**Xiaoyao powder alleviates the hippocampal neuron damage in chronic unpredictable mild stress-induced depression model rats in hippocampus via connexin 43Cx43/glucocorticoid receptor/brain-derived neurotrophic factor signaling pathway**

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**ABSTRACT**

**Xiaoyao Powder (XYP) has been widely applied in China to treat stress-related illnesses, such as migraine, depression, Parkinson’s disease, insomnia, and hypertension. Herein, this study aims to explore the effect of XYP on chronic unpredictable mild stress (CUMS)-induced depression and its underlying mechanisms. CUMS-induced depression rat models were established, they were subsequently randomly divided and treated with various conditions. Results of this study indicated that supplementation of XYP observably abolished CUMS-induced hippocampal damage and serum corticosterone (CORT) elevation. In mechanism, we discovered that CUMS induction could cause a prominent downregulation in glucocorticoid receptor (GR), phosphorylated-GR (p-GR), connexin 43 (Cx43), and brain-derived neurotrophic factor (BDNF), a remarkable upregulation in c-Src. While the introduction of XYP could reverse the changes in all of these indicators mediated by CUMS. Furthermore, we proved that Cx43 could interact with GR, and the protective effect of XYP on hippocampal neurons is realized by up-regulating GR. Summarized, this study indicated that XYP could ameliorate hippocampal neuron damage in CUMS-induced depression model rats through acting on Cx43/GR/BDNF axis.**

**Introduction**

Depression is one of the most common mental disorders, manifested by low emotion and slow thinking, speech, or movements [1]. Studies predicted that depression will be the second disease and one of the most serious diseases in developing countries [2,3]. It is reported that stressful events with the characteristics of chronic, low intensity, long-term existence are the most familiar inducing factors of depression [4,5]. And hippocampus is an organ vulnerable to stress and also a high regulating center of the hypothalamic-pituitary-adrenal (HPA) axis [6]. And the main pathological changes of depression mainly appear in the central nervous system, including decreased plasticity of neurons, decreased density of temporal cortex, and neuronal necrosis [7]. Therefore, the structural and functional damage of hippocampal neurons is the main cause of depression.

According to the literatures, stress-induced hippocampal nerve damage have attracted scholars’ attention for decades [8,9]. The hippocampus, which can differentiate into multiple nerve cell lines, is mainly involved in memory and performance on emotional information [10]. Accumulating evidence proved that hippocampus is directly related to cognition and emotion [11]. It is known that the hippocampus is important for the regulation of the hypothalamic pituitary adrenal axis which is very sensitive to glucocorticoids (GC). A study proved that GC binds to the glucocorticoid receptor (GR) to affect people’s cognitive...
behaviors [12]. GR, as the main mediator of stress response in neuronal progenitor cells and hippocampal neurogenesis, has been proved to directly regulate the balance of excitation or inhibition, and de-regulated expression of GR may lead to paroxysmal disorders [13]. GR is highly expressed hippocampus and it is very sensitive to the fluctuations of corticosterone (CORT) [14,15]. Brain-derived neurotrophic factor (BDNF) is another important factor to regulate the synaptic structure of hippocampal neurons in stress caused depression [16]. BDNF can protect the division and survival of neurons during stress and promote the growth of nerve fibers [17]. Several studies show that the number of neurons and the length of dendron both decrease in BDNF knockout mice [18,19]. Moreover, the BDNF knockout mice show obvious depression such as lack of pleasure and lower exploration competence. Therefore, deletion of BDNF has a strong enhancing effect on depression. Gap junctions (GJs) are forms of cell junction mostly composed of connexin 43 (Cx43), which plays an important role in regulating the intercellular signal transmission [20]. Previous studies show that overexpression of Cx43 protected astrocyte cells from apoptosis. C-Src, a member of the non-receptor tyrosine kinase family, has been shown to regulate cell proliferation, metastasis and also affect Cx43 channel functionality through a variety of mechanisms. The study also showed c-Src can directly phosphorylate Cx43 at Tyr247 and Tyr265 to cause the closure of the GJs channel [21].

