Anti-allodynic and Neuroprotective Effects of Koumine, a Benth Alkaloid, in a Rat Model of Diabetic Neuropathy

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Diabetic neuropathy is characterized by progressive degeneration of nerve fibers associated with diabetes mellitus. Antidepressants and anticonvulsants are the mainstay of pharmacological treatment, but are often limited in effectiveness against the core clinical feature of pain. In the current study, we examined the potential effects of koumine, a Gelsemium elegans Benth alkaloid, using a rat model of diabetic neuropathy. Rats were administered intraperitoneally a single dose of streptozocin (60 mg/kg) to induce type 1 diabetes. Koumine was given at a dose range of 0.056–7 mg/kg subcutaneously for one week starting 3 weeks after streptozocin administration. Behavioral responses to mechanical stimuli were evaluated every day after streptozocin injection. At 4 weeks after streptozocin injection, sensory nerve conduction velocity (SNCV) and morphological alternation of sciatic nerves were assessed by electron microscopy. Diabetic rats developed mechanical hyperalgesia within 3 weeks after streptozocin injection and exhibited reduced SNCV and impaired myelin/axonal structure. Koumine treatment of diabetic rats decreased neuropathic pain behavior as early as after the first administration. At a dose of 7 mg/kg, koumine was more effective than gabapentin (100 mg/kg), and decreased mechanical sensitivity threshold to a level comparable to healthy control. Repeated treatment of koumine significantly reduced the damage to axon and myelin sheath of the sciatic nerve and increased SNCV, without affecting body weight and blood glucose. These findings encourage the use of koumine in the treatment of diabetic neuropathy.

Key words  diabetes; neuropathy; koumine; pain; myelin; axon

Diabetic neuropathy is characterized by progressive peripheral neuropathy, and causes neuropathic pain and sensory/motor loss in the extremities. The mainstay of pharmacological treatment against neuropathic pain includes anesthetics, analgesics, anticonvulsants, and antidepressants. But these agents are typically limited in efficacy in addition to a variety of side effects. Chronically elevated blood glucose in diabetes is associated with damaged mitochondria, increased production of reactive oxygen species, decreased nerve blood flow, reduced supply of trophic factors and slowed nerve conduction. If not corrected, these defects lead to degenerative abnormalities in peripheral/central nervous system. We have recently reported promising analgesic action of koumine, a major alkaloidal constituent of Gelsemium elegans Benth in a variety of animal models, including writhing test, formalin test, sciatic nerve chronic constriction injury (CCI), and spinal nerve ligation (SNL) model. Here we report potent activity of koumine against allodynia in a rat model of streptozocin-induced diabetic neuropathy. Sensory nerve conduction speed increased by koumine. Histological examination of the sciatic nerve revealed mitigated nerve damage. Surprisingly, blood glucose was not affected despite of clear evidence of attenuated nerve damage. These encourage the development of koumine and its structural analogues for the treatment of peripheral neuropathy.

MATERIALS AND METHODS

Animals  Male Sprague-Dawley rats (220–250 g) purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China) were housed in plastic cages (5 rats per cage) with free access to food and water at 22–25°C under a 12-h light/dark cycle. All experiments were conducted in compliance with the international regulations on animal experimentation and approved by the Committee of Ethics of Fujian Medical University. All efforts were made to minimize the animal suffering and the number of animals. All behavioral experiments were performed between 9:00 and 17:00 h. Each rat was used in only one experiment.

Drugs and Treatments  Koumine (99% purity, specimen number: KM201308) was isolated from Gelsemium elegans Benth, using pH-zone-refining counter-current chromatography, as previously described. Gabapentin (95%, Shanghai Sunheat Chemicals Co., Ltd., Shanghai, China) was included in the behavioral test as a positive control. Koumine and gabapentin were dissolved in sterile physiological saline before use and were administered subcutaneously (s.c.) at a dose volume of 4 mL/kg rat body weight.

