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Study on the effects of parecoxib on hypothalamus orexin neuron of cerebral infarction rats

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Abstract. – OBJECTIVE: To explore the effect of parecoxib on cerebral infarction in rats and the regulatory mechanism on hypothalamus orexin neurons (orexin) and protein expression.

MATERIALS AND METHODS: 60 SD male rats were randomly divided into sham operation group, model group and treatment group (20 rats in each group). Cerebral infarction model was established by modified Longa method. Rats in the treatment group were given parecoxib (2.5 mg kg^{-1}) in tail by intravenous injection, while both the sham operation group and the model group were given the equal volume of sterile PBS solution in the tail vein. Continuous intervention of 72h was carried out in the three groups. Immunofluorescence staining and Western blot were used to detect the expression of orexin neurons and orexin protein in the hypothalamus of rats, respectively.

RESULTS: Immunofluorescence staining showed that the number of orexin positive cells in the model group was significantly less than that in the sham-operated group ($p < 0.01$). After treatment intervention, the number of orexin positive cells in the hypothalamus was significantly increased compared to that in model group ($p < 0.01$). Western blot analysis showed that compared with sham operation group, the expression of orexin in the hypothalamus of model group was significantly decreased ($p < 0.01$), whereas the expression of orexin protein was significantly elevated after parecoxib intervention ($p < 0.01$).

CONCLUSIONS: Parecoxib plays a therapeutic effect on cerebral infarction by up-regulating the orexin neuron.

Key Words:
Cerebral infarction, Orexin neuron, Rats.

Introduction

Cerebral infarction is a type of common disease in clinical neurology, accounting for 75% of the total cerebrovascular disease[1]. The direct cause of cerebral infarction is the disorder in local cerebral blood circulation, leading to high morbidity and high mortality. The sequelae of movement disorders, aphasia, and disturbance of consciousness is often observed a small number of patients after discharge[2]. At present, the pathogenesis of cerebral infarction is mainly related to local cerebral ischemia, hypoxia, apoptosis, and necrosis of brain cells and excitatory toxicity, etc.[3]. In recent years, orexin has been shown to exert a protective effect on the brain through the promotion of hypoxia-induced pathway[4].

Parecoxib is a new developed, non-steroidal anti-inflammatory drug, a highly selective COX-2 inhibitor in clinical application only by injection, which cures cerebral infarction by scavenging free radicals, anti-oxidation, inhibiting excitatory amino acid release and inhibiting neuronal apoptosis and other ways[5]. However, in the treatment of cerebral infarction, regulating mechanism of parecoxib on hypothalamic orexin neurons remains unclear. In this study, we aimed to investigate the role of parecoxib on orexin neuron within the rat model of cerebral infarction.

Materials and Methods

Materials

Male clean grade SD rats are provided by the Experimental Animal Center (300-325 g, free drinking and eating, 22 ± 0.5°C, humidity 60 ± 2%, daylight 7AM-7PM). All animal feeding procedures are strictly in accordance with the provisions of the Animal Protection Committee established by the Animal Management Committee. Parecoxib for injection was purchased from Shineway Pharmaceutical (approval number: Z13020795) (Shijiazhuang, Hebei, China); goat serum, DAPI fluorescent dyes and fluores-

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cent sealing agents were purchased from Vector Laboratories Corporation (Burlingame, CA, USA); primary antibody of Orexin polyclonal rabbit antibody was purchased from Santa Cruz Biotechnology with Lot SC-289375 (Santa Cruz, CA, USA), and secondary antibody of Alexa fluor® 594-goat anti-rabbit IgG was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). Lot A-11072, rabbit anti beta-Actin polyclonal antibody, bicinchoninic acid (BCA) protein kit and ECL Kit were purchased from Biyuntian Biotech Corp. (Beijing, China); horseradish peroxidase (HRP) labeled Goat anti-rabbit IgG was purchased from Beijing Jinqiao Technology Co., Ltd., (Beijing, China).