Xiaoyao powder (XYP), a traditional Chinese, has been widely used by clinicians. It shows good clinical effects on mental disorders and has been proved to have an excellent effect on corticosterone-induced stress injury and is beneficial to nerve growth [22]. According to the latest research, we discovered that XYP has the effect of enhancing depressive-like behaviors in chronic unpredictable mild stress (CUMS)-induced depression model rats [23-25]. However, it is not completely clear the effect and mechanism of XYP on hippocampal neuron injury in UMS-induced depression model rats.

In our study, the aim of this study was to explore the effect of XYP on the expression of BDNF, glucocorticoids receptor (GR), Cx43, and c-Src in the hippocampus of rats with depression, and further investigated the mechanism of XYP regulates GR/BDNF signaling pathway and gap junction in hippocampal neurons of rats with chronic stress.

**Materials and methods**

**Drugs and reagents**

Consistent with previous research [23], XYP included Bupleurum chinense 30 g, Angelica sinensis 30 g, Radix Paeonae Rubra 30 g, Atractylodes macrocephala 30 g, Poria umbilus 15 g, Radix glycyrrhizae 15 g, Zingiber officinale Rosc 10 g, Mentha haplocalyx 10 g, which were all purchased from Taiping Welfare Pharmacy (Jiuzhitang, China). The XYP solution was prepared by dissolving the powder in distilled water to obtain final concentrations of 5.0 g/kg (low dose), 10.0 g/kg (middle dose) and 20.0 g/kg (high dose). Imipramine (20 mg/kg; Pharmaceuticals Sine, China) and RU-486 (25 mg/kg; Biyuntian, Shanghai, China) were selected as the depression antagonist and GR-receptor antagonist, respectively.

**Animal grouping and treatments**

In line with previous research [26], the current study is conducted at the Guangzhou University of Chinese Medicine from May 2019 to June 2021. Procedures were approved by the Institutional Animal Care Committee of Guangzhou University of Chinese Medicine (IACUC number: 2,018,146). Forty-eight male Sprague Dawley (SD) rats (8 weeks, weighting 250–300 g) were obtained from the Tongji University, Shanghai, China. Chronic unpredictable mild stress (CUMS) depression model were established and the rats were randomly divided into eight groups: a) control group; b) normal stressed group (NS); c) stress-induced depression group (SD); d) stress-induced depression with low dose XYP (SDL) group; e) stress-induced depression with middle dose XYP group (SDM); f) stress-induced depression with high dose XYP group (SDH); g) stress-induced depression with high dose XYP group (for control); and h) stress-induced depression
with high dose XYP group and RU-486. Rats in d), e), f) and g) groups d, e, f, and g were given indicated amount of XYP intragastrically every day, and rats in h group were received RU-486 (25 mg/kg).

**Histopathological evaluation**

On the basis of the study [27], brain tissues were fixed for 3 days in 10% formaldehyde, soaked in 30% sucrose and stored at 4°C until infiltration was complete. Ten-micrometer-thick coronal brain sections were prepared with the help of a stereotaxic atlas. The sections were used for Nissl (cresyl violet) staining and HE (hematoxylin-eosin) staining to visualize cytoarchitecture of the hippocampal cell layers and neuron counts. Histological evaluation was accomplished using three to four sections per hippocampus (n = 6) for each rat (n = 6) by an image analyzer (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA) and transmission electron microscope (TEM; HT7700, Hitachi, Japan).

**ELISA assay**

In accordance with the research [28], serum corticosterone was measured with commercial ELISA kit according to the manufacturer’s instructions (SU-BN30679, Reagent biology. China). The lower assay limit of detection was 0.5 ng/mL for corticosterone. Absorbance was measured by a microplate reader (Multiscan Mk3, Thermo Fisher Scientific).

