

## Original Article

# Mechanism of calcium channel blockers in relieving pathological pain of diabetic peripheral neuropathy in rats

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**Abstract:** Diabetic neuropathic pain (DNP) is complex and refractory. Commonly used calcium channel blockers (CCBs) can be divided into L type and T type. T type CCB mibefradil can alleviate DNP. However, the role of a combined application of L type and T type CCBs in DNP has not been reported. Thirty-six rats were used to establish a type I DNP model using streptozotocin (STZ), and were equally divided into four groups, including DNP group, mibefradil group, verapamil group, and mibefradil and verapamil group. Paw withdrawal mechanical threshold (PWMT) was evaluated by the von Frey method. Paw withdrawal latency (PWL) was determined by thermal radiation. PKC $\gamma$  expression in cornu dorsale medullae spinalis was detected by real-time PCR and Western blot. Inflammatory cytokines TNF- $\alpha$  and IL-2 levels were tested by ELISA. Mibefradil and verapamil reduced PWMT and PWL values, down-regulated PKC $\gamma$  expression, and inhibited TNF- $\alpha$  and IL-2 secretion ( $P < 0.05$ ). The combined treatment group demonstrated stronger analgesic effects than mibefradil or verapamil alone group ( $P < 0.05$ ). Mibefradil and verapamil combined application can alleviate DNP by inhibiting inflammation and reducing PKC $\gamma$  expression which showed better effects than drug single application.

**Keywords:** Calcium channel blocker, mibefradil, verapamil, PKC $\gamma$ , diabetic neuropathic pain

## Introduction

Diabetes is an endocrine disease that seriously threatens human health. As one of the most common chronic complications of diabetes, diabetic neuropathic pain (DNP) refers to the pathological changes of the nervous system induced by diabetes. DNP is divided into diabetic neuralgia and neuropathy [1, 2]. DNP seriously influences quality of life because of it is complex and refractory [3, 4]. DNP involves several peripheral nervous systems, including autonomic nerves, motor nerves, and sensory nerves [5]. DNP may appear as hypoesthesia, numbness, burning, spontaneous pain such as needle-like pain, burning pain, dull pain, or drilling pain, pain reaction enhancement, pain hypersensitivity, paresthesia, hyperalgesia, and limb pain [6]. Pathological pain is relatively high in DNP, and may be aggravated in chronic cases and with poor blood glucose control [7, 8]. Diabetic patients are liable to suffer from vitamin deficiencies, ischemia, metabolic distur-

bance, and neurothliposis; leading to neurapraxia and sensory innervation pain. However, diabetes does not affect the nervous system itself [9, 10]. The mechanisms of DNP are complex and still unclear. In spite of medical developments, current therapeutic methods show poor efficacy to DNP. It is urgently needed to find effective methods for treatment of DNP [11, 12].

According to the different voltage gaited calcium channels, they can be divided into high voltage activated calcium channels (L-type calcium channel) and low voltage activated calcium channels (T type calcium channel) [13, 14]. It was shown that T type calcium channel blocker (CCB) mibefradil can be activated below the resting potential since it cannot pass the blood-brain barrier. It is featured as rapid activation and slowly closing, thus can be used in DNP treatment [15, 16]. However, the role of L-type CCBs combined with T-type CCBs in DNP treatment and related mechanisms have not been reported.

**Table 1.** Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGAGTACCTTGCTTCTGGG	TAATGATAGGTGACCCCTGGT
PKC $\gamma$	TGCTTATGCATGATGCCGACT	CGCTTCTTCGTCAACTCTTATC

## Materials and methods

### Experimental animals

A total of 36 healthy male SD rats aged 3 months and weighing 250±30 g were purchased from and raised in an experimental animal center of Hubei Province. The raising conditions maintained temperatures at 21±1°C, relative humidity at 50-70%, and a 12 h day/night cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Huangshi Central Hospital of Hubei Province.

### Main reagents and instruments

Streptozotocin (STZ) was purchased from Sigma (USA). Trizol was purchased from Invitrogen (USA). PVDF membrane was acquired from Pall Life Sciences. Western blot related reagents were provided by Beyotime (Shanghai, China). ECL reagent was obtained from Amersham Biosciences. Mouse anti rat PKC $\gamma$  monoclonal antibody and goat anti mouse HRP labeled IgG secondary antibody were provided by Cell Signaling (USA). RNA extraction kit, reverse transcription kit, and IL-2 and TNF- $\alpha$  ELISA kits were purchased from R&D (USA). The microplate reader was provided by BD (USA). DNA amplifier was obtained from PE Gene Amp PCR System 2400. The microscopic surgery instrument was purchased from Suzhou medical apparatus factory. The electronic glucometer was provided by Advantage (USA). ABI 7500 real time PCR amplifier was derived from ABI (USA). Glucose test paper was obtained from Zhujiang Chemical Reagent co., Ltd (China). Other reagents were purchased from Sangon (China).

