Original Paper

Gentiopicroside Ameliorates Diabetic Peripheral Neuropathy by Modulating PPAR- γ /AMPK/ACC Signaling Pathway

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Key Words

Diabetic peripheral neuropathy (DPN) • Gentiopicroside • PPAR- γ • AMPK

Abstract:

Background/Aims: Gentiopicroside is promising as an important secoiridoid compound against pain. The present study aimed to investigate the analgesic effect and the probable mechanism of Gentiopicroside on Diabetic Peripheral Neuropathy (DPN), and to figure out the association among Gentiopicroside, dyslipidemia and PPAR- γ /AMPK/ACC signaling pathway.

Methods: DPN rat models were established by streptozotocin and RSC96 cells were cultured. Hot, cold and mechanical tactile allodynia were conducted. Blood lipids, nerve blood flow, Motor Nerve Conduction Velocity (MNCV) and Sensory Nerve Conduction Velocity (SNCV) were detected. Gene and protein expression of PPAR- γ /AMPK/ACC pathway was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Westernblot. Besides, PPAR- γ antagonist GW9662 and agonist rosiglitazone, AMPK antagonist compound C and activator AICAR as well as ACC inhibitor TOFA were used to further confirm the relationship between PPAR- γ and AMPK. **Results:** The results demonstrated that Gentiopicroside markedly ameliorated hyperalgesia with prolonged paw withdrawal latency to heat and cold stimuli and fewer responses to mechanical allodynia compared with DPN model group. Gentiopicroside regulated dyslipidemia, enhanced nerve blood flow and improved MNCV as well as SNCV. Gentiopicroside suppressed ACC expression through the activation of AMPK and PPAR- γ mediated the activation of AMPK and subsequent inhibition of ACC expression. **Conclusion:** In conclusion, the present study demonstrated that Gentiopicroside exerted nerve-protective effect and attenuated experimental DPN by restoring dyslipidemia and improved nerve blood flow through regulating PPAR- γ /AMPK/ACC signal pathway. These results provided a promising potential treatment of DPN.

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Introduction

Diabetic peripheral neuropathy (DPN) is characterized by pain, paraesthesia, sensory loss and affects up to 50% of diabetes patients with considerable morbidity, mortality and diminished quality of life [1]. Moreover, DPN is also associated with substantial morbidity including depression, anxiety, sleep disturbances, susceptibility to foot or ankle fractures, ulceration and lower-limb amputations [2, 3].

The pathogenesis of DPN remains controversial. Chronic hyperglycemia is widely acknowledged as the major culprit in the initiation and maintenance process of DPN for years. Recently, increased evidences have shown that dyslipidemia, characterized by elevated plasma levels of total cholesterol (TC), low density lipoprotein cholesterol (LDL) and triglycerides (TG) and low level of high density lipoprotein cholesterol (HDL), plays an important role in pathogenesis of DPN [4, 5]. Dyslipidemia is suspected to be a cause in manifestation and development of premature atherosclerosis leading to decreased blood flow which impairs nerve perfusion and further to nerve dysfunction [6]. However, the specific mechanisms leading to dyslipidemia remains unclear.

Peroxisome proliferator activated receptors- γ (PPAR- γ), a ligand-dependent nuclear receptor that has vital roles in adipogenesis and glucose metabolism, is highly expressed in nerve tissue [7]. AMP-activated protein kinase (AMPK) stimulates the β -oxidation of fatty acids for lipid utilization by inhibiting the activity of acetyl-CoA carboxylase (ACC) by phosphorylation [8, 9]. Both PPAR- γ and AMPK are involved in the maintenance of lipid and cholesterol homeostasis. Nevertheless, the link between PPAR- γ and AMPK in regulating lipid metabolism has not been thoroughly studied.