**Immunohistochemical assay**

As described in previous research [29], Brain sections were incubated with H_{2}O_{2} (10%) for 30 min to eliminate endogenous peroxidase activity and blocked with 10% normal goat serum for 1 h at room temperature. Subsequently, sections were incubated with primary antibodies against GR and phosphorylated GR (p-GR) (Abcam, Cambridge, MA; 1/1000) for 24 h at 4°C. Antibody detection was carried out with the Histostain-Plus Bulk kit (Invitrogen), and 3,3-diaminobenzidine was used for visualization with an Olympus C-5050 digital camera.

### Table 1. Primer sequences in RT-qPCR experiments.

| ID   | GenBank   | Sequences (50-30) | Product Length (bp) |
|------|-----------|-------------------|---------------------|
| GR. F | M14053.1  | GTCCATGGGCTGTATATGG | 184                 |
| GR. R |           | TGCAGACTTGAACCTTGG |                     |
| BDNF. F | M61178.1  | GCGGCCATATAAAAAAGCTGC | 238                 |
| BDNF. R |           | GCCAGCAAATCTCTCTTGG |                     |
| Actin. F | V01217.1  | AGCCATGTCGATGCACTCC | 228                 |
| Actin. R |           | CTCTGAGTCGTTGAGAA |                     |

F, forward; R, reversed.

**RT-qPCR**

By referring to previous study, RNA was extracted from the hippocampi using Tri-Reagent (Invitrogen, USA) according to the manufacturer’s instructions. RNA (1–2 μg) was then reverse transcribed to cDNA using Reverse Transcription Kit (Promega, USA). RT-PCR was performed on ABI Prism 7500 Real-Time PCR System (ABI PRISM™ 7500). In brief, 5 μL of cDNA were used in a 20 μL reaction system, containing 10 μL of SYBR Green qPCR SuperMix (Invitrogen, USA), 0.5 μL of forward primer, 0.5 μL reverse primer, and 4 μL of water. The reaction conditions were 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 32 s. Each experiment was done in triplicate and β-actin was used as an internal control. The genes specific primers sequence were shown in Table 1.

**Western-blotting**

Based on previous research [30], total protein of rat hippocampus was extracted with RIPA lysis buffer and quantified by BCA (Keygene biotech, Nanjing) kit. The protein was subjected to 8% SDS PAGE, followed by transfer onto to PVDF membrane (IPVH00010, MILLIPORE). Then, the membrane was blocked by 5% BSA in TBS-T (TBS containing 0.1% Tween-20), and incubated overnight at 4°C with the following primary antibodies: anti-GAPDH, anti-actin, anti-GR, anti-p-GR, anti-BDNF, anti-Cx43 and -c-Src (all 1:1000 dilution). The membranes were then incubated with HRP-conjugated secondary antibody (Goat Anti-Rabbit IgG, 1:20,000, southern biotech). After incubation with the secondary antibodies for 1 h at room temperature, the membranes were scanned and the integrated optical density (IOD) was calculated using
Co-immunoprecipitation assay

Refer to the reported literature [31], HEK293T cells were lysed with radioimmunoprecipitation (RAPI) buffer, and the extracted and quantified proteins were added with the Pierce™ Protein GPlusAgarose (Invitrogen, USA) and incubated at 4°C for 1 h. Partial supernatant (60 μL) was applied as negative control. The supernatant was then added with anti-GR (1:200) or anti-CX43 (1:200) at 4°C overnight, respectively. All protein samples were then added into Protein-G-Agarase, overturned at 4°C, centrifuged, and supernatant discarded. After washing with pre-cooled PBS, the mixture was also added with 60 μL RIPA buffer, 4 μL DTT and 16 μL sample loading buffer. Subsequently, the protein samples were collected for western blot detection.