Diabetic Neuropathic Pain  Rats were given a single intraperitoneal (i.p.) injection of streptozocin (STZ, 60 mg/kg, Sigma) or vehicle (0.01 M citrate buffer, pH 4.5), as described previously. Seventy-two hours later, blood (tail vein) glucose was measured using a commercial glucose meter (One-Touch Ultra, Lifescan). Rats with a minimum of 16.67 mmol/L glucose were used for further experiments. Starting from day 22 after the STZ injection, rats received daily s.c. injection of koumine (0.056, 0.28, 1.4, or 7 mg/kg), gabapentin (100 mg/kg) or vehicle s.c. for 7 consecutive days. The dose selection of koumine was based on our previous report, and we defined gabapentin dose by previous reference. Pain behavior was assessed, as described below, prior to the STZ injection (baseline), before drug treatment (pre-dosing), and 60 min after each drug injection (post-dosing). We chose 60 min after drug injection to measure pain behavior because we had done several experiments to confirm the best timing koumine ex-
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Mechanical allodynia was measured using a commercially available electronic Von Frey apparatus (Model 2390; IITC Life Science Inc., Woodland Hills, CA, U.S.A.) as described before with minor modifications. Briefly, rats were placed into a Plexiglas box on a steel mesh floor. Increasing pressure (maximum: 55 g) was delivered to the center of the hind paw with an upward motion of the von Frey filament until foot withdrawal. The pressure that resulted in foot withdrawal was automatically recorded and used to reflect pain threshold. The procedure was repeated twice at approximately 10 min intervals for each hind paw to calculate the average. Rats were habituated to the test environment for at least 60 min prior to each session. The observer scoring the behaviors was blind to the treatment condition.

Nerve Conduction Velocity Upon completion of the behavior test, rats were anesthetized with pentobarbital (100 mg/kg, i.p.). A stimulating electrode was inserted intramuscularly into the tuber ischiale. A recording electrode was placed between the malleolus medialis and the second toe. A reference electrode was located between the recording electrode and the stimulating electrode. The stimulation to the sciatic nerve (square-wave; 2.0 mV in intensity, 3.0 ms in width) was applied using an electromyography recording system (ADInstrument, U.K.). The action potential latency (L) of sciatic nerve and the distance (D) between the stimulating and recording electrodes were measured to calculate the sensory nerve conduction velocity (SNCV) using the following formula: SNCV

Fig. 1. Effects of Repeated Subcutaneous Koumine Administration on Blood Glucose (a) and Body Weight (b) in Diabetic Rats

KM: koumine, GP: gabapentin. Data are presented as mean±S.E.M. ***p<0.001, diabetic model group versus normal control group (one-way ANOVA with post hoc Bonferroni test for each time point). Each group consisted of 6–9 rats.
In each experiment, SNCV measurement was repeated three times to calculate the mean value. The limb temperature was kept at 37°C during the experiment.

**Histological Evaluation by Electron and Light Microscopy**

The morphological evaluation was performed after the electrophysiological assessment. Rats were transcardially perfused with saline and then 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). The mid-thigh sciatic nerve was rapidly excised, fixed with pre-cooled 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide solution, dehydrated stepwise in increasing concentrations of ethanol, and embedded in Epon 812 epoxy resin. Ultrathin sections were stained with lead citrate and uranyl acetate for examination under a transmission electron microscope (EM208, PHILIPS, Netherlands). Another part of sciatic nerve sections was independently processed, stained by toluidine blue and examined on light microscopy (CX21, Olympus, America).

**Statistical Analysis**

Effects of koumine on allodynia were evaluated by the increment of withdrawal threshold after koumine treatment and expressed as percentage of maximal possible effect (%MPE): MPE% = \( \frac{(\text{postdose threshold}) - (\text{predose threshold})}{(\text{baseline threshold}) - (\text{predose threshold})} \times 100 \). It is possible to obtain a negative MPE if the mechanical withdrawal threshold decreased after treatment. Dose–response curves were generated by fitting the data to a logistic equation. Drug effects were analyzed using a one-way or two-way ANOVA followed by the Bonferroni test for post hoc analysis. All data are presented as mean±S.E.M. (standard error of measurement). All statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, U.S.A.). A p value of <0.05 was considered statistically significant.
**RESULTS**

**Effects of Koumine on Blood Glucose and Body Weight**
Koumine treatment group did not alter blood glucose (Fig. 1a) or body weight (Fig. 1b).