Nowadays, effective treatments for DPN are limited. Traditional drugs such as antidepressants, anticonvulsants, antispasmodic or combinations of these drugs are based on tight glucose controls. However, various side effects and low analgesic efficacy have limited the utilization of medications mentioned above. Approximately 2/3 cases of diabetes have intractable neuralgia and are even forced to choose denervation treatment [10, 11]. Therefore, clinicians have never stop seeking other effective multimodal therapies for DPN, among which traditional Chinese medicine catches our attentions for achievements it has already gained in the field of analgesia.

Gentiopicroside is a secoiridoid compound isolated from *Gentiana lutea* which is called *Qin Jiao* in Chinese. It is one of the most common herbal medicines in China [12]. Animal experiments have revealed its pharmacological effects such as choleric, antihepatotoxic, adaptogenic and anti-inflammatory activities [12-14]. Recently, Gentiopicroside is found to exert on the central nervous system with analgesic effect [15]. Lei Chen et al. reported that Gentiopicroside significant inhibited inflammation-related allodynia and decreased NR2B receptors expression in the anterior cingulate cortex [16]. It was considered to be one of the various mechanisms of Gentiopicroside exerting analgesic effects. Furthermore, in the previous research we conducted on DPN rat models, Gentiopicroside was found to effectively regulate dyslipidemia [17], which we suspected to be one of the important mechanisms of Gentiopicroside in the treatment of DPN. The current research aimed to further investigate the analgesic effect and the probable mechanism of Gentiopicroside on DPN, and to figure out the association among Gentiopicroside, dyslipidemia and PPAR- γ /AMPK/ACC signaling pathway through a series of experiments *in vivo* and *in vitro*.

Materials and Methods

DPN rat models establishment

The animal experiments were performed in conformity with NIH guidelines [18], ARRIVE (Animal Research: Reporting *In vivo* Experiments) guidelines [19] and were approved by the Animal Care and Use Committee of the Sun Yat-Sen University (No. 201616778). Fifty-six specific pathogen free female Sprague Dawley rats (6-week-old, weighing from 200g to 220g) were obtained from the Animal Experiment Center

of the Sun Yat-Sen University. Animals were maintained on a 12:12-h artificial light-dark cycle and housed in 8 cages with 7 rats each. DPN rat models were induced by single intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, MO, USA) at the dose of 55mg/kg. Rats with random blood glucose level >16.7 mmol/L after 24h were considered as diabetic rats and randomly divided into 4 groups. Rats intraperitoneally injected the same volume/kg saline and with random blood glucose level <7 mmol/L after 24h were taken as control group and forward divided into 3 groups.

Grouping

Rats were randomly divided into seven groups of eight rats each: control group, Gent control group (intragastric Gentiopicroside, 50mg/kg·d), Oxcarbazepine control group (intragastric oxcarbazepine, 25mg/kg·d), DPN model group (intragastric distilled water + STZ), Gent low dose model group (intragastric Gentiopicroside, 50mg/kg·d + STZ), Gent high dose model group (intragastric Gentiopicroside, 100mg/kg·d + STZ) and Oxcarbazepine model group (intragastric oxcarbazepine, 25mg/kg·d + STZ). Gentiopicroside (Nanjing Jingzhu Biological Science and Technology Co., Ltd., Nanjing, Jiangsu, China) and oxcarbazepine (Novartis pharmaceutical co., Ltd. Switzerland) were intragastrically given every day from the very beginning of the protocol till the end (from day 1 to day 14). We calculated optimal dose for rats according to the following formulas: Dose for rats = (Xmg/kg × 60kg × 0.018) / 0.2kg = 6.3 Xmg/kg (X: the effective dose for man; 60kg: the standard weight for man; 0.018: ratio of equivalent dose between man and rats based on body surface area; 0.2kg: the standard weight for rat) [20].

Hot plate test

Rats were placed in a box with thermal radiation source at room temperature. Paw withdrawal latency (PWL) were recorded after heat stimulation. Tests were repeated three times with 5 minutes interval and each stimulation process lasted for 30 seconds according to the procedure described by Hargreaves [21] on day 1, 7 and 14 after DPN models established.