Transmission electron microscope (TEM)

Based on the reported literature [31], each group of hippocampal neurons was immobilized with 3% glutaraldehyde for 2 h. After washing, the hippocampal neurons were addressed with 1% osmium for 1.5 h. Subsequently, the hippocampal neurons were subjected to a series of treatments, including uranium dioxy acetate staining for 1 h, progressive dehydration with 50%–100% alcohol, and dehydration with pure acetone. After embedding in epoxy resin, the Leica ultra-thin slicer was applied for slicing (50–70 nm). Then the ultrastructural changes of hippocampal neurons were observed by JEM-100 electron microscope after lead staining.

Statistical analysis

By referring to relevant literature [32], the data was analyzed using the statistical software GraphPad Prism 7.0 (GraphPad Software, Inc.). A two-way analysis of variance (ANOVA) with post hoc Tukey’s test was performed for comparisons of two or more groups (Barnes maze test). The level of significance was set at ^p < 0.05. All data were expressed as mean ± SEM (standard error of the mean).

Results

YXY protected hippocampus against CUMS-induced damage

To explore the influence of YXY on hippocampus neuron damage in depressed rats, a rat model of depression was established through CUMS induction. Then rat brains were sectioned and used for Nissl staining and HE staining. In control group and NS group, results shown that hippocampus have intact and regular hierarchical cell arrangement, all cells also present the normal nucleus and intact neuron membrane (Figure 1(a)). In SD group, the structure of hippocampus was obviously damaged and the cells were also reduced and indistinct; Specifically, pyramidal cells and granulosa cells became disordered and the cell structure was disorganized (Figure 1(a)). In SDL, SDM and SDH groups, the structures of hippocampal region were basically intact, cells were in a more regular hierarchical structure compared with SD group, and the cell membrane of neurons was basically intact. Only a small number of cells showed vacuolation and karyopyknosis (Figure 1(a)). Meantime, quantitative analysis indicates that CUMS also caused significant decrease of neuron numbers, which could be ameliorated by supplement of YXY with a dose-dependent manner (Figures 1(b-c)). The CORT were also investigated by ELISA assay, serum CORT was significantly increased in the SD group (p < 0.01). These CUMS-induced CORT elevations were also significantly inhibited by administration of YXY in a dose-dependent manner (Figure 1(d)). Moreover, transmission electron microscopy (TEM) results indicated that cell nuclei become smaller with wrinkly nuclear membranes and Nissl’s bodies were also dissolved in SD group, compared to control or NS group (Figure 2(a-c)). Similarly, length and density of synapses in SD group were significantly decreased compared to control group or NS group. Interestingly, administration of YXY relieved these morphologic changes in a dose-dependent manner (Figure 2(d-f)).

XYP reversed GR, p-GR, and BDNF expression suppressed by CUMS

To further investigate the possible mechanism by which YXY affects hippocampal neuron injury, we
further identified the expression of hippocampal survival-related proteins (GR and BDN). As shown in Figure 3, both GR, p-GR and BDNF were significantly down-regulated in SD group compared to control group or SN group, while these down-regulated expression of GR, p-GR and BDNF could be gradually
reversed by supplement with gradient dose of XYP (Figure 3).

Cx43 interacted with GR, and XYP abolished Cx43/GR/BDNF expressions inhibited by CUMS

Next, we further confirmed the interaction between GR and Cx43 and the regulation of Cx43, GR and BDNF proteins by XYP. First, western-blotting and RT-PCR were employed to investigate the role of XYP in Cx43/GR/BDNF signaling axis. As shown in Figure 4, the expression of GR, p-GR, BDNF, Cx43 were significantly down-regulated in SD group, while the expression of c-Src was significantly up-regulated. In the groups supplemented with different doses of XYP, this CUMS-caused suppression of GR, p-GR, BDNF, Cx43 were significantly ameliorated compared to the SD group. Meanwhile, CUMS-increased expression of c-Src was also abolished with XYP in a dose-dependent manner (Figure 4). What is more, we next want to investigate the interaction between Cx43 and GR. We firstly checked the binding of CX43 and FLAG tagged GR in overexpression system. As shown in Figure 5(a), CX43 were interacted with FLAG tagged GR in overexpression system. To further confirm this interaction, FLAG Tagged CX43 and GFP tagged GR were co-transfected into HEK293T cells. The data showed that FLAG Tagged CX43 was interacted with GFP tagged GR (Figure 5(b)). Taken together, results of this study indicated that GR interacted with Cx43.
XYP induced up-regulation of Cx43/GR/BDNF could be blocked by GR antagonist

