

Original Research Article

3-Cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one ameliorates diabetic peripheral neuropathy in type 2 diabetes mellitus rats via PI3K/Akt signaling pathway

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Abstract

Purpose: To investigate the curative effects of 3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one (CHMP) on streptozotocin (STZ)-induced model of diabetic SD rats, and the underlying mechanism.

Method: Diabetes was induced in rats using single intraperitoneal injection of STZ. Subsequently, diabetic and non-diabetic rats were randomly grouped into five experimental groups. Six weeks after the STZ-injection, the diabetic animals were orally administered test compound (CHMP) at two doses of 10 and 20 mg/kg body weight for 6 weeks. Thereafter, the rats were anesthetised, and body weight, blood sugar, and motor nerve conduction velocity (MNCV) were determined. Moreover, real time-polymerase chain reaction (RT-PCR) and western blot analysis were used to assay the expression levels of genes in PI3K/Akt pathway and Glut4.

Results: Treatment of diabetic rats with CHMP significantly reduced levels of fasting blood glucose and enhanced average rat body weight, relative to diabetic control ($p < 0.05$). Motor nerve conduction velocity (MNCV) was remarkably increased in CHMP-treated rats (54.2 ± 2.2), when compared to the diabetic control rats (46 ± 4.1 , $p < 0.01$). Results from RT-PCR and western blot indicated increased expressions of PI3K, Akt and IRS-1, and down regulation of GSK-3B expression in skeletal muscle. The CHMP treatment also upregulated the Glut4 expression in skeletal muscle.

Conclusion: These findings show that CHMP may be beneficial in the management of diabetic neuropathy.

Keywords: Diabetic neuropathy, 3-Cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one, Motor nerve conduction velocity, Streptozotocin, Hyperglycaemia

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INTRODUCTION

Diabetes is among the most important global health-care problems worldwide. The incidence of diabetes has reached 10% in the US, and is increasing at the rate of 5% annually, according

to reports from Centre for Disease Control and Prevention [1]. It is predicted that the number of diabetic patients will double and may reach a pandemic level of 366 million between 2000 and 2030 [2]. It is estimated that about 50% of the diabetic patients will sooner or later develop

peripheral neuropathy, the most prevalent and debilitating microvascular issue attributed to the disease [3]. Peripheral neuropathy is due to damage caused to nerves as a result of chronic hyperglycaemia in the diabetic state. Depending on the onset form of neuropathy, the effect of diabetic neuropathy may range from distress to death. The most common symptoms of peripheral diabetic neuropathy are numbness, pain in legs and hands, foot ulceration, loss of sensation and susceptibility to amputation [4].

Diabetes induces a variety of neuropathy syndromes ranging from acute, chronic, focal and diffuse. Peripheral diabetic neuropathy significantly reduces the quality of life of the patient, and it is associated with huge financial burden [5]. Diabetic neuropathy (DNP) is diagnosed in about 30 to 40% of type 1 diabetic patients, and in 25 to 40% of type 2 patients [6, 7]. A study has indicated a reduction in the cumulative frequency of microangiopathy in type 1 diabetes in the last four decades, owing to several control measures [8]. The number of patients with type 2 diabetes is growing globally and DNP prevalence is high among type 2-diabetic patients [9].

Diabetic patients and experimental animals manifest high level of oxidative stress because of continuous and chronic hyperglycaemia which diminishes anti-oxidative defence, resulting in *de novo* free radical generation [10]. It has been suggested that chemotypes with antioxidant potential and free radical-scavenging action may facilitate the renewal of β -cells and may help in protection of pancreatic islets of Langerhans [11]. The present study was carried out to investigate the antidiabetic property of 3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one (CHMP) in streptozotocin-induced diabetic rats.

EXPERIMENTAL

Chemicals and reagents

Streptozotocin and CHMP were obtained from Sigma Chemical Co. (St Louis, MO, USA), Carboxy methyl cellulose (CMC) and pentobarbital were purchased from Merck & Co (USA), while TRIZOL reagent and BCA Kit were products of Invitrogen Life Technologies, USA.