Cold allodynia

Rats were placed on cold stimulation instruments (Panlab Haard LE - 7420, Spain) at 4°C and PWL were recorded according to the procedure described by Sayyed [22] on day 1, 7 and 14 after DPN models were established.

Mechanical tactile allodynia

Rats were placed in a cage with an iron wire net. Von Frey filament (Semmes-Weinstein Monofilaments A 835, Sammons Preston, USA) was used to stimulate rat legs and the positive responses were recorded within 10 mechanical stimuli according to the procedure described by Detloff [23] on day 1, 7 and 14 after DPN models were established.

Blood lipid detection

At the end of the experiment (day 14), rats were anesthetized and blood samples were collected in tubes by cardiac puncture. Serum contents of total cholesterol (TC), triglycerides (TGs), low density lipoprotein (LDL) and high density lipoprotein (HDL) were determined using the Olympus AU400 Clinical Chemistry analyzer (Olympus Corporation, Tokyo, Japan).

Nerve blood flow detection

At the end of the experiment (day 14), rats were anesthetized and sciatic nerves were exposed for nerve blood flow detection using a laser Doppler flowmetry system (PeriFlux System 5000, Perimed AB, Sweden) according to procedure described by Naruse [24]. The blood flow was reported as arbitrary perfusion units.

Moter nerve conduction velocity (MNCV) determination

At the end of the experiment (day 14), rats were anesthetized and stimulation was given from the sciatic notch and the knee. Compound muscle action potentials were recorded. MNCV could be calculated by the following formula: MNCV (m/s) = distance between both sides of stimulation (cm) × 10 / differences between proximal and distal latency (ms) [25].

Sensory nerve conduction velocity (SNCV) determination

SNCV was measured at the end of the experiment using the Neuropack Σ orthodromic recorder (Nihon Kohden, Tokyo, Japan). Sensory nerve action potential was recorded. SNCV was calculated by the following formula: SNCV (m/s) = distance between stimulation electrode and recording electrode / latency measured between stimulation and peak amplitude [26].

Reverse transcription-quantitative polymerase chain reaction analysis (RT-qPCR)

Sciatic nerve was isolated, frozen in liquid nitrogen and stored at -80°C for RT-qPCR analysis. Total RNA was extracted from sciatic nerve using TRIzol (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to manufacturer protocol. RT-qPCR was performed in triplicate for each specimen using SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR cycling conditions were as follows: 10 min pre-denaturation at 95°C followed by 35 or 36 cycles of 10 sec denaturation at 95°C and 60 sec annealing at 52°C . Sequences of the primers used and each number of cycles are listed in table 1. The relative levels of the target mRNAs were normalized to the corresponding levels of GAPDH mRNA in the same cDNA sample using a standard curve method recommended in the LightCycler Software version 3.5 (Roche Molecular Diagnostics, Pleasanton, CA, USA).

Culture of Schwann cells (SCs)

RSC96 cells were purchased from ScienCell Research Laboratories and cultured in Dulbecco's Modified Eagle Medium (DMEM, purchased from Herndon, Virginia, USA) containing 1% penicillin/streptomycin solution and 10% fetal bovine serum (FBS, purchased from Herndon, Virginia, USA) in a humidified 5% CO_2 , 37°C incubator. Medium was changed once every 3 days. Rosiglitazone (PPAR γ agonist), GW9662 (PPAR γ -antagonist), compound C (AMPK inhibitor), 5-aminoimidazole-4-carboxamide- β -D-ribofuranoside (AICAR, AMPK activator) and 5-(tetradecyloxy)-2-furoic acid (TOFA, ACC inhibitor) were purchased from Sigma Aldrich (St. Louis, MO, USA) and added at the beginning of the cultures.

