Antidepressant effect of Xingnao Jieyu decoction mediated by alleviating neuroinflammation in a rat model of post-stroke depression

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Abstract

OBJECTIVE: To identify the antidepressant effect of Xingnao Jieyu (XNJY) decoction on a post-stroke depression (PSD) rat model and the underlying molecular mechanism.

METHODS: We established a rat PSD model by middle cerebral artery occlusion (MCAO) combined with chronic unpredictable mild stress (CUMS). Healthy SD rats were randomly divided into six groups: sham, PSD, fluoxetine (Flu), and XNJY groups at low, middle, and high doses. The sham group underwent sham operation, while the other groups underwent MCAO+CUMS. The Flu and XNJY decoction groups were intragastrically administered with Flu or different doses of XNJY for 21 consecutive days. Histopathological changes in the cortex and hippocampus were observed by staining with hematoxylin and eosin and Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling. Iba1 positive cells were evaluated by immunofluorescence assay. The expressions of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), 5-hydroxytryptamine (5-HT), and norepinephrine (NE) in the cortex and hippocampus were measured by enzyme linked immunosorbent assay.

RESULTS: The PSD group rats had a significant decrease in body weight, consumption of sucrose water, and locomotor activity but an increase in immobility time during a forced swimming test (P < 0.01) compared with sham group. Flu and different doses of XNJY significantly recovered these indices (P < 0.01). XNJY also inhibited neuronal damage and apoptosis in the cortex induced by PSD (P < 0.01). Furthermore, XNJY reduced the number of Iba1 positive cells and the expressions of TNF-α, IL-6, and IL-1β, in addition to recovered the levels of 5-HT and NE in the cortex and hippocampus (P < 0.01).

CONCLUSION: The alleviation of neuroinflammation might be an important mechanism of the XNJY decoction against PSD. Thus, XNJY might be a promising candidate for the treatment of PSD.

Keywords: Depression; Stroke; Infarction, middle cerebral artery; Stress; Inflammation; Antidepressive agents; Xingnao Jieyu

INTRODUCTION

Post-stroke depression (PSD) affects one-third of stroke survivors and leads to greater disability, and in some cases increased mortality. This behavior is the most common emotional disorder after stroke. The pathological mechanisms of PSD involve neurotrans-
mitter deficiency, hypothalamic-pituitary-adrenal axis deregulation, neuroinflammation, and oxidative stress, of which inflammation in the brain is a crucial process linking stroke and depression. Neuroinflammation after stroke influences the repair of neural damage and subsequent pathologies. Extensive studies have illustrated that chronic and excessive inflammation throughout stroke exacerbate vascular dysfunction and lead to severe neuronal cell death. This phenomenon causes dementia or depression. Thus, alleviating neuroinflammation may be a potential therapeutic intervention for PSD. Pharmacotherapy is currently the main antidepressant treatment, and alleviating inflammation contributes to the clinical efficacy of antidepressants. Clinical trials of conventional antidepressant medications showed significant beneficial effect but with toxic and side effects and drug withdrawal syndrome. Traditional Chinese Medicine (TCM), which induces few side effects and has multiple targets, was reported to have potential in the treatment of PSD. Xingnao Jieyu (XNJY) decoction, a TCM preparation, has been shown to alleviate the PSD model rats through BDNF/ERK/CREB pathway. To further clarify the effect of XNJY on neuroinflammation, middle cerebral artery ischemia combined with chronic unpredictable mild stress (CUMS) is used to establish a PSD model in rodents, which is the most commonly-used model that mimics the clinical features of PSD. In the present study, we established a rat model of PSD using middle cerebral artery occlusion (MCAO) combined with CUMS to investigate the antidepressant effects and the influence of XNJY on the inflammatory response in rat PSD.