In vivo evaluation of CHMP

Experimental animals

Two-month-old male Sprague-Dawley (SD) rats (mean weight = 190 ± 20 g) were procured from Vital River Laboratory Animal Technology Co.

Ltd ((Beijing, China). The rats were kept in the institutional animal holding facility for about 7 days to allow them to acclimatise to the local environment at a temperature of 22 ± 2 °C and relative humidity of 62%, prior to commencement of the study. Two animals were accommodated in one cage. All rats were given free access to water and pellet diet. The experiments and animal care were conducted in accordance with the institutional animal ethical committee (IAEC) (approval number = HCTM/C66/2019), and in conformity with international guidelines [12].

Diabetes induction

Following overnight fast, diabetes was induced in 36 rats via intraperitoneal injection of streptozotocin (STZ). The STZ was solubilised in sodium citrate buffer, pH 4.4, and was administered at a dose 60 mg/kg of body weight.

Treatments

After the induction of diabetes, the rats were grouped and treated as shown in Table 1.

Table 1: Grouping and treatment of SD rats

Treatment	Condition	Dosage
NC	Normal	Vehicle
NT	Normal	CHMP at 100 mg/kg body weight
DC	Diabetic	Vehicle
DL	Diabetic	CHMP at 10 mg/kg body weight
DH	Diabetic	CHMP at 20 mg/kg body weight

(NC = normal control; NT = normal control treated with CHMP at the highest tested dose i. e. 100 mg/kg; DC = untreated diabetic control; DL and DH = diabetic rats treated with low dose i.e. 10 mg/kg, and high dose i.e. 100mg/kg of CHMP, respectively)

The diabetic rats were assigned to three different groups using the random table method, with 12 animals per group, and were treated with homogeneous suspension of CHMP prepared in 0.5% CMC viz: diabetic control (DC) received equivalent volume of 0.5 % CMC (vehicle) in place of CHMP, diabetic group (DL) received CHMP at low dose (10 mg/kg), and the diabetic group (DH) received CHMP at higher tested dose (100 mg/kg). Rats in the normal control (NC) group were orally gavaged with 1.0 mL 0.5 % CMC, while those in the NT group received CHMP at the highest tested dose (100 mg/kg body weight).

Determination of blood glucose levels

After 72 h, blood was taken from the tail of each rat and used for glucose determination with a

glucometer. Blood glucose levels >16.6 mmol/L (> 5%) were taken as indicative of diabetes. Blood glucose measurement was done 2 weeks after diabetes induction to confirm the diabetic status of the animals. Treatment with CHMP started after six weeks of STZ-induced diabetic condition, and lasted for six weeks.

Furthermore, levels of blood glucose were determined at the end of the study 1h after the administration of last dose of CHMP or vehicle. The rats were euthanized within 2 h from the last dose of CHMP via intraperitoneal injection of pentobarbital at a lethal dose of 150 mg/kg. Samples of plasma and sciatic nerves from the rats were used in subsequent assays.

Measurement of body weight of rats

Using digital weighing balance, the body weights of the rats were determined three times during the course of study i.e. at the start of experiment, 6 weeks after STZ-induced onset of diabetes, and 6 weeks post-treatment with CHMP.

Measurement of motor nerve conduction velocity (MNCV)

The MNCV values of the rats was measured under pentobarbital anaesthesia using Power Lab 8sp instrument (Chengdu Instrument factory, China). The measurement was done through stimulation of the sciatic nerve with 2 volts and 5 stimuli, and MNCV was calculated as shown in Equation 1:

$$\text{MNCV} = d/s \dots\dots\dots (1)$$

where *d* is the distance between sciatic nerve stimulation point and toe skin recording point, and *s* is the sciatic M wave latency.