### Methods

**Experimental animal grouping:** The rats were randomly divided into four equal groups, including DNP group, mibefradil group, verapamil group, and mibefradil and verapamil group. For DNP induction the rats were treated with STZ to establish diabetic nephropathy model.

**DNP model establishment and treatment:** DNP rats were fasted for 12 h and treated with 0.5% STZ prepared by sterile citric acid-sodium citrate buffer with caudal vein injection at 40 mg/kg. Blood glucose, urine glucose, and urine volume were tested after two weeks. DNP modeling success was defined as blood glucose >16.7 mmol/L, urine glucose <++, and urine volume increasing for two measurements [17]. The rats in the mibefradil, verapamil, and combined verapamil and mibefradil groups were treated with 1.5 mg mibefradil and/or 1.5 mg verapamil via intraperitoneal injection based on the DNP model after two weeks.

### Sample collection

The blood was collected from the aorta using the negative pressure method. The blood was set at room temperature for 30 min and centrifuged at 4°C and 3600 rpm for 10 min. The supernatant was stored at -20°C. The rat's left renal tissue was obtained and stored at -80°C.

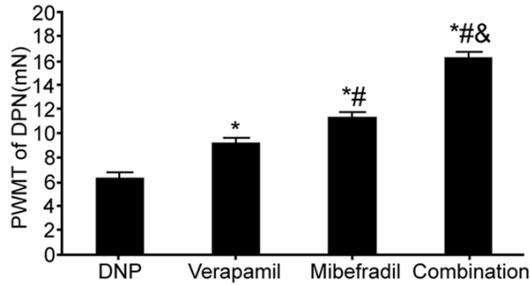
### Real-time PCR

Total RNA was extracted from the cornu dorsale medullae spinalis by trizol and reverse transcribed to cDNA. The primers were designed using PrimerPremier 6.0 software (**Table 1**) and synthesized by Sangon. Real-time PCR was performed at 56°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as the internal reference. The relative expression of mRNA was calculated by 2<sup>- $\Delta$ Ct</sup> method.

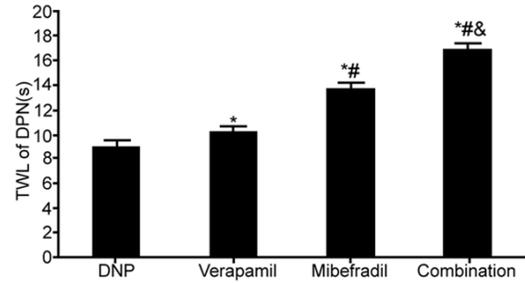
### Western blot

The cornu dorsale medullae spinalis was mixed with RIPA buffer containing protease inhibitors and homologized on ice for 15-30 min. Next, the tissues were sonicated for 5 s, 4 times and centrifuged at 10,000 g for 15 min. The protein was transferred to a new Eppendorf tube and stored at -20°C. The protein was separated on a 10% SDS-PAGE and transferred to PVDF membrane with voltage at 100 mA for 1.5 h. After being blocked by 5% skim milk for 2 h, the membrane was incubated in Rattin, Bcl-2, and Bax monoclonal antibodies (1:1000, 1:2000, 1:1000) at 4°C overnight. Then the membrane was incubated in goat anti rabbit secondary antibody (1:2000) at room temperature for 30 min. Next, the membrane was treated by devel-

## CCB combination in DNP



**Figure 1.** The impact of CCB on PWMT in DNP rats. \*P<0.05, compared with DNP group; #P<0.05, compared with verapamil group; &P<0.05, compared with mibefradil group.



**Figure 2.** The influence of CCB on TWL in DNP rats. \*P<0.05, compared with DNP group; #P<0.05, compared with verapamil group; &P<0.05, compared with mibefradil group.

oper for 1 min and exposed to observe the result. The film was scanned by Quantity One software and analyzed by a protein image processing system. Each experiment was repeated four times.