MATERIALS AND METHODS

Materials
The XNJY decoction consisted of Shichangpu (Acorus gramineus, 12 g), Yuanzhi (Radix Polygala, 12 g), Danshen (Radix Salviae Miltiorrhizae, 30 g), Chaihu (Radix Bupleuri Chinensis, 12 g), Hehuannpi (Silktree Albizzia Bark, 15 g), Yujin (Radix Curcumae, 10 g), and Baji tian (Radix Morindae Officinalis, 10 g). Fluoxetine hydrochloride (Flu) was obtained from Lilly Company (d) were inserted in the right internal carotid artery, and Poly-l-lysine-coated monofilament nylon sutures (#2534-50, Hanan, China) were inserted in the right internal carotid artery, advanced to occlude the right middle cerebral artery, and then withdrawn 60 min after occlusion. After MCAO surgery, all rats were kept in cages without any stress and had free access to food and water for 2 d. Then, the rats were equally and randomly allocated into the sham, PSD, Flu, and XNJY (low, middle, or high dose) groups. The sham group was intragastrically administered with saline for 21 d. The PSD group was treated with unpredictable mild stress and intragastrically administered with saline for 21 d. The Flu group was treated with unpredictable mild stress and intragastrically administered with Flu (2.08 mg/kg) for 21 consecutive days. The XNJY groups were treated with unpredictable mild stress and intragastrically administered with different doses of XNJY decoction (XNJY-L, 10.5 g/kg; XNJY-M, 21 g/ kg; and XNJY-H, 42 g/kg) for 21 consecutive days.

Middle cerebral artery ischemia and psd models
The model of the middle cerebral artery ischemia in rats was prepared by MCAO. Poly-l-lysine-coated monofilament nylon sutures (#2534-50, Hanan, China) were inserted in the right internal carotid artery, advanced to occlude the right middle cerebral artery, and then withdrawn 60 min after occlusion. After MCAO surgery, all rats were kept in cages without any stress and had free access to food and water for 2 d before further experiments. The neurological deficit score was determined using the Zea-Longa5 method to determine the successful establishment of the stroke model. Stroke model rats were prepared by CUMS to establish the PSD model.

Neurological deficit score
Neurological deficit scores were determined after...
MCAO surgery and evaluated on a five-point scale system: 0, no neurologic deficit; 1 (failure to fully extend left forepaw), mild focal neurologic deficit; 2 (circling to the left), moderate focal neurologic deficit; 3 (falling to the left), a severe focal deficit; and 4 (no spontaneous locomotor activity or barrel rolling), a critical focal neurologic deficit. Rats with no neurologic deficit were removed from further study.

**Chronic unpredictable mild stress**

Rats underwent the CUMS procedure following Willner’s method with some modifications. Rats were exposed to 7 different stressors for 21 consecutive days to induce a depressive-like state. The stressors included food and water deprivation (24 h), shaking (200 Hz for 5 min), inversion of the light/dark cycle (24 h), tail nipping (2 min), soiled cage (100 mL of water spilled onto the bedding, 24 h), forced swimming in cold water (4 °C) for 5 min, and electric shock (0.9 mA, 15 s × 4 times). Each random stressor was applied three times during the CUMS procedure, and the same stressor was not applied consecutively.

**Body weight assessment and sucrose preference test**

Body weight and sucrose water consumption of rats were evaluated on days 1 and 22 of the experiment. After 24 h of water and food deprivation, we conducted a sucrose water consumption test by providing one bottle of 1% sucrose water and one bottle of standard drinking water. The consumption of sucrose water in 1 h was calculated. The result was calculated according to the following equation: sucrose intake/(sucrose intake + water intake).

**Forced swimming test**

The forced swimming test was conducted to evaluate the depressivelike behavior of rats after the last treatment. Rats were forced to individually swim in a glass cylinder (15 cm in height and 10 cm in diameter) filled with water [(25 ± 1) °C]. All animals were forced to swim for 5 min, and the immobility time (including passive swimming) during the test was recorded by two blinded observers.

**Open field test**

The open field test was performed using an open box (80 cm × 80 cm × 50 cm) to investigate rat locomotor activity. The box floor was divided into 25 equal squares, and the rats were quickly placed at the center of the box. After a 5-min test, the horizontal score was recorded when the lines were crossed by four paws, and the vertical score was calculated when the two forelimbs left the ground.

**He staining and TUNEL staining**

Rats were anesthetized intraperitoneally with 10% chloral hydrate and perfused with cold heparinized saline after treatment. Then, the brains were removed and fixed in 4% formaldehyde in PBS for 24 h and embedded in paraffin for sectioning. The brains were cut into 3-5-μm thick sections in the coronal plane and stained with HE and TUNEL. The slides were observed by optical microscopy (DP73, Olympus). Apoptotic neurons in the ischemic cortex and hippocampus were counted in five fields per section under high-power magnification (400 ×) by a blinded operator.