Experimental Grouping

60 male SD rats were randomly divided into 3 groups: sham operation group, model group, and treatment group, 20 rats each group. Rats in model group were treated with modified Longa method to establish model of cerebral infarction; according to the early research results, after 3 hours of successful model, the rats were given intravenous injection of parecoxib (2.5 mg·kg-1) in tail, while both the sham operation group and the model group were given the equal volume of sterile phosphate-buffered saline (PBS) solution into the tail vein, but the sham group do artery separation in surgery, without ligation of plug wire. The three groups were administered 1 times per 12 h, administered continuously for 6 times.

Establishment of Cerebral Infarction Model in Rats

The specific method of cerebral infarction model is established according to the modified Longa method[6], and a permanent cerebral infarction model (MACO) was established by suture method. Rats were anesthetized with 10% chloral hydrate (0.35 g/kg, i.p.), the supine position of which was fixed, after disinfection in the middle of neck, right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were collected and observed under microscope. Horizontal and sequentially ligation of CCA and ECA were carried in the near heart, which were clamped with an arterial clamp. A small incision was cut near the heart of CCA, the bolt was inserted from the CCA to the ICA direction, until there was a sense of resistance (the depth is about 20 mm); the thread end was cut and fixed, while skin suture and disinfection were carried out with penicillin.

Immunohistochemistry

Tissue sections: the rats in each group were anesthetized with 10% chloral hydrate, saline and 4% paraformaldehyde were used for perfusion and fixation. The brain tissue was taken out rapidly, after immobilization, sucrose dehydration and OCT embedding, the slices were cut by frozen section machine (Leica, Wetzlar, Germany). The thickness of the film was 30 μm. Tissue sections were collected for future test with the buffer. Slices were rinsed into PBS, closed solution of 10% goat serum and 0.3% Triton-X-100 were treated at room temperature for 1 h, mixed liquor of primary (5% Donkey serum, 0.3% Triton-X-100, Rabbit anti hrGFP PBS buffer) was added for overnight at 4°C; slices were rinsed with PBS solution for 3 times (10 min/time), PBS solution (5% Donkey serum, 0.3% Triton-X-100, Alexa Fluor®488 Donkey anti-rabbit IgG fluorescent secondary antibody (1:500) was added for the incubation of 2 h under the condition of avoiding light. After phosphate-buffered saline (PBS) solution for 3 times (10 min/time), the slices were mounted with anti-fluorescence quenching sealing agents, which were observed under the fluorescence microscope.

Image analysis: 5 photos were taken for each rat. 5 visual field were randomly selected under each photo and processed with Image-proplus6.0 professional image analysis software (MediaCybernetics, Rockville, MD, USA). The average optical density (OD) and surface density (AD) were calculated.

Detection the Expression of Orexin Protein by Western Blot

Total protein extraction: the whole hypothalamus tissue was obtained after aseptic sampling, which was quickly put on ice for homogenate. After the lysis, BCA kit was adopted for quantitative protein, and then the protein was separated by electrophoresis. The protein was enclosed for 2 h after transferring, and then primary antibody of orexin (1:1000) was incubated overnight; horseradish peroxidase (HRP) labeled Goat anti-rabbit IgG was added for incubation with 2 h. Finally, protein bands were revealed by chemiluminescence method. With β-actin as the reference, average absorbance (AA) of each band was detected. Each specimen was repeated 3 times to get the average value. NIH Image J image software was adopted to analyze the pictures.

