PREGABALIN TREATMENT OF PERIPHERAL NERVE DAMAGE IN A MURINE DIABETIC PERIPHERAL NEUROPATHY MODEL

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Abstract

Context. Peripheral nerve lesions are a major complication of diabetes mellitus, the main clinical manifestations of which are numbness and pain involving the limbs.

Objective. To determine the correlation between pregabalin treatment and diabetic peripheral neuropathic pain.

Design. An experimental animal study in BALB/c mice.

Subjects and Methods. Diabetes models are established by injecting streptozotocin (STZ) into the abdominal cavities of mice. The correlation between the treatment effect, time, and dosage of pregabalin was determined. The effect of a type 1 organic cation transporter (Octn1) in the absorption of pregabalin was evaluated.

Results. Pregabalin reduced tactile allodynia in diabetic mice. The best analgesic effect occurred when intestinal absorption was increased. Octn1 mediated pregabalin entry into intestinal epithelial cells, which influenced the absorption of pregabalin with a time-dependent fluctuation in the small intestine. Peripheral nerve damage caused by diabetes was dependent on time and dose of pregabalin, which was related to the regular expression of Octn1 in small intestinal epithelium.

Conclusions. Peripheral nerve damage caused by diabetes was dependent on time and dosage of pregabalin, which was related to the regular expression of Octn1 in small intestinal epithelium.

Key words: curative effect, diabetes mellitus, Octn1, peripheral lesion, pregabalin.

INTRODUCTION

Peripheral nerve lesions are a major complication of diabetes mellitus, the main clinical manifestations of which are numbness and pain involving the limbs. Many patients with diabetic peripheral neuropathy feel severe limb pain, and medications are the main therapy (1, 2). Currently, pregabalin (PGN) is widely applied for relieving

the symptoms of diabetic peripheral neuropathy and fibromyalgia. It has been reported that pregabalin can change cellular calcium ion currents, adjust neurotransmitter release, and alter neuronal excitability by combining voltage-dependent calcium channels (2); however, pregabalin is not suitable for all patients. The tolerance of organisms plays an important role in the clinical efficacy of drugs. The related gene expression, protein synthesis, and other factors also have an influence on the effect of drugs. Some proteins have an effect on drug sensitivity and drug dynamic characteristics, which are related to the circadian rhythm and are time-dependent (3-5).

In this study, mice were used to establish a diabetes model, and the correlation between the time and dosage of pregabalin administration and the analgesic effect was determined. The expression of small intestinal epithelium-associated transport proteins was investigated microscopically to provide a guidance for the specific drug dosage regimens and to achieve maximal therapeutic effects.

MATERIALS AND METHODS

Animals and model construction

Forty 5-week-old adult male BALB/c mice were bought from Beijing Biological Technology Co., Ltd. (Beijing, China). All of the mice were raised under standard conditions. ZT0 was defined as daylight hours and >ZT12 was darkness. Pregabalin was dissolved in normal saline, and the injected dose was 0.1 mL/10 g of body weight.

During the study, streptozotocin, which has a toxic effect on pancreatic β cells, was used to induce the diabetes model in mice ($n=20$) by intraperitoneal injection of 200 mg/kg (6). Three days later, the blood glucose level was measured using a glucometer (Accu-Chek Active, GC model; Roche, Germany). The weights were also measured after the injection. The

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observation time was 3 weeks. All mice were randomly divided into control (physiologic saline, n=20) and experimental groups (pregabalin, n=20). All of the operations in the studies were approved by the Ethics Committee of our hospital and complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving animals.

Assessment of diabetic peripheral neuropathic pain

A fiber thread measuring instrument (Shanghai Jade Research Scientific Instrument Co., Ltd., Shanghai, China) was used to detect and evaluate diabetes-induced neuropathic pain. The mice were placed in a plastic cage with a wire net on the bottom of the cage. The mice adapted to the environment for 30 min, then the paw withdrawal threshold (PWT) was determined using a method described previously (7). To evaluate the treatment of pregabalin on diabetic peripheral neuropathic pain, the area under a curve (AUC) was calculated using the trapezoidal rule of the entire PWT duration.

Concentration of pregabalin detection

The concentration of pregabalin in serum and cell lysates was detected using high performance liquid chromatography (HPLC). Gabapentin is derived from phthalic aldehyde and was designated as the internal standard. Phosphate buffer solution was used for the mobile phase (pH 3.0, 20 mmol) and acetonitrile (volume ratio, 3:1) passed through a 5C18-MS-II chromatographic column (4.6 x 150 mm) at a flow rate of 1.0 mL/min. The separated analyte was detected with a RF-20AXs fluorescence detector. Pregabalin and gabapentin were used with a total running time of 30, 20, and 28 min. The serum concentration of pregabalin was expressed as μM , and the accumulation in enterocytes was marked as nmol/mg of protein.