**Immunofluorescence assay**

Rats were anesthetized intraperitoneally with 10% chloral hydrate and perfused with cold heparinized saline after treatment. Then, the brains were removed and fixed in 4% formaldehyde in PBS for 24 h and embedded in paraffin for sectioning. The brains were cut into 3-5-μm thick sections in the coronal plane, permeabilized with 0.3% Triton X-100 and 1% bovine serum albumin for 1 h, and incubated for 1 h at room temperature. After washing with PBS, the brains were incubated overnight with Iba1 antibody (1: 1000) at 4 °C. Then, the brains were incubated in the dark with Cy3 conjugated goat anti-rabbit IgG (1: 200) for 2 h at room temperature and stained with DAPI for 10 min. Images were acquired by a Pannoramic® MIDI digital slide scanner (3D HISTECH, Hungary). Iba1 positive cells were counted in three random images from each brain section.

**ELISA**

Inflammatory cytokines TNF-α, IL-6, and IL-1β, and neurotransmitters 5-HT and NE, in brains were measured by ELISA. Rats were anesthetized intraperitoneally with 10% chloral hydrate and perfused with cold saline. The cortex and hippocampus were quickly separated on ice. After homogenization and centrifugation, the test was immediately conducted. The standard dilutions and samples were added to enzyme-coated plates according to the ELISA assay protocol. The reagent was added to the plate, and the samples were incubated for 30 min at 37 °C. After the ending solution was added into the plate, the absorbance at 450 nm was measured in a microplate reader (Bio-Rad, Hercules, USA). The results were expressed as the ratio to the sham group.

**Statistical analysis**

Data were expressed as the mean ± standard error of mean (SEM). Statistical significance was analyzed by one-way or two-way analysis of variance (ANOVA) in SPSS version 19.0. The Tukey test was used as a post-hoc test to perform multiple comparison analyses. P values less than 0.05 were considered statistically significant.

**RESULTS**

**XNJY ameliorates the depression symptoms in a rat PSD model**

Figure 1A shows the time schedule of the experimental procedures. The body weight measurement (Figure 1B) and sucrose preference test (Figure 1C) were conducted...
on days 1 and 22 of the experiment. The forced swimming test (Figure 1D) and open field test (Figure 1E and F) were performed on day 22. Compared with sham group, there was a significant reduction in the body weight and sucrose consumption in the PSD group ($P<0.01$). These indices recovered after Flu and XNJY treatments. Figure 1D shows that the PSD model had a significantly increased immobility time during forced swimming ($P<0.01$) compared with sham group. By contrast, XNJY and Flu treatments decreased the immobility time during forced swimming ($P<0.05$). Moreover, PSD group rats exhibited a significant reduction in locomotor activity (including horizontal and vertical movements) compared with the sham group ($P<0.01$). Flu and XNJY treatments reversed this reduction in locomotor activity compared with PSD group rats (Figure 1E and F).

### XNJY protects neurons from PSD-induced injury and apoptosis

We investigated the neuroprotective effects of XNJY against PSD-induced injury and apoptosis by staining with HE and TUNEL. Histopathological changes in the cortex and CA1 region of the hippocampus were evaluated by HE staining (Figure 2). The cellular structure of the cortex and hippocampus was organized in the sham group. Moreover, the neurons were round or oval with clear nuclei. By contrast, the PSD group showed many damaged neurons with karyopyknosis, cell gaps, and debris. Compared with the PSD group, these histopathological changes were recovered to different degrees after treatment with Flu and different doses of XNJY. TUNEL staining was used to detect apoptotic cells. Numerous apoptotic neurons were observed in the cortex of the PSD group ($P<0.01$). However, no significant apoptotic injury was found in the CA1 region of the hippocampus. After treatment with different doses of XNJY and Flu, numbers of TUNEL-positive cells were significantly decreased in the cortex ($P<0.01$).