Analysis the Level Change of Bcl-2 and Bax with RT-PCR

Total RNA from 0.1 g of cerebral tissues of rats in each group was extracted according to the instructions for RNA extraction. In brief, cerebral tissues were cooled in liquid nitrogen for cutting into pieces, which were then homogenized with 1 ml TRIzol. 0.2 ml chloroform was added and mixed, and upper aqueous phase was collected by 4°C of centrifugation. 0.5 ml isopropyl alcohol was added, and the supernatant was discarded and added into 1 ml 75% ethanol for 2 times washing. After centrifugal and drying, 20 μl DEPC H_2O was added for RNA dissolution. RNA was converted to cDNA by reverse transcription kit, which was regarded as PCR template. According to the mRNA sequence provided by GeneBank to design primers, (bacx primers, sense chain: 5'-TCCACCAAGAAGCTGAGCGAG-3', antisense: 5'-GTCCAGCCCATGATGGTTCT-3', the length of amplified product was 257 bp. Bcl2: upstream sequence: 5'-TTCTTTGAGTTCGGTGGGGTC-3', downstream sequence: 5'-TGCATATTTGTTTGGGGCAGG-3', amplified fragment length was 304bp 941-1244. GAPDH sequence F: CGGAGTCAACGGATTTGGTCGTAT, R: AGCCTTCTCCATGGTGGTGAAGAC), which was diluted to 10 μmmol/L. The reaction conditions of primer specificity and annealing temperature were optimized, and the corresponding reaction system with 20 μl total volume was established. After centrifugation with 1500 rpm, PCR was carried out under the following condition: 95°C pre-denaturation for 30 s, 95°C denaturation for 30 s, 60°C annealing elongation for 30 s. Real-time quantitative PCR reaction was obtained by using SYBR Green I fluorescent dye technique. The standard curve of each sample was obtained, and the CT value was calculated by the computer.

Behavioral Score

Behavioral observation includes: after 2 h cerebral ischemia followed by 4 h and 22 h reperfusion, the rat's tail was hanged at 35 cm high. Two limbs activity status were observed. Behavioral score[7] ranged from 0 to 4 points. High score refers to serious neurobehavioral damage.

Statistical Analysis

The experimental data were analyzed by SPSS17.0 statistical software (SPSS Inc., Chicago, IL, USA). Measurement data were recorded with mean ± standard deviation ($\bar{x} \pm s$), while multiple comparisons among groups were analyzed using ANOVA analysis. $p < 0.05$ for the difference was statistically significant.

Results

Immunofluorescence Staining Results

Immunohistochemistry showed that the hypothalamic orexin neurons were densely distributed. Cells were oval or fusiform with evident protruding, which mostly were bipolar neurons (Figure 1A). Image analysis showed that the number of orexin neurons in model group was significantly less than that in sham-operated group ($p < 0.01$) (Figure 1B, Table I). However, the number of orexin neurons in the treatment group was significantly higher than that in the model group ($p < 0.01$) (Figure 1C, Table I).