Drug kinetic analysis

A non-linear mixed effect model (NONMEM) was adopted for analyzing the serum concentration of pregabalin, which was the model for studying pharmacokinetic parameters in the population. The calculated pharmacokinetic parameter in the population based on the pregabalin concentration in the serum was obtained using a one compartment model with first-order absorption. Individual pharmacokinetic parameters were obtained from NONMEM post-hoc analyses. The changes, which were evaluated by adding covariate or object function (OBJ), and the changes

in OBJ values for each model were compared. The difference was statistically significant when the OBJ value was 3.841 and the degree of freedom was 1.

Transfer of pregabalin

Immortalized small intestinal epithelial cells (aMoS7) on small intestinal crypts of mice were built. The functional expression of this cell was Octn1; thus, whether or not intestinal absorption of pregabalin was mediated by Octn1 was determined. The cells were cultured in Eagle culture media (DMEM) which was improved by Dulbecco and supplemented with 10% fetal bovine serum, 25 $\mu\text{g/mL}$ green enzyme, 25 $\mu\text{g/mL}$ *Streptomyces griseus*, and 10 $\mu\text{g/mL}$ of insulin. Lipofectamine 2000 reagent was used for transfecting oligonucleotides in aMoS7 cells at a final concentration of 1,050,100 pmol/ well. To make the overexpression in Octn1 cells, a sub-clone cDNA overall length of SLC22A4 to the BamHI and XhoI position was made with pcDNA 3.1 as the carrier. Then, the construction body was transferred to aMoS7 cells. Transgenic-expressing cells were transferred by G418 (Wako) and a single colony was amplified in 4 $\mu\text{g/mL}$ of G418 culture media. The primary cultures of aMoS7 cells were kept at 37°C for 30 min, and the content of the transport medium included the following: 115mM NaCl; 2.5mM KCl; 1mM CaCl_2 ; 1mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 10mM HEPES; and 5mM amylaceum (pH 7.4). After pre-incubation, the cells were cultured in 2 mL of pregabalin solution. Then, the cells were twice-washed at a low temperature in PBS, a homogenate of cells was made in PBS, and the pregabalin concentration was determined by HPLC.

Detection of pregabalin intestinal accumulation in the intestine

Related tissues were obtained from the proximal jejunum in diabetic mice at ZT2 and ZT14. The separated tissues were washed in low-temperature salt water and incubated in Krebs-Ringer buffer solution for 20 min at 37°C. The contents of the solution were 128.9 mM NaCl, 4.2 mM KCl, 1.5 mM CaCl_2 , 22.4 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 1.3 mM MgSO_4 , and 10 mM D-amylaceum. Pregabalin (the concentration was 0.5 mM) was then added and incubated for 30 min at 37°C. The tissues were washed in low-temperature PBS after culturing and homogenized in 300 mL of PBS. The concentration of pregabalin in the homogenate was determined by HPLC.

Immune imprinting experiment

Small intestinal tissues were obtained from

mice. CellLyctic™ MT cell lysis solution (Sigma-Aldrich, St. Louis, MO, USA) was used for tissue homogenization and centrifuged at 12,000g for 10 min.

Figure 1. Changes in blood glucose, weight, and PWT during the observation period of control and experimental groups. *, p<0.05.

Figure 2. Effect of dosing time on analgesic results of pregabalin. Changes in PWT and blood serum PGN at ZT2 and ZT14 for the experimental group on the 21st day. Horizontal ordinate was the time pregabalin was given.

Supernatant protein was separated on lauryl sodium sulfate-polyacrylamide gels and transferred to PVDF membranes. The PVDF membranes HAVE reacted with anti-mouse Octn1 or actin. Visualization of specific antigen-antibody complexes was facilitated by horseradish peroxidase conjugated secondary antibody and Chemi-Lumi One.

Statistical analysis

Variance analysis and Fisher's least significant difference test were applied for evaluating the differences between groups. A p<0.05 was considered to represent statistical significance.

RESULTS

Establishment of STZ-induced diabetic peripheral neuropathy model

A single dose of STZ (200 mg/kg) was injected into the abdominal cavities of mice to establish the diabetic peripheral neuropathy model. This toxin of bacterial origin destroys pancreatic islets β cells and induces insulin deficiency. Thus, STZ-induced hyperglycemia is always used as a diabetes model in animals. The blood glucose level increased significantly after 3 days of oral intake (Fig. 1A), the weight was reduced (Fig. 1B), and the PWT was gradually reduced and reached the lowest point on the 14th day (Fig. 1C).

Effect of time and dosage of pregabalin on analgesic efficacy

Pregabalin (50 mg/kg) was injected into the abdominal cavities of the experimental group at ZT2 and ZT14 on the 21st day. PWT appeared to increase at two time points, but there was no significant difference in dosage and time (p>0.05; Fig. 2A). Drug kinetics showed that the serum concentration of pregabalin reached a peak value of 0.5 h after injection at ZT2 and

Figure 3. Octn1-mediated pregabalin entry into intestinal epithelial cells. The left chart was immunoblotting, which analyzed the expression of Octn1 on aMoS7 cells (applied Octn1 carrier and blank carrier for transfecting aMoS7 cells, respectively). The right chart showed the increased absorption of pregabalin with Octn1 over-expression. *, $p < 0.05$.