Cytotoxicity assay

In order to reveal the protective effect of Gentiopicroside on RSC96 cells, WST-1 was used to measure cell viability. 10 μL of WST-1 reagent (Beijing Bioco Laibo Technology Co. Ltd., Beijing, China) and 90 μL of fresh medium were added to each well, which were then incubated at 37°C for 4h. Optical density of sample bearing wells were measured at 450 nm with a reference wavelength of 640 nm a multi plate reader (Varioskan Flash Multimode Reader, Thermo Scientific, Chicago, IL, USA). Ratio of sample wells to non-treated control cells were reported as cell viability.

Westernblot analysis

Proteins were prepared using the lysis buffer and separated by 8% SDS-PAGE gel and further transferred to PVDF membranes. After blocking with 5% nonfat milk for 2 h, the PVDF membranes were incubated with specific primary antibodies overnight at 4°C . After being washed, membranes were incubated with secondary antibodies for 1h. Detection was visualized using the Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NA, USA).

Table 1. Primers and product size for each target gene

Gene	Primer	Length (bp)	Cycles	Annealing temperature($^{\circ}\text{C}$)
PPAR- γ	Forward: 5'-ATTACGATAGCCGTTCC-3'	190	36	52 $^{\circ}\text{C}$
	Reverse: 5'-TAATGCTATCGGCAATT-3'			
AMPK	Forward: 5'-GTACATCGTAGCGTTCA-3'	189	36	52 $^{\circ}\text{C}$
	Reverse: 5'-CATGTAGCATCGCAAGT-3'			
ACC	Forward: 5'-ACGCTATCATCAGTGCATA-3'	192	36	52 $^{\circ}\text{C}$
	Reverse: 5'-TGCGATAGTAGTCACGTAT-3'			
GAPDH	Forward: 5'-GAATCTGGTGGCTGTGGA-3'	202	35	52 $^{\circ}\text{C}$
	Reverse: 5'-CCCTGAAAGGCTTGGTCT-3'			

Statistical analysis

Data are reported as means $\pm$ standard deviation. Tests for Homoscedasticity and normality distribution were performed. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparisons test. $P < 0.05$ was considered statistically significant different. All statistical analyses were performed using SPSS version 20.0 (IBM Inc., USA). GAPDH was used as an internal control.

Results

Gentiopicroside ameliorated hyperalgesia

DPN rat models were successfully established with fasting glucose level higher than 16.7 mmol/L. All rats survived. Stimulation tests including hot plate test, cold allodynia and mechanical tactile allodynia were conducted to manifest the therapeutic effect of Gentiopicroside on DPN rats. Results in Fig. 1 showed that paw withdrawal latencies to heat and cold stimuli in DPN model group were significantly shorter, and the positive responses to mechanical allodynia were significantly longer than those in control group ($P < 0.05$), which further confirmed the successful establishment of DPN model. Gentiopicroside and the known effective drug oxcarbazepine were given as intervention. There was significantly longer paw withdrawal latency in treatment group after heat and cold allodynia ($P < 0.05$). However, there was no difference in therapeutic effect between Gentiopicroside and oxcarbazepine. 50 mg/kg-d Gentiopicroside seems to be the sufficiently effective dose for DPN rats.

Gentiopicroside regulates dyslipidemia

DPN rat models showed lipid metabolism disorder characterized by elevated levels of TC, TG as well as LDL and decreased level of HDL compared with three control groups ($P < 0.05$). After the intervention of high-dose Gentiopicroside, dyslipidemia was well regulated with increase in HDL levels and decrease in TG, TC as well as LDL levels ($P < 0.05$). Low-dose Gentiopicroside slightly decreased TG only ($P < 0.05$). However, effects of oxcarbazepin on lipid metabolism seemed insignificant in DPN rats (Fig. 2).

Fig. 1. Gentiopicroside alleviates heat, cold and mechanical hyperalgesia in STZ-induced DPN rats ($n=8$ in each group). * $P < 0.05$ versus control group; # $P < 0.05$ versus DPN model group by one-way ANOVA followed by Bonferroni tests.