### ELISA

ELISA was used to test TNF- $\alpha$  and IL-2 levels in the serum. A total of 50  $\mu$ l diluted standard substance was added to each well to establish the standard curve. The OD value of the standard substance was used to prepare the linear regression equation, which was adopted to calculate the concentration of samples.

### Von Frey method evaluation of paw withdrawal mechanical threshold (PWMT)

The rat was put into the behavior detection cage with a perspex hood. Von Frey hairs were used to stimulate the posterior limb pedis for 6-8 s to observe paw withdrawal response. Rapid paw withdrawal response within a short time after the stimulus was defined as positive. The stimulus was repeated every five seconds, 10 times. PWMT was the smallest stimulus intensity to induce paw withdrawal frequency 50% of the time.

### Thermal radiation detection of paw withdrawal latency (PWL)

The rat was put into the behavior detection cage with a perspex hood at 20-22°C. The thermal stimulator was applied to irradiate the pad of posterior limb. Rapid paw withdrawal response within a short time after the stimulus was defined as positive. PWL referred to the irradiation time of the thermal stimulator.

### Statistical analysis

All data were presented as mean  $\pm$  standard deviation and compared by t-test or ANOVA. All data analyses were performed with SPSS 11.5 software. P<0.05 was adopted as statistical significance.

## Results

### CCB increases PWMT in DNP in the rat

DNP rats were treated by mibefradil, verapamil, or mibefradil combined with verapamil to test the impact of CCB on PWMT. It was shown that CCB treatment significantly increased PWMT compared with the DNP group (P<0.05). The combined group and mibefradil group demonstrated stronger intensity than verapamil (P<0.05). The combined group exhibited the most obvious analgesic effect (P<0.05) (**Figure 1**).

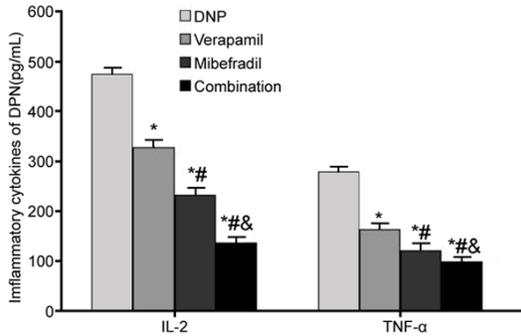
### CCB prolongs TWL in DNP rat

DNP rats were treated by mibefradil, verapamil, or mibefradil combined with verapamil to test the impact of CCB on TWL. It was shown that CCB treatment significantly prolonged TWL compared with the DNP control group (P<0.05). The combined group and mibefradil group demonstrated stronger intensity than verapamil (P<0.05). The combined group exhibited the most obvious analgesic effect (P<0.05) (**Figure 2**).

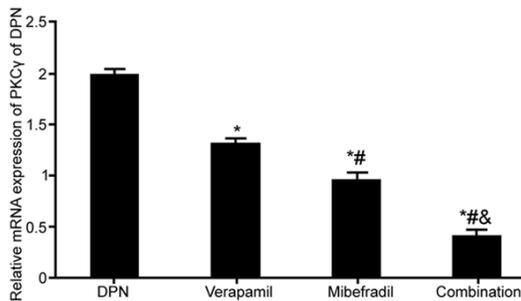
### Reduced serum inflammatory cytokines in DNP rats after CCB treatment

DNP rats were treated by mibefradil, verapamil, or mibefradil combined with verapamil to test

## CCB combination in DNP



**Figure 3.** The effects of CCBs on serum inflammatory cytokines in DNP rats. \* $P < 0.05$ , compared with DNP group; # $P < 0.05$ , compared with verapamil group; & $P < 0.05$ , compared with mibefradil group.

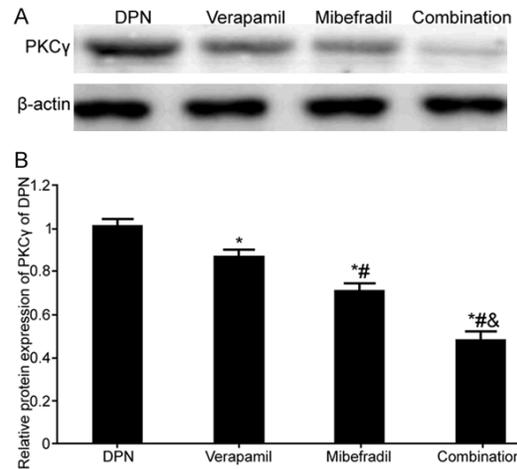


**Figure 4.** The effect of CCBs on PKC $\gamma$  mRNA expression in DNP rats. \* $P < 0.05$ , compared with DNP group; # $P < 0.05$ , compared with verapamil group; & $P < 0.05$ , compared with mibefradil group.