#### XNJY inhibits microglial activation in a rat PSD model

We determined the numbers of Iba-1-labeled microglial cells in the cortex and hippocampus to further clarify the underlying antidepressant mechanisms of XNJY. Figure 3A and B show that the numbers of Iba-1-labeled microglial cells in the cortex and hippocampus of the PSD group were significantly increased compared with those of the sham group. After treatment with different doses of XNJY and Flu treatment, the numbers of Iba-1-labeled positive cells in the hippocampus were decreased ($P<0.05$). Moreover, compared with the Flu group, different doses of XNJY inhibited microglial activation in the cortex ($P<0.05$).

#### XNJY alleviates inflammatory responses and recovers 5-HT and NE levels in a rat PSD model

We measured the levels of TNF-$\alpha$, IL-6, and IL-1$\beta$ in the cortex and hippocampus (Figure 4A-C). The PSD group had a significant increase in the levels of the three proinflammatory cytokines in the cortex and hippocampus ($P<0.01$). The different doses of XNJY sig-

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**Figure 1** XNJY decoction ameliorates depression symptoms in a rat PSD model

**A**: time schedule of experimental procedure; **B**: body weight measurements; **C**: sucrose preference test; **D**: forced swimming test; **E**: open field test. MCAO: middle cerebral artery occlusion; XNJY: Xingnao Jieyu; PSD: post-stroke depression; Sham: sham operated; Flu: the rats underwent middle cerebral artery occlusion combined with unpredictable mild stress, and then was intragastrically administered with Fluoxetine hydrochloride (2.08 mg/kg) for 21 d; XNJY-L/XNJY-M/XNJY-H: the rats underwent middle cerebral artery occlusion combined with unpredictable mild stress, and then was intragastrically administered with different doses of XNJY decoction (XNJY-L, 10.5 g/kg; XNJY-M, 21 g/kg; and XNJY-H, 42 g/kg) for 21 consecutive days. Data are presented as the mean ± standard error of mean. Multiple comparison analysis was conducted by Tukey test after analysis of variance. $P$ values less than 0.05 were considered statistically significant. *$P<0.01$ versus the Sham group; **$P<0.05$; ***$P<0.01$ versus the PSD group.
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Flu: the rats underwent middle cerebral artery occlusion combined with unpredictable mild stress, and then was intragastrically administered with Fluoxetine hydrochloride (2.08 mg/kg) for 21 d; XNJY-L/XNJY-M/XNJY-H: the rats underwent middle cerebral artery occlusion combined with unpredictable mild stress, and then was intragastrically administered with different doses of XNJY decoction (XNJY-L, 10.5 g/kg; XNJY-M, 21 g/kg; and XNJY-H, 42 g/kg) for 21 consecutive days.

significantly downregulated these cytokines in the cortex and hippocampus ($P < 0.05$). Flu treatment reversed these abnormal indices in the hippocampus. However, compared with the XNJY groups, Flu had no effect on the levels of proinflammatory cytokines in the cortex. Levels of 5-HT and NE in the hippocampus and cortex were significantly reduced in the PSD group compared with the sham group ($P < 0.01$) (Figure 4D and E). After treatment with Flu and different doses of XNJY, the levels of 5-HT and NE in the hippocampus and cortex were significantly recovered ($P < 0.01$).

**DISCUSSION**

We explored the antidepressant effects of the XNJY decoction and its underlying mechanisms in a rat PSD model. The results showed that XNJY ameliorated the depression symptoms and protected the cortex neurons against PSD-induced neuronal injury and apoptosis. Furthermore, XNJY alleviated inflammatory responses by inhibiting microglial activation and downregulating levels of TNF-α, IL-6, and IL-1β in the cortex and hippocampus. XNJY also recovered the levels of 5-HT and NE in the cortex and hippocampus. These results indicated that alleviating inflammatory responses might be an important mechanism associated with the beneficial effects of the XNJY decoction against depression.
PSD delays rehabilitation after stroke and affects the quality of life of stroke patients. PSD, which contributes to an increase in suicide, is a frequent and important complication of stroke. Strong evidence has revealed that the occurrence of depression after stroke is closely associated with many factors, such as genetic variation, severity of stroke, lesion location, degree of disability, and social support. Pharmacotherapy, which includes tricyclic and tetracyclic antidepressants, neurotransmitter inhibitors, and monoamine oxidase inhibitors remain the main approaches for treating PSD. However, all these approaches induce significant adverse effects and drug withdrawal syndrome. The XNJY decoction, a TCM compound, has great potential for the treatment of PSD. This study revealed that XNJY and Flu attenuated depression symptoms by improving body weight, sucrose preference, and locomotor activity as well as reducing immobility time. These results are in agreement with previous findings. Moreover, studies demonstrated that CUMS in rodents suffering from cerebral ischemia induced neuronal cell death via apoptosis and necrosis. We observed injury and apoptosis in the cortex of the PSD group and XNJY significantly protected neurons against PSD-induced injury and apoptosis. Neuroinflammation involves cellular and biochemical responses to numerous insults that occur within the central nervous system. This process is a prominent pathological feature in ischemic stroke, depression, and PSD. Regulating neuroinflammation offers a potential therapeutic approach against PSD.
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Figure 4 XNJY decoction alleviates inflammatory responses in a rat PSD model