Gentiopicroside enhanced nerve blood flow

As shown in Fig. 3 panel A, sciatic nerve blood flow was significantly reduced in DPN rats ($P < 0.05$). After Gentiopicroside intervention, sciatic nerve blood flow was significantly enhanced ($P < 0.05$). There seemed a dose-dependent effect of Gentiopicroside on nerve blood flow. However, oxcarbazepin, a known drug for DPN, did not improve the nerve blood flow as shown by our data ($P > 0.05$).

Gentiopicroside improved nerve conduction velocity

There was an obvious fall in Motor Nerve Conduction Velocity (MNCV) and Sensory Nerve Conduction Velocity (SNCV) in DPN rats according to Fig. 3 panel B and C ($P < 0.05$). Gentiopicroside as well as oxcarbazepin showed improvement in raising MNCV and SNCV ($P < 0.05$). There was no statistical difference between different drug group and different dose group.

Gentiopicroside regulated PPAR- γ /AMPK/ACC gene expression

Expression of key genes in PPAR- γ /AMPK/ACC signaling pathway was clearly shown in Fig. 4. DPN rats induced by STZ manifested decreased levels of PPAR- γ as well as AMPK and

Fig. 2. Regulation of dyslipidemia by Gentiopicroside on STZ-induced DPN rats ($n=8$ in each group). * $P < 0.05$ versus control group; # $P < 0.05$ versus DPN model group; & $P < 0.05$ versus oxcarbazepin model group by one-way ANOVA followed by Bonferroni tests.

Fig. 3. Effect of Gentiopicroside on sciatic nerve blood flow and nerve conduction velocity. ($n=8$ in each group). Panel A showed enhancement of Gentiopicroside on nerve blood flow. Panel B and C showed improvement of Gentiopicroside and oxcarbazepin on MNCV and SNCV respectively. * $P < 0.05$ versus control group; # $P < 0.05$ versus DPN model group; & $P < 0.05$ versus oxcarbazepin model group by one-way ANOVA followed by Bonferroni tests.

increased levels of ACC ($P < 0.05$), which suggested an important role of PPAR- γ /AMPK/ACC signaling pathway in pathogenesis of DPN. Both low-dose and high-dose Gentiopicroside group were found to distinctly up-regulated expressions of PPAR- γ as well as AMPK and down-regulated that of ACC ($P < 0.05$). Nevertheless, oxcabazepin has shown no effect on the changes of PPAR- γ /AMPK/ACC signaling pathway expressions. Results above suggested that analgesic effect of Gentiopicroside and oxcabazepin on DPN rats had totally different mechanisms. Regulating PPAR- γ /AMPK/ACC signaling pathway seemed to be the treatment mechanism by Gentiopicroside. Thus, we would further confirm the hypothesis by the following cell experiment. (Fig. 4)

Gentiopicroside protected RSC96 cell

According to the results of WST-1 shown by Fig. 5, Gentiopicroside (3 and 10 μ M) exerted protective effect on RSC96 cell cultured in a high concentration of glucose. The nerve protective effect of Gentiopicroside in cell experiment is consistent with that of animal experiment. Gentiopicroside at the dose of 3 μ M were then used as an intervention to Fig. out its effect on expressions of PPAR- γ /AMPK/ACC signaling pathway.

Gentiopicroside inhibited ACC expression by activating AMPK

Gentiopicroside enhanced expression of AMPK and inhibited that of ACC, which was in accord with results found in animal experiments. In order to Fig. out the further relationship between AMPK and ACC expressions, AMPK antagonist named compound C, activator AICAR and ACC inhibitor TOFA were used. Results showed that the inhibitory effect of Gentiopicroside on ACC expression was weakened by AMPK antagonist compound C under hyperglycemia condition. In contrast, Gentiopicroside-induced activation of AMPK was not affected by ACC inhibitor TOFA, which suggesting that Gentiopicroside suppressed ACC expression through the activation of AMPK. (Fig. 6)

Fig. 4. Gentiopicroside regulated expression of key genes of PPAR- γ /AMPK/ACC signal pathway. (n=8 in each group). * $P < 0.05$ versus control group; # $P < 0.05$ versus DPN model group; & $P < 0.05$ versus oxcabazepin model group by one-way ANOVA followed by Bonferroni tests.