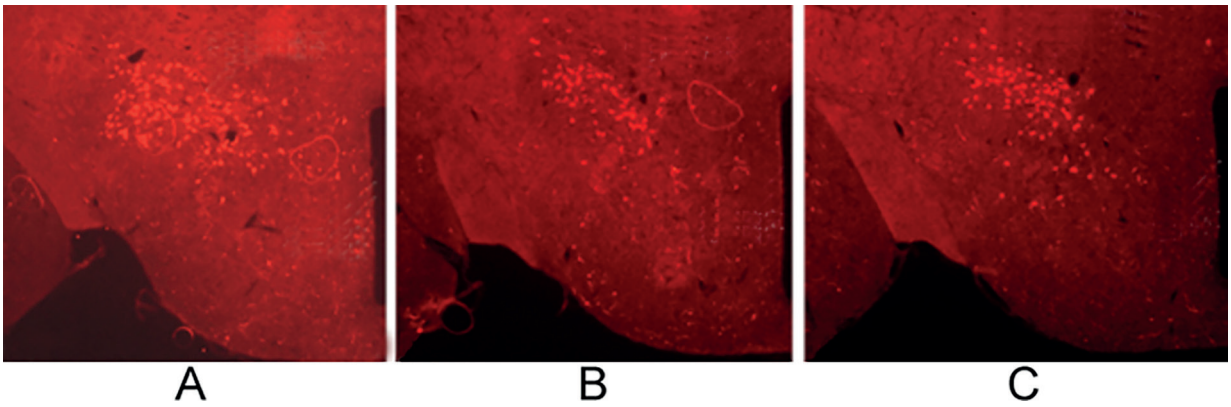

Figure 1. Effect of parecoxib on expression of hypothalamic orexin neurons in rats with cerebral infarction. ***A,*** Sham operation group. ***B,*** Model group. ***C,*** Treatment group (The scale are both 500 μm). The lateral hypothalamic area and the third ventricle were clearly seen in each group.

Table I. Comparison of expression of orexin neurons in hypothalamus of three groups ($\bar{x} \pm s$).

Group	n	OD value	SD value
Sham operation group	20	0.89 ± 0.05	0.91 ± 0.09
Model group	20	0.39 ± 0.07*	0.41 ± 0.06*
Treatment group	20	0.61 ± 0.08*,#	0.65 ± 0.04*,#

*Compared with sham operation group, $p < 0.01$; #Compared with sham model group, $p < 0.01$.

Detection the Expression of Orexin Protein in Hypothalamus of Three Groups with Western Blot

The results of Western blot showed that the expression of orexin in hypothalamus of model group was significantly decreased, compared to that of sham operation group ($p < 0.01$), whereas the orexin protein content in the treatment group was significantly elevated compared to that in the model group ($p < 0.01$; Figure 2, Table II).

The Changes of Caspase-3 mRNA, Bcl-2, Bax

Fluorescent quantitative PCR analysis showed that compared with sham operation group, the levels of Caspase-3 and Bax in brain tissue of model group and treatment group were significantly increased ($p < 0.05$). Of note, compared with the model group, the levels of Caspase-3 and Bax were significantly reduced in the treatment group ($p < 0.05$); moreover, compared with that in the sham operation group, Bcl-2 level was significantly inhibited the model group and the treatment group. Notably, Bcl-2 level in the treatment group was significantly up-regulated compared to that in model group ($p < 0.05$) (Figure 3).

Behavioral Scoring Results

Compared with the model group, the behavioral scoring results of sham operation group and the treatment group were significantly lower at 4 h after reperfusion, but there was no significant difference between the two groups ($p > 0.05$). Intriguingly, the behavioral score of the treatment group was statistically increased after reperfusion 22 h, compared with sham operation group ($p < 0.05$) (Table III).

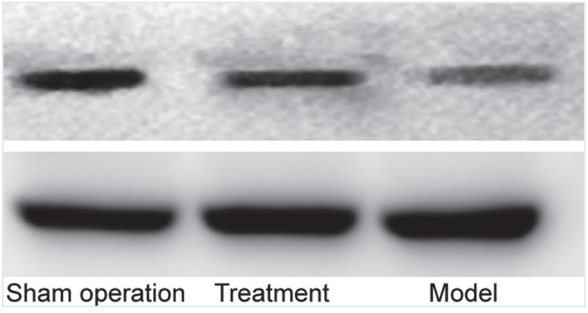

Figure 2. Expression of orexin protein in hypothalamus of three groups.

Discussion

Cerebral infarction is commonly found in the elderly, posing a serious threat to human health. It is generally believed that cerebral infarction is mainly due to the disorder of local blood supply in the brain tissue, leading to the corresponding neurological damage and signs[8]. The main cause of cerebral infarction includes ischemic injury, abnormal blood rheology, and equilibrium destruction of the fibrinolytic system. The main treatment methods of cerebral infarction are thrombolytic, anti coagulation, and anti neuronal apoptosis; however, drawbacks still exists[9]. Orexin neurons is mainly located in the hypothalamus, which contains 2 components of orexin-A and orexin-B with 2 corresponding G protein receptor of OX1R and OX2R. It has been found that the hypothalamic orexin system in cerebral injury plays an important regulating role[10]. This study primarily focuses on orexin-A. Parecoxib is a specific inhibitor of COX-2, which shows good curative effect on the treatment of cerebral infarction.