**Effects of Koumine on Mechanical Allodynia**
To evaluate the impact of koumine on mitigating diabetic neuropathic pain, we measured the effects of koumine using the STZ-induced diabetes model. A two-way ANOVA on mechanical withdrawal threshold (MWT) in the right hind-paw demonstrated a significant treatment effect (control vehicle vs. koumine) between subjects ($F_{6.594}=2560.6$, $p<0.001$) and treatment time ($F_{10,594}=1511.7$, $p<0.001$). Post hoc tests showed diabetes significantly decreased MWT to mechanical stimulation ($p<0.001$ vs. normal control group), demonstrating the development of mechanical allodynia which persisted for the entire observation period (Fig. 2). Gabapentin (100 mg/kg) also significantly reduced mechanical allodynia ($p<0.001$ vs. diabetic model rats). Koumine attenuated diabetes-induced allodynia in a dose-dependent manner ($p<0.001$ vs. vehicle; Fig. 2). On day 28 (one week after koumine administration), the MPE was 42.96$\pm$1.32, 89.16$\pm$0.9, 94.92$\pm$0.73 and 99.72$\pm$0.76 for koumine at 0.056, 0.28, 1.4, and 7 mg/kg, respectively (across the doses; Figs. 2c, d). ED$_{50}$ (50% effective dose) of koumine was 0.063 mg/kg. The MPE for gabapentin at a dose of 100 mg/kg was 90.53$\pm$1.43 (Fig. 2c). The MPE for koumine at a dose of 7 mg/kg is significantly higher than that for gabapentin at 100 mg/kg ($p<0.001$) (Fig. 2c).

**Effects of Koumine on Nerve Conduction Velocity**
SNCV in diabetic rats was significantly lower than that in the healthy control rats ($p<0.001$; Fig. 3). Repeated koumine treatment for one week at 1.4 and 7 mg/kg, but not 0.056 and 0.28 mg/kg, increased SNCV in diabetic rats ($p<0.05$ vs. vehicle).

**Effects of Koumine on Sciatic Nerve Morphology**
Finally, we used transmission electron microscopy to examine the ultra-structure of the sciatic nerve. In healthy rats, the nerve fiber displayed uniform and dense myelination with structural integrity. Lamellar structures also showed concentric light and dark circles and axonal structure (Figs. 4a, b). In diabetic rats, the myelin structure was disorganized and expanded toward both the axonal and the stromal sides (Figs. 4c, d). There was a visible lamellar fracture, demyelination, and separation of myelin sheath. The nuclei of Schwann cells appeared irregular, with enlarged endoplasmic reticulum, vacuolization of mitochondria and ruptured basement membrane. At a dose of 0.28, 1.4 and 7 mg/kg, but not 0.056 mg/kg koumine alleviated the damage to myelin structure and vacuolar defects, significantly mitigated the lamellar separation, increased the size of myelin and axon and normalized Schwann cells (Figs. 4, 5).

**DISCUSSION**
STZ-induced diabetes in rats causes both mechanical hyperalgesia and tactile allodynia. In the current study, koumine attenuated neuropathic pain in diabetic rats. The effect of koumine on tactile was dose-dependent, and occurred as early as after the first s.c. injection, with higher efficacy than that of gabapentin. In addition, the trend of mechanical withdrawal threshold curves of koumine treated groups suggest that consecutive administration of koumine allows different doses of koumine to reach higher effective levels. Repeated administration of koumine did not produce sedative activity and physical/psychological dependence (data not shown). The ED$_{50}$ of koumine (0.063 mg/kg) is more than 1500-fold lower than the reported 50% lethal dose (LD$_{50}$) 99 mg/kg. The lowest effective dose of koumine for neuropathic pain (0.056 mg/kg) in the current study was much lower than previously reported for CCI and SNL models (0.28 mg/kg) suggesting koumine is selective for diabetic neuropathic pain.