Fig. 5. Gentiopicroside protected RSC96 cell detected by WST-1. * $P < 0.05$ versus control group; # $P < 0.05$ versus high concentration of glucose group by one-way ANOVA followed by Bonferroni tests.

Gentiopicroside promoted AMPK expression by activating AMPK

PPAR- γ antagonist GW9662 and agonist rosiglitazone were used to indentify the key role that PPAR- γ play when induced by Gentiopicroside in RSC96 cell. The results showed that Gentiopicroside-induced AMPK activation and ACC inhibition were inhibited by GW9662. Conversely, neither antagonist of AMPK nor ACC affected the expression of PPAR- γ induced by Gentiopicroside. In a word, Gentiopicroside exerted nerve cell protective effects through regulating PPAR- γ /AMPK/ACC signaling pathway. Specifically, PPAR- γ mediated the activation of AMPK and subsequent inhibition of ACC expression. (Fig. 7)

Fig. 6. Protein expressions of PPAR- γ /AMPK/ACC signal pathway detected by westernblot. Gentiopicroside inhibited ACC expression by activating AMPK. * $P < 0.05$ versus control group; # $P < 0.05$ versus DPN model group (RSCs cultured in high concentration of glucose); & $P < 0.05$ versus Gentiopicroside group (Gentiopicroside +Glu) by one-way ANOVA followed by Bonferroni tests.

Fig. 7. Protein expressions of PPAR- γ /AMPK/ACC signal pathway detected by westernblot. Gentiopicroside promoted AMPK expression by activating AMPK. * $P < 0.05$ versus control group; # $P < 0.05$ versus DPN model group (RSCs cultured in high concentration of glucose); & $P < 0.05$ versus Gentiopicroside group (Gentiopicroside +Glu) by one-way ANOVA followed by Bonferroni tests.

Discussion

Diabetic peripheral neuropathy, the most common chronic complication of diabetes mellitus with an incidence of almost 50% [27], is a heterogeneous group of disorders with extremely complex pathophysiology and affects both somatic and autonomic components of the nervous system, reducing quality of life and increasing mortality. The abnormalities underlying DPN appear to be multiple and involve metabolic disturbance such as hyperglycemia and dyslipidemia [28]. Deficiency of insulin in the circulation system induces lipase activation and increases fatty acid mobilization from white adipose tissue. Hepatic lipid synthesis is found to increase due to the enhanced *de novo* lipogenesis mediated by increased fatty acid fluxes. Activity of lipoprotein esterase (LPL), the main enzyme of triglyceride hydrolysis, is decreased due to insulin resistance, leading to the deposition of blood lipid [29]. Dyslipidemia is recognized as an independent risk factor for the development of neuropathy in patients with diabetes [30]. Besides, reduced nerve perfusion is another important factor in the etiology of DPN which could be enhanced by dyslipidemia. Although efforts have been made to clarify the pathogenic mechanism of DPN and its ideal therapeutic drugs, neither verified pathogenesis nor effective medications have been established [31, 32].

Available treatments to date consist of improved metabolic control and a focus on symptoms but do not concentrate on fundamental mechanisms in the pathogenesis of neuropathy. Traditional Chinese Medicine named Qinjiao shows effective analgesic effect on DPN [33]. Gentiopicroside, a main component of Qinjiao has been used in Chinese herbalism as an ingredient in several traditional formulae for over 2000 years. Previous researches have shown therapeutic effect of Gentiopicroside on DPN rats with unclear mechanism [15, 34, 35]. Based on this, the present study aimed to prove protective effect of Gentiopicroside on DPN and Figure out the underlying molecular mechanism.