the impact of CCB on serum inflammatory cytokines TNF- $\alpha$  and IL-2 expression. It was shown that CCB treatment significantly reduced TNF- $\alpha$  and IL-2 expression in the serum compared with DNP control group ( $P < 0.05$ ). The combined group and mibefradil group demonstrated stronger efficacy than verapamil ( $P < 0.05$ ). The combined group exhibited the most obvious analgesic effect ( $P < 0.05$ ) (Figure 3).

### CCB downregulates PKC $\gamma$ mRNA expression in DNP rats

DNP rats were treated by mibefradil, verapamil, or mibefradil combined with verapamil to test the impact of CCB on PKC $\gamma$  mRNA expression in the cornu dorsale medullae spinalis. It was shown that CCB treatment significantly downregulated PKC $\gamma$  mRNA expression compared with the DNP control group ( $P < 0.05$ ). The combined group and mibefradil group demonstrated stronger efficacy than verapamil ( $P < 0.05$ ). The combined group exhibited the most clear analgesic effect ( $P < 0.05$ ) (Figure 4).



**Figure 5.** The effect of CCBs on PKC $\gamma$  protein expression in DNP rats. A. Western blot detection of PKC $\gamma$  protein expression; B. PKC $\gamma$  protein expression analysis. \* $P < 0.05$ , compared with DNP group; # $P < 0.05$ , compared with verapamil group; & $P < 0.05$ , compared with mibefradil group.

### CCB decreases PKC $\gamma$ protein expression in DNP rats

DNP rats were treated with mibefradil, verapamil, or mibefradil combined with verapamil to test the impact of CCB on PKC $\gamma$  protein expression in the cornu dorsale medullae spinalis. It was shown that CCB treatment significantly downregulated PKC $\gamma$  protein expression compared with the DNP control group ( $P < 0.05$ ). The combined group and mibefradil group demonstrated stronger efficacy than verapamil ( $P < 0.05$ ). The combined group exhibited the most clear analgesic effect ( $P < 0.05$ ) (Figure 5).

## Discussion

DNP shows numerous pathogenesis, including impaired glucose metabolism induced by glucose toxicity and abnormal protein glycosylation, arachidonic acid decrease, prostaglandin reduction, and dyslipidemia [18, 19]. Vascular endothelial cell proliferation caused by abnormal nerve and blood vessel activation can also induce DNP [20]. Oxidative stress and inflammation are causes that can aggravate DNP. Inflammatory cytokines TNF- $\alpha$  and IL-2 secretion enhancement has been found in DNP. Multiple methods have been applied to treat DNP, such as anticonvulsants, antidepressant drugs, and opioids. In addition, glucose control, nerve repair by mecobalamine and growth factors, anti-oxidative stress, and neu-

rotrophic factors have also been adopted [21, 22]. Sodium channels can be blocked to inhibit presynaptic glutamate release and stabilize nerve cell membranes to relieve pain [23]. Recently, it was found that blocking T-type calcium channels can also provide a pain relief effect [24]. However, whether L-type CCBs can relieve DNP, and its mixed effects with T-type CCBs are still unclear.

This study established a DNP rat model and treatment plan using mibefradil, verapamil, and mibefradil combined with verapamil.

We show that CCB clearly prolonged PWMT and PWL. The combined group and mibefradil group demonstrated stronger efficacy than verapamil. The combined group exhibited the most clear analgesic effect. This suggested that L-type CCBs also plays a role in relieving DNP. The combination with T-type CCBs showed stronger analgesia effects in DNP. Moreover, we verified that CCBs inhibited serum inflammatory cytokines release in DNP rats, revealing that CCBs can relieve pain by regulating inflammation. A previous study demonstrated that PKC $\gamma$  levels were significantly increased in spinal dorsal horn neurons that affected calcium channels, thus aggravating pain in DNP rat [25].

### Conclusion

Mibefradil and verapamil combined application can alleviate DNP through inhibiting inflammation and reducing PKC $\gamma$  expression; which showed better effects than single drug application.

### Disclosure of conflict of interest

None.

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