Figure 4. Day and night expression changes of Octn1 protein in intestinal epithelial cells of the experimental and control groups. There were significant time-dependent differences between these two groups.

ZT14 and there was no significant difference between the two time points ($p > 0.05$). Thus, the analgesic effect of dosing time-dependent differences for pregabalin was related to pharmacokinetics (Fig. 2B).

Octn1 mediated pregabalin to enter the small intestine

Immortalized mouse intestinal epithelial cells (aMoS7) were used and protein expression of Octn1 by vector-transfected cells was increased. There was no apparent change in protein content by using an empty vector. The accumulation of pregabalin in aMoS7 cells further promoted the expression of Octn1 ($p < 0.05$). Octn1 facilitated the transfer of pregabalin entry into the cells, and the level of expression was related to the absorption and analgesic effect of pregabalin (Fig. 3).

Dosing time regularity of Octn1 expression

On the 21st day after STZ treatment, the

expression of Octn1 protein demonstrated apparent day and night changes, which usually reached a maximum value in the early stages of the dark (ZT12-ZT14). This rhythmicity was also found in the control group (Fig. 4). Therefore, the dosing time dependence of absorption in the small intestine might be caused by the day and night regularity of Octn1.

DISCUSSION

As people have different metabolic features at different time points every day, the curative effects of drugs at different time points also exhibit differences. Some studies have shown that organs have day and night sensitivity differences to drugs, and the pharmacokinetics have an effect on the efficacy of drugs (8-10).

In the current study, an analgesic effect produced by pregabalin in the diabetic mouse model induced by chain urease was evaluated, and the analgesic effect of ZT14 was stronger than ZT12. The peak concentrations of pregabalin at ZT14 were clearly higher than ZT12. A dosing time-dependent difference and serum concentration of the pregabalin analgesic effect were not demonstrated in this study. The kinetic differences of oral pregabalin could cause a dosing time-dependent difference with respect to the analgesic effect.

Pharmacokinetics includes absorption, decentralized transportation, metabolism, cleaning, and other processes. To analyze the diurnal variation of pregabalin pharmacokinetics, the NONMEN program was used to analyze the serum concentration

of pregabalin after oral administration. A dosing time-dependent difference existed in the diurnal variation of the absorption rate constant (K_a), but the related feature did not exist in the mice in which drugs were injected into the abdominal cavities.

Intestinal epithelial cells express many kinds of transporters, which have positive or negative effect on drug absorption of the small intestine. It was shown that there are three kinds of Octn transporters that participate in drug absorption of pregabalin, and the related effect of Octn1 was evident. Octn1 takes part in mediating gabapentin, a pregabalin analogue, to combine with intestinal epithelial cells (5,11-13). With respect to the up-and-down regulation of Octn1 expression, it was also shown that Octn1 mediate pregabalin absorption in aMoS7 cells. At the same time, it was shown that the substrate, ERGO of Cctn1, could control the absorption of pregabalin in cultured rat small intestinal epithelial cells (14-16). In experimental animals, the function and expression of many intestinal epithelial transporters show the features of diurnal variation. Octn1 presented the features of diurnal cycle time variation. If Octn1 was incubated with 0.5 mM jejunum segment cells together, then the absorption regularity of pregabalin dosing time-dependence was lost. Although there are other transporters that can mediate the absorption of pregabalin to remain the same in one day, then the most important transporter was Octn1. The expression of Octn1 was effectively increased, which was derived from phthalic aldehyde absorption of pregabalin (17,18). PPAR α showed a time-dependent regularity of expression by the time-dependent inhibition of bile acid expression (19). Streptozotocin induced an increased food intake of diabetic mice, but the consumption during the dark time also increased. Thus, aggregation of bile acids in the small intestine had an effect on day and night activity of PPAR α , which caused a time-dependent expression of Octn1.

With respect to pharmacokinetics, some studies have mentioned that temporary expression analysis of rat spinal $\alpha 2\delta$ -1 subunit induced by streptozotocin showed that day and night time-dependent expression of this subunit could influence the analgesic effect of gabapentin (20-22). The analgesic effect of pregabalin at ZT14 was higher than ZT2 after oral administration. Expression of Octn1 protein in small intestinal epithelium cells showed day and night time variation regularity, with the peak value occurring during the early period of the dark cycle. Thus, day and night regularity changes in the small intestinal absorption process may directly influence the analgesic effect

of pregabalin on related dosing time-dependence of diabetic mice.

A limitation in this study is that the glycemia was detected by glucometer, which might cause unpredictable errors.

In conclusion, the results of this study preliminarily showed that the analgesic effect of oral pregabalin on peripheral nerve pain in diabetic mice was directly related to the dosing time variation. Further research on the intestinal epithelial absorption process would be helpful to improve the analgesic effect of pregabalin. Adjusting the existing time-dosing plan will improve the analgesic efficacy of pregabalin on diabetic peripheral neuropathic pain.

Conflict of interest

The authors declare that they have no conflict of interest.

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