Streptozotocin (STZ) application results in high blood glucose, reduced paw withdrawal latency to heat and cold stimuli and increased responses to mechanical allodynia, which suggested DPN rat models were successfully established and exhibited thermal, cold as well as mechanical hyperalgesia respectively. In addition, a decline of motor and sensory nerve conduction velocity was showed in DPN rat groups verifying injuries of sciatic nerves. It is well-established that STZ-diabetic rats have been increasingly used as a model of DPN for its similar-to-human pathophysiological process [34]. Interestingly, dyslipidemia characterized by elevated plasma levels of TC, LDL and TG and low concentration of HDL was found in DPN rat models, suggesting a potential therapeutic target.

In vivo, Gentiopicroside exerted analgesia and nerve protective effects proven by elevated paw withdrawal latency to heat and cold stimuli, less responsive to mechanical allodynia, increased MNCV and SNCV, which was in line with previous studies [15, 16, 35]. Besides, Gentiopicroside regulated dyslipidemia, improved nerve perfusion, up-regulated expressions of PPAR- γ as well as AMPK and up-regulated expression of ACC in PPAR- γ /AMPK/ACC signaling pathway.

Oxcarbazepin is an anticonvulsant drug that has been reported to be efficacious in the treatment of DPN and has been recommended by the NeuPSIG (Special Interest Group on Neuropathic Pain of the International Association for the Study of Pain). It converts to active metabolite named 10-hydroxy-oxcarbazepin with an 8-to-10-hour half-time and is excreted by urine and metabolized in liver by glycosylation. Oxcarbazepin relieves pain by blocking voltage-dependent sodium channels, stabilizing membrane of nerve cell, inhibiting repetitive discharge of neurogen and reducing synaptic transmission of nerve impulses [36]. According to our results, oxcarbazepine exerted no effect in regulation of dyslipidemia, improvement of nerve perfusion and regulation of expressions of PPAR- γ /AMPK/ACC pathway. Gentiopicroside seemed to exert nerve protective effect in a different mechanism other than oxcarbazepine. In order to Figure out the close link between regulation of dyslipidemia of Gentiopicroside and PPAR- γ /AMPK/ACC signaling pathway, we further referred to experiments *in vitro*.

According to the *in vitro* experiment results, retention of proliferation activity of RSC96 cells suggested that Gentiopicroside protected Rat Schwann cells, which was in accord with results *in vivo*. Gentiopicroside was shown to accelerate AMPK activation, and compound C abolished Gentiopicroside-induced activation of AMPK and downregulation of ACC expression. In contrast, ACC antagonist TOFA had no influence in AMPK expression. These finding indicated that Gentiopicroside downregulated ACC expression by activating AMPK in RSC96 cell under high glucose condition. AMPK was an upstream regulator of ACC.

PPAR- γ is expressed in various tissues including nerve. It contributed to the balance between lipid influx and efflux and maintaining the lipid metabolism balance [37]. Intense clinical studies have highlighted that PPAR- γ activator named rosiglitazone conferred protection against diabetes mellitus. Gentiopicroside was shown to activate AMPK in a PPAR- γ -dependent manner. Inhibitor of AMPK such as compound C did not inhibit Gentiopicroside-induced elevation of PPAR- γ expression. These finding suggested that AMPK might be a downstream effector of PPAR- γ and Gentiopicroside acted as a PPAR- γ agonist in regulating PPAR- γ /AMPK/ACC signaling pathway, leading to dyslipidemia regulation, improvement of nerve perfusion and eventually amelioration of DPN

Conclusion

In conclusion, Gentiopicroside effectively alleviated DPN by regulating dyslipidemia and enhancing nerve perfusion via the PPAR- γ /AMPK/ACC pathway. This study was the first to our knowledge to affirm the protective effect of Gentiopicroside on DPN rats and show the underlying mechanisms involving dyslipidemia and PPAR- γ /AMPK/ACC pathway. Gentiopicroside may be a novel therapeutic approach for DPN patients. A better understanding in the mechanism as well as more comprehensive data from well-designed clinical studies will lay the groundwork for accelerating the development of Traditional Chinese Medicine against DPN.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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