Table II. Expression of orexin protein in hypothalamus of three groups ($\bar{x} \pm s$).

Group	n	Orexin protein level
Sham operation group	20	0.91 ± 0.08
Model group	20	0.62 ± 0.04*,#
Treatment group	20	0.33 ± 0.06*

*Compared with sham operation group, $p < 0.01$; #Compared with the spinal cord injury group, $p < 0.01$.

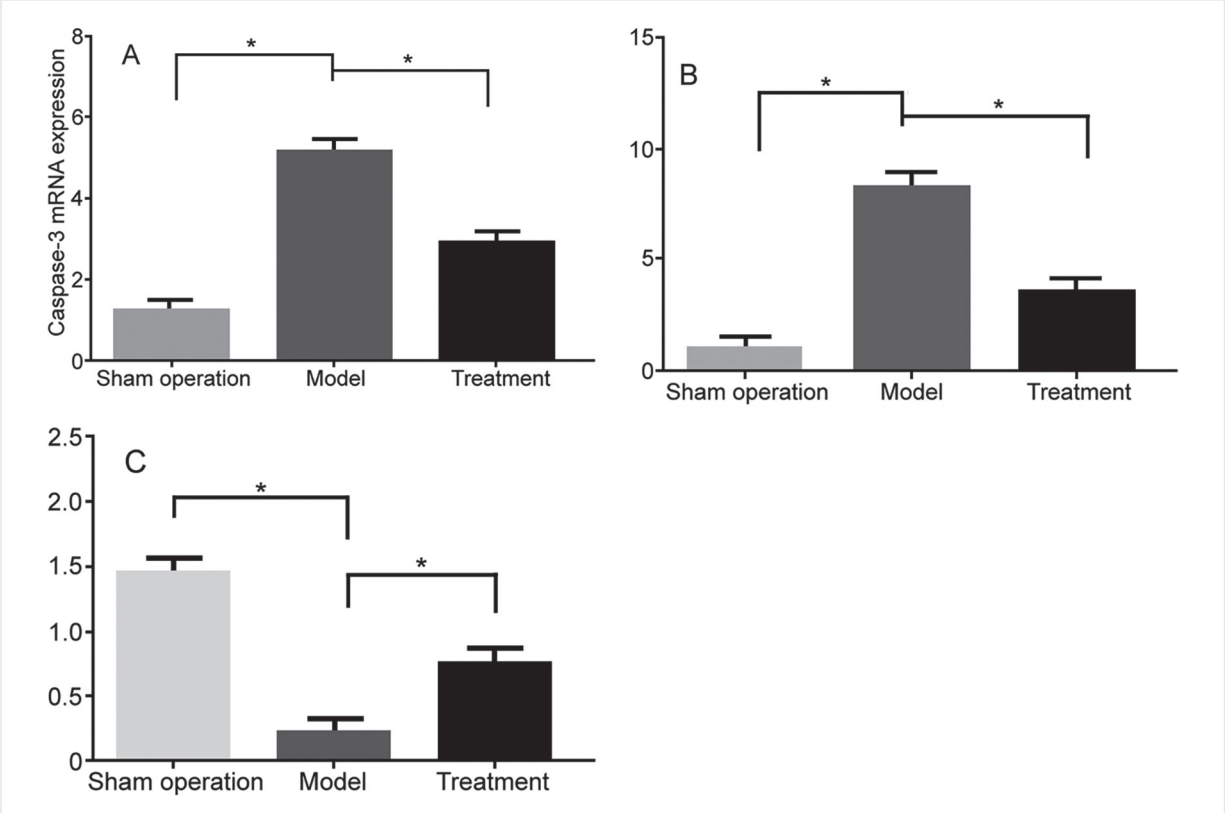

Figure 3. Statistical comparison of Bcl-2 and Bax levels in three groups of rat models. ***A,*** Represents caspase-3 mRNA level. ***B***, Indicates Bax level. ***C,*** Indicates Bcl-2 level).

