: 5′-CTG GAA GGT GGA CAG TGA G-3′   |         |       |                     |

Tm: Temperature; F: forward primer; R: reverse primer.
The PCR conditions were: 94°C for 2 minutes, 94°C for 30 seconds, 50-65°C for 30 seconds, and 72°C for 1 minute. Amplification products (5 μL) and DNA Ladder (5 μL; Beijing Taigemeng) were subjected to 2% agarose gel electrophoresis (90 V, 40 minutes) and visualized using a ZF UV transmittance and reflection analyzer (Jiapeng Technology Co., Ltd., Shanghai, China). Quantitative analysis was performed using Quantity One-4.6.2 software, and the relative mRNA expression levels were represented as the ratio of target band absorbance to β-actin absorbance [24, 65-66].

Statistical analysis
Data were analyzed using SPSS 15.0 software (SPSS, Chicago, IL, USA) and are expressed as mean ± SD. Differences between groups were compared using one-way analysis of variance, and pairwise comparisons were performed using the q test. A P value < 0.05 was regarded as statistically significant.

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