Real-time PCR

The mice from the various groups were euthanized, and soleus muscle biopsies weighing approximately 100 mg were collected with aseptic techniques, and stored at -80 °C. The tissues were thawed and homogenised. Total RNA was isolated from muscle tissues with TRIzol reagent. Impurities of DNA, if any were removed by subjecting the isolated RNA samples to DNase I digestion. Thereafter, 3 µg RNA was reverse-transcribed to cDNA. The PCR mixture was prepared according to the instructions in the supplier's manual. The primer sequences used for mRNA quantification were:

IRS-1 gene: forward (3'→5'): CAGGCACCA
TCTCAACAATC, reverse (3'→5'): GTTCCAC

CCACCATACTG;

Akt1 gene: forward (5'→3'): GGCAGGATG
TGTATGAGAAGAA, reverse (3'→5'): GTGATCA
TCTGAGCTGTGAACT;

Glut4 gene: forward (3'→5'): CCCACAGAAA
GTGATTGAACAG, reverse -(3'→5'): AGAGAGC
CCAAAGGGTAGTGAG;

Pik3r1 gene: forward (3'→5'): CCTTGGAGAG
GTTTGACCATTA, reverse (3'→5'): CATCCAG
CTATGCTGTATCTATC;

GSK-3β gene: forward (3'→5'): GCCAATGCA
GAGGTCCTAAA, reverse (3'→5') CGAAAGG
GAAGAGAAGGTAACA, and

β-actin: forward (5'→3'): CACCCGCGAG
TACAACCTTC reverse (3'→5'): CCCATACCCA
CCATCACAC

Western blotting analysis

The skeletal muscle tissues were lysed and the protein content of the lysates were estimated using BCA assay. Similar quantities of the proteins from each sample were loaded and subjected to SDS-PAGE. After transferring the gels to nitrocellulose membranes, the membranes were incubated with primary antibodies for 55 min at 23 °C. This was followed by incubation with secondary antibody. The visualisation of the bands was carried out using chemiluminescence reagent.

Statistical analysis

Three independent experiments were performed for data validation. The results are expressed as mean ± SD. One-way ANOVA and student's *t*-test were employed for statistical analysis.

Values of *p* < 0.05 and < 0.01 were taken as statistically significant and very significant, respectively.

RESULTS

Effect of CHMP on fasting glucose levels in rats

The fasting blood glucose levels of rats after STZ-induced diabetes were monitored over a period of six weeks before the treatment, during the treatment, and post-treatment (Table 2). The diabetic rats (DC) had significantly higher blood levels than rats in NC group. However, CHMP treatment markedly reduced the level of fasting blood glucose in DL and DH rats. Nonetheless, there were significant differences between DL and DC groups, as well as DC and DH groups.

Table 2: Effect of CHMPI on the levels of fasting blood glucose (mM) of rats

Group	First week	Week 2	Week 6	Week 12
NC	4.85 ± 0.481**	4.95 ± 0.480**	4.95 ± 0.482**	4.90 ± 0.481**
NT	4.80 ± 0.485	5.70 ± 0.450	5.40 ± 0.430	4.8 ± 0.640
DC	18.95 ± 2.00	24.11 ± 7.35	22.9 ± 3.25	23.3 ± 3.91
DL	19.7 ± 1.15	23.4 ± 3.55	19.8 ± 1.43	16.8 ± 3.09*
DH	20.0 ± 0.880	23.4 ± 3.43	16.8 ± 1.55**	14.6 ± 3.86**

Values are presented as mean ± SD (n = 12); **p* < 0.05 vs. NC; ***p* < 0.01 vs. DC

Effect of CHMP on body weight of rats

Body weight was monitored before STZ injection, on 6th week of STZ injection, and on 12th week of CHMP administration (Table 3). The body weight of the animals in NC and NT groups were gradually increased during the study period, while the body weight of rats in DC group was significantly lower than that of the NS group. There was no significant difference in body weight between NC and NT groups. Rats in the DL and DH groups treated with CHMP had significantly increased body weights, relative to rats in DC group.