Therefore, the anti-inflammatory, antioxidant and immunomodulatory effects of parecoxib are being paid more and more attention. Cerebral ischemic injury is associated with many pathological processes, and it is considered that typical infectious inflammatory injury, the release of oxygen free radical and inflammatory mediators are the most important factors in the progress of ischemic injury. Thus, it is mandatory to study the effect of parecoxib on cerebral infarction[11]. Previous studies have shown that parecoxib can reduce the infarct volume of rats, inhibit the expression of Caspase-3 proteins, thereby protecting neurons in brain tissue[12]. The results showed that the death of the penumbra was mainly mediated by apoptosis, and clinicians have realized that early prevention and timely treatment will directly affect the prognosis of patients[13,14]. It has been proved that parecoxib has antioxidant, anti-aging, reduce ischemia-reperfusion injury and enhance memory function in animal body level, the possible mechanism is that flavonoids can neutralize oxygen free radicals, prevent vascular wall thickening and hardening, and improve the supply of oxygen and nutrients to the brain[15]. This research employed the rat MCAO model, and observed that after parecoxib intervention, orexin neurons and their protein levels in hypothalamus were changed. Our pre-experiment found that parecoxib (2.5-3 mg•kg^{-1}) was used for 2-3 d, which can reduce the animal behavior changes after cerebral infarction, and improve the neuronal dysfunction. Our data showed that, compared with the model of cerebral infarction group, parecoxib may significantly increase the number of hypothalamic orexin neurons ($p < 0.01$), which suggests that it can resist the apoptosis of neurons. In addition, Western blot

Table III. Behavioral score statistics of 3 groups rat model.

Group	Reperfusion 4 h	Reperfusion 22 h
Model group	2.72 ± 1.12	2.74 ± 1.02
Shame operation group	1.41 ± 1.19	2.16 ± 0.95
Treatment group	1.44 ± 1.26	1.35 ± 1.19

results showed that, after parecoxib intervention, hypothalamic orexin expression was significantly higher than that in the model group ($p < 0.01$), suggesting that the protective effect of parecoxib on brain tissue after cerebral infarction may be associated with the up-regulated expression of orexin in hypothalamus. Apoptosis is regulated by multiple genes. Studies have shown that mitochondrial pathway plays an important role in the mechanism of apoptosis[16]. It is known that Bcl-2 and Bax belong to a pair of important regulatory genes, which have important influence on cell apoptosis[17]. Caspase-3 is the most important apoptotic protease in apoptosis. Its activation largely depends on the release of Cyt-c, the Bcl-2 and Bax genes in the Bcl-2 family, which can be used to mediate the release of Cyt-C[18]. From the data of this study, compared with sham operation group, the levels of Caspase-3 and Bax in brain tissue of model group and treatment group were significantly increased; and compared with the treatment group, the levels of Caspase-3 and Bax were significantly increased in the model group. Conversely, the level of Bcl-2 presented opposite result, indicating that parecoxib can effectively reduce brain cell apoptosis in rats with cerebral infarction. Neuroanatomical studies[19-21] found that the fibers of orexin neurons in the hypothalamus were wildly projected into the various regions of the brain, in which regulation effect is related to arousal, diet, addiction, endocrine and cardiovascular system. However, in the process of cerebral disease, the exact mechanisms of projections of orexin neurons in the hypothalamus change as well as the efficacy of parecoxib on animal after cerebral infarction require further investigation.

Conclusions

Parecoxib increases the number of orexin neurons, inhibits the apoptosis of orexin neurons and plays a therapeutic role in rats with cerebral infarction.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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