In addition to the behavioral effects, koumine restored sensory nerve conduction and alleviated nerve pathology in STZ-induced diabetic neuropathy. Slowing of sensory nerve conduction is an early sign of neuronal dysfunction in both diabetic rats and humans. We also observed lamellar fracture, demyelination, and separation of myelin sheath in sciatic nerves in diabetic rats which is also an sign of diabetic neuropathy. The slowing of sensory nerve conduction in diabetes might directly linked to glucose neurotoxicity and could be reversed by establishing normoglycemia. The fact that koumine did not restore glucose level in the current study suggests that the action against neuropathic pain and
associated electrophysiological nerve damage is not due to a normoglycemic effect. It is interesting to note that the slowing of sensory nerve conduction might correlate with altered ion fluxes and currents, but not any structural impairment in axonal diameter or in myelin sheath. So it is possible that koumine might restore sensory nerve conduction by altering ion fluxes and currents, however, mitigate nerve pathology by other mechanism(s).

Neuroactive steroids could provide protection against acquired and inherited peripheral neuropathy. In experimental models of diabetic neuropathy, neuroactive steroids have been shown to be neuroprotective. A previous study from this laboratory has revealed that koumine could increase allopregnanolone, a type of neuroactive steroids, in the spinal cord of CCI rats, raising the possibility that koumine alleviates diabetes-induced neuropathic pain by increasing neuroactive steroids in the peripheral nervous system. Therefore, different effects of koumine on diabetic neuropathy such as analgesic activity, sensory nerve conduction restoring activity and nerve pathology recovering activity might be mediated by neuroactive steroid system. More interestingly, we found that repeated koumine could significantly reduce the elevated total blood cholesterol levels in STZ-induced diabetic rats (data not shown) without affecting blood glucose and body weight. Koumine’s abilities to regulated blood cholesterol suggested that the activating of liver X receptor (LXR), which could control cholesterol homeostasis, elevated neurosteroids levels and protect diabetic neuropathy, might be involved in koumine’s analgesic and neuroprotective mechanism. Moreover, our team have recently found koumine could regulate mitochondrial

Fig. 4. Effects of Koumine on Sciatic Nerve Morphology Assessed with Transmission Electron Microscopy

(a, b) Normal control; (c, d): Diabetic peripheral neuropathy model group (DPN); (e, f): Koumine 7 mg/kg group; (g, h): Koumine 1.4 mg/kg group; (i, j): Koumine 0.28 mg/kg group; (k, l): Koumine 0.056 mg/kg group; (m, n): Quantification of myelin thickness (m) and number of normal Schwann cells. Left panel (a, c, e, g, i, k) 4000X magnification (scale bar=5 µm); right panel (b, d, f, h, j, l) 12000X magnification (scale bar=2 µm). Data are presented as mean±S.E.M. ***p<0.001, diabetic model group vs. normal control group; #p<0.05, ###p<0.001, koumine treated group vs. model group (one-way ANOVA with post hoc Bonferroni test). Each group consisted of 6–9 rats.
translocator protein (18 kDa) (TSPO), an outer mitochondrial membrane protein (data not shown). Koumine’s ability to regulate TSPO is similar to that of olesoxime, a cholesterol-like compound which could bind TSPO to increases conductance through mitochondrial permeability transition pore (mPTP), a channel that spans the mitochondrial inner membrane and opens when mitochondria are damaged by excessive calcium, ADP and ATP depletion, and oxidative stress. Like koumine, olesoxime’s analgesic actions in diabetic neuropathy model are significantly stronger than that in CCI model, which suggests koumine and olesoxime might share the same mechanism involving TSPO and mPTP modulation. The potencies of koumine on mechanical allodynia, SNCV and sciatic nerve morphology were different, which also suggested that koumine might exert anti-allodynic and neuroprotective effects by different mechanisms.

In conclusion, the current study demonstrated that koumine, a main alkaloidal constituent of Gelsemium elegans Benth., could attenuate tactile allodynia, improve sensory nerve conduction, and mitigate the pathology of sciatic nerves in streptozocin-induced diabetic rats. The antinoceptive effects of koumine in diabetic neuropathy seem to be more powerful than previously reported in other models for neuropathy, indicating selectivity for diabetic neuropathy. In addition to pain relief, koumine could also slow or even block the progression of nerve damage associated with diabetes.

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