A: levels of TNF-α in the cortex and hippocampus were measured by ELISA; B: levels of IL-6 in the cortex and hippocampus were measured by ELISA; C: levels of IL-1β in the cortex and hippocampus were measured by ELISA; D: neurotransmitter NE in the cortex and hippocampus were measured by ELISA; E: neurotransmitter NE in the cortex and hippocampus were measured by ELISA. Cor: Cortex; Hip: Hippocampus; XNJY: Xingnao Jieyu; PSD: post-stroke depression; TNF-α: tumor necrosis factor-α; ELISA: enzyme linked immunosorbent assay; IL: interleukin; 5-HT: 5-hydroxytryptamine; NE: norepinephrine. Sham: sham operated; Flu: the rats underwent middle cerebral artery occlusion combined with unpredictable mild stress, and then was intragastrically administered with Fluoexetine hydrochloride (2.08 mg/kg) for 21 d; XNJY-L/XNJY-M/XNJY-H: the rats underwent middle cerebral artery occlusion combined with unpredictable mild stress, and then was intragastrically administered with different doses of XNJY decoction (XNJY-L, 10.5 g/kg; XNJY-M, 21 g/kg; and XNJY-H, 42 g/kg) for 21 consecutive days. Data are presented as the mean ± standard error of mean. Multiple comparison analysis was conducted by Tukey test after analysis of variance. P values less than 0.05 were considered statistically significant. *P < 0.01 versus the Sham group; **P < 0.05; †P < 0.01 versus the PSD group.

IL-1β. Moreover, activated microglia also release reactive oxygen species and other free radicals/oxidants that induce neural injury and apoptosis. Iba1, a microglial cell surface protein marker, is commonly utilized to investigate microglial activation. The current study showed a prominent increase in the number of Iba1 positive cells in the cortex and hippocampus of PSD rats. Numbers of Iba1 positive cells were significantly decreased after XNJY treatment. In addition, Flu inhibited microglial activation in the hippocampal region but had no influence in the cortex. Increasing evidence has shown that the overproduction of proinflammatory cytokines is key for the occurrence and development of depression. Recent studies showed that TNF-α, IL-6, and IL-1β are important proinflammatory cytokines involved in neuroinflammation and play major roles in mood disorders. Our findings revealed that levels of TNF-α, IL-6, and IL-1β were significantly upregulated in the cortex and hippocampus of rats following CUMS after ischemic stroke. However, XNJY treat-
ment downregulated the levels of these proinflammatory cytokines. Flu intervention downregulated the levels of TNF-α, IL-6, and IL-1β in the hippocampus but had no effect in the cortex. Furthermore, XNJY recovered the decreased levels of 5-HT and NE induced by PSD in the cortex and hippocampus, with effects similar to those of Flu. These findings suggest that compared with Flu, XNJY is more effective against depression symptoms, and that this is associated with the alleviation of neuroinflammation.

In conclusion, the XNJY decoction, a TCM compound, improved depressive-like behavior in rats by protecting neurons against injury and apoptosis, inhibiting microglial activation, downregulating levels of TNF-α, IL-6, and IL-1β, and recovering the levels of 5-HT and NE in the cortex and hippocampus. The results of the present study indicated that alleviating neuroinflammation may be an important mechanism of XNJY decoction against PSD. Thus, the XNJY decoction might be a promising candidate for the treatment of PSD.

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