Table 3: Effect CHMP on mean body weight of the STZ-induced diabetic rats

Group	Body weight before STZ injection (first week)	Body weight at the start of CHMP treatment (6 weeks)	Body weight at sacrifice (12 weeks)
NC	2005.5 ± 3.3	390.5 ± 4.5**	440.3 ± 5.2**
NT	200.0 ± 4.2	387.8 ± 5.2	438.4 ± 3.3
DC	197.4 ± 6.3	340.5 ± 4.1	380 ± 9.3
DL	203.6 ± 5.1	380.4 ± 6.3*	435.7 ± 4.3*
DH	205.3 ± 7.2	382.6 ± 7.1*	437.4 ± 1.1*

Values are presented as mean ± SD (n = 12); **p* < 0.05 vs. NC; ***p* < 0.01 vs. DC

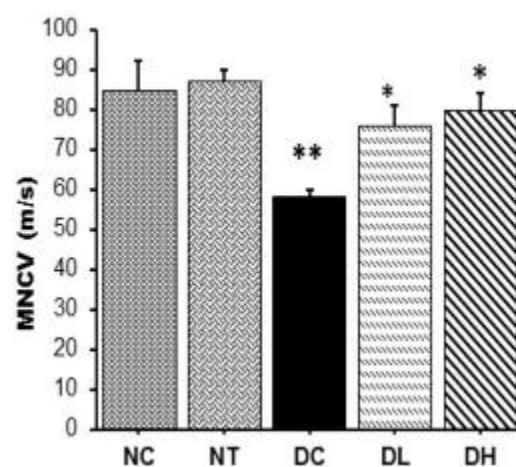
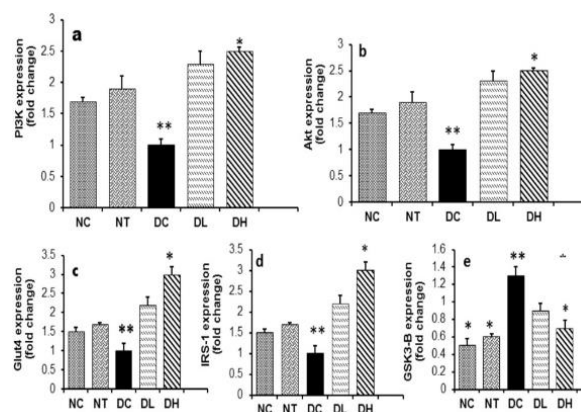
Effect of CHMP treatment on MNCV

The results of MNCV determined after 6 weeks of CHMP treatment, are shown in Figure 1. Treatment with CHMP at a dose of 100 mg/kg body weight led to significant reversal of MNCV in DH rats (54.2 ± 2.2), when compared to DC rats (46 ± 4.1; *p* < 0.01). In the NC rats, the MNCV value was 60.2 ± 2.1. Treatment of diabetic rats (DL) with CHMP at a dose of 10 mg/kg body weight did not significantly modify MNCV (45 ± 3.3).

Effect of CHMP on the mRNA levels of PI3K/Akt pathway genes.

Figure 2 shows the mRNA expression levels of PI3K/AKT cascade. Treatment of diabetic rats with CHMP significantly upregulated the expressions of PI3K (2a), Akt (2b), IRS-1 (2d) and Glut4 (2c). In contrast, the mRNA

expression of GSK3β (2e) was significantly downregulated, relative to DC rats (*p* < 0.01). The CHMP-induced increases in mRNA expressions of PI3K, Akt, IRS-1 and Glut4, and decrease in mRNA expression of GSK3β in diabetic rats were dose-dependent.

**Figure 1:** Effect of CHMP treatment on MNCV**Figure 2:** Effect of CHMP on the transcription levels of different genes of PI3K/Akt pathway in skeletal muscles

Effect of CHMP on expressions of key proteins of PI3K/Akt cascade

Protein expressions of PI3K, Akt, p-Akt, p-Akt/Akt, IRS and Glut4 in diabetic rats were also significantly increased by CHMP treatment. These increases in protein expression correlated with the mRNA expression of the respective

genes (Figure 3). Moreover, the protein expression of GSK3 β was downregulated in the CHMP treatment groups.