Furthermore, the present study further confirmed whether XYP affects hippocampal neuron injury by regulating GR through the rescued experiment in CUMS-induced depression model rats. And ELISA, histopathological evaluation, WB, and RT-PCR were performed to confirm whether XYP could alleviate hippocampal injury via Cx43/GR/BDNF pathway in vivo. As shown in Figure 6(a), the hippocampus cells were not arranged tightly when SD mice treated with XYP and GR antagonist (RU486), TEM imaging further confirmed that hippocampus cells presented vacuoles and their layers were also not clear in
Moreover, immunofluorescence results shown that both expression of GR and p-GR in SD_XYP_RU486 group were also significantly down-regulated compared to SD_XYP group. Next, the result of quantitative analysis confirmed that administration of RU486 also resulted in significantly decrease of neuron numbers compared to SD_XYP group (Figures 6 (b-c)). Meanwhile, ELISA results shown that serum CORT in SD_XYP_RU486 group was significantly higher than that in SD_XYP group (Figure 6(d)). Interestingly, the expression of GR,
p-GR, BDNF and Cx43 were dramatically down-regulated when RU486 were added, but the level of c-Src was up-regulated after SD-XYP were treated with RU486 (Figure 6(e-f)). Similarly, qPCR results also confirmed that the mRNA level of GR and BDNF were significantly suppressed after blocking GR with RU486 (Figure 6(g)).

Discussion

Depression, as a comprehensive disease with complex etiology, has a great impact on the physiology and psychology of patients [33,34]. Depression can be caused by many factors, of which chronic stress is considered as the major factors that have significant effect on depression [22]. Previous study uncovered that various stress hormones, endocrine changes, and immune inflammatory responses accompanied by chronic stress can affect the process of stress-induced depression [35]. At present, CUMS model has been widely used for the study of depression [36]. In order to find potential drugs that can alleviate hippocampal neuron damage in depressed rats, we also adopted the same method to establish CUMS-induced depression rat model. This study showed that CUMS could cause a significant increase of serum CORT and damage of hypothalamus, suggesting the successfullness of establishment of CUMS model.

XYP is made up of eight kinds of Chinese herbs [37]. Radix Bupleurum saponins, the effective components of Radix Bupleurum, have obvious anti-inflammatory effect, and its mechanism is related to preventing the release of inflammatory mediators (IL-1, IL-6, TNF-α) [38]. Both Angelica sinensis and Radix Paeonie Rubra have anti-inflammatory effects [39]. Ferulic acid in Angelica sinensis can have a neuroprotective effect by improving the phagocyte function and inhibiting IL-β and JNK pathway [39]. Paeoniflorin and pagononol Radix Paeonie Rubra can restrain the secretion of IL-6, TNF-α and other inflammatory factors [40]. Atractylodes macrocephala, Poria umbellus, Radix glycyrrhiza, Zingiber officinale Rosc, Mentha haplocalyx all exert anti-inflammatory effects by reducing the release of inflammatory factors, and the active ingredients include atractylodes lactone, Poria cocos polysaccharide, curcumin, menthol, and glycyrrhiza flavone [41-44]. Currently, XYP has been widely used in China to treat migraine, depression, Parkinson’s disease, insomnia, and hypertension related to stress [45-47]. Recent research testified that XYP was relevant to the regulation of glucocorticoid receptor under chronic stress condition [37]. Meanwhile, XYP has been proved to have antidepressant effects by several studies [48,49]. Besides, studies revealed that XYP has a certain protective effect on hippocampal neuron injury [50,51]. In our study, we further confirmed that administration of XYP could alleviate hippocampal neuron injury in CUMS-induced depression model rats. Moreover, we further demonstrated that XYP also could upregulate GR, p-GR, and BDNF in CUMS-induced depression model rats. Among them, GR is the primary mediator for stress-induced response in neuronal progenitor cells and hippocampal neurogenesis. Expression of GR in hippocampus region is particularly sensitive to fluctuations in corticosterone [14]. Studies showed the changes of GR expression play an important role in regulation nuclear translocation, cofactor binding, and gene transcription. Clinical and animal studies have shown that mRNA of BDNF was down-regulated in depressed patients or rats [52]. In animal depression model, the expression of BDNF was decreased in hippocampal neurons and cerebral cortex [53]. Dolcet X. et al showed that hybrid BDNF knockout mice and TrkB mutant mice were resistant to depression. Gray et al demonstrated that GR regulated the expression of BDNF in hippocampus under chronic stress [54]. Studies also proved that in chronic stress test of GR knockout mice, the hippocampal BDNF expression could not change with stress, which proved that GR had an important role in BDNF regulation [55-57]. Therefore, our current research further revealed that XYP can improve hippocampal neuron injury in CUMS-induced depression model rats, and its mechanism might be in connection with GR and BDNF.

On the other hand, more and more evidence suggest that gap junction is closely related to the incidence of depression. Sun et al. found that injection of GJ inhibitor glycyrrhizic acid and Cx43 mimic, Gap26, and Gap27, into the anterior marginal cortex induced depression in rats [58]. Further research showed that in CUMS rats, the
expression of Cx43 were significantly down-regulated which could be reversed by fluoxetine. As one of the conjugated proteins, c-Src could bind to phosphorylated Cx43 to shut off the intercellular GJ channel [59]. In this study, we further investigated that XYP could downregulate Cx43 and upregulate c-Src in CUMS-induced depression model rats with a dose-dependent manner. Therefore, we speculated that CUMS increased c-Src expression to bind to Cx43 and shut off intercellular GJ channel, leading to damage of hypothalamus. Moreover, we proved that Cx43 could interact with GR. To confirm that XYP against hippocampal injury via Cx43/GR/BDNF pathway in CUMS-induced depression model rats, the GR specific inhibitor RU486 was introduced in this study. As expected, the RU486 present could reverse the biological effect of XYP, which further proved that XYP protected rats hippocampal from stress-induced brain injury by acting on GR and its downstream pathway.

Conclusions

In summary, the present study found that stress-induced depression could cause hippocampus damage, elevation of serum CORT, decrease of neurotrophic factors (GR, p-GR, and BDNF) and Cx43 expressions, and increase of c-Src expression. While XYP could obviously ameliorate these changes in model rats with CUMS-induced depression. Therefore, we proved that the traditional Chinese medicine XYP could protect rats against stress-induced brain injury by regulating Cx43, GR, BDNF expressions. However, there are also several limitations to the current study. Such as the use of large numbers of rats. For example, more rats should be applied to confirm the current conclusion; the specific mechanism of XYP regulating Cx43, GR, BDNF also needs to be further explored; it is also necessary to further investigate the specific active components of XYPP in the repair of hippocampal neuron injury of CUMS-induced depression rat models.

Authors’ contribution

Yuanyuan Zhang, Baoying Yang and Yi Qin conceived and designed the experiments; Yuanyuan Zhang, Yong Luo, Xuanan Hou performed the experiments; Kang Lu and Yanhong He contributed reagents/materials/analytical tools; Yuanyuan Zhang, Yong Luo, Baoying Yang, and Yi Qin wrote and revised the paper.

Ethics approval

Procedures were approved by the Institutional Animal Care Committee of Guangzhou University of Chinese Medicine (IACUC number: 2018146).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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