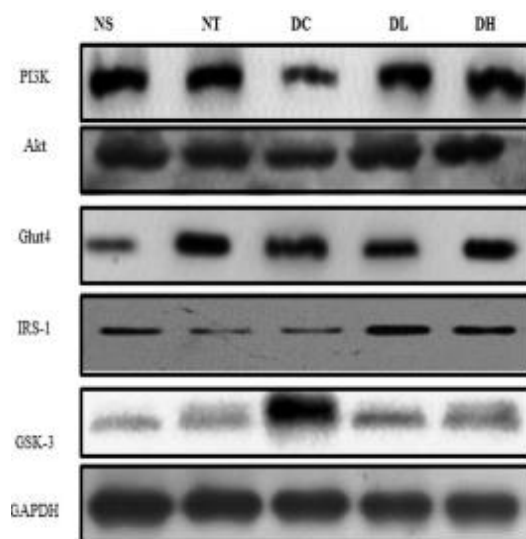


Figure 3: Western blots showing the effect of CHMP on the expression levels of the PI3K/Akt proteins in skeletal muscles

DISCUSSION

It has been reported that high levels of fasting blood glucose which are within normoglycemic range contribute to an autonomous risk for the development of type 2 diabetes. This factor, together with body mass index, may lead to type 2 diabetes in future in individuals at high risk of developing the disease [12,13].

The present study revealed that plasma glucose levels in the normal control rats i.e. NS and NT groups remained almost unaffected, relative to that in DC group. In contrast, there was an upsurge of fasting glucose in the diabetic rats. In addition, the plasma glucose levels were high, relative to NT or NS rats. However, treatment of diabetic rats with CHMP for 6 weeks led to significant reduction in fasting blood levels in DL and DH groups, when compared to DC group.

Determination of MNCV is the most sensitive test for the diagnosis of diabetic neuropathy. Moreover, MNCV measurement is repeatable, and it is considered a specific test for neurological disorder [14]. Treatment of diabetic rats with CHMP significantly reduced MNCV, when compared with untreated diabetic control rats.

Type 2 diabetes is a very complicated disease with characteristic features such as insulin resistance, deficiency in insulin secretion and high levels of endogenous glucose. Aside from

the failure of β -cells of pancreas, insulin resistance by target tissues is the major contributing factor to the development of type 2 diabetes mellitus [15]. Among the target tissues, skeletal muscle is a major tissue that accounts for insulin-reliant glucose consumption [16].

Changes in insulin levels trigger initiation of the PI3K/Akt pathway, resulting in the development of insulin-resistance as well as type 2 diabetes [17,18]. In the current study, CHMP treatment for 6 weeks significantly reduced the protein expressions of the key genes of the PI3K/AKT cascade in the skeletal muscle of type 2 diabetic SD rats. This is in agreement with the literature. It was observed that CHMP markedly downregulated mRNA and protein expressions of IRS-1, Akt Glut4 and PI3K, and suppressed those of GSK3 β in diabetic rats.

From these results, it can be inferred that CHMP exerts potent anti-diabetic effects and may prove beneficial in the development of drugs for management of diabetes.

CONCLUSION

This study has shown, for the first time, that 3-CHMP, an α -pyrone-based compound, exhibits remarkable anti-diabetic effects in a rat model of experimental diabetic neuropathy. The results indicate that CHMP attenuates diabetic neuropathy in rats by regulating the PI3K/Akt signalling pathway in skeletal muscle. Thus, CHMP is a good candidate for development of anti-diabetes drugs.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. XL, GZ, JY, SR and JW performed the animal studies, MW performed the RT-PCR, SZ designed the experiments and performed the western blot analysis. WX analyzed the data. XF, HH and LT manuscript. All authors read and approved the manuscript.

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