MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

# QUALIFICATION THESIS

on the topic **Immunofluorescence analysis of the antidepressant activity of Xiaoyao Pill**

First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

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Educational and professional program Biotechnology

**APPROVE**
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Doctor of Technical Science
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_____
«___»_____2024

**ASSIGNMENTS**
**FOR THE QUALIFICATION THESIS**
**Yan Ge**

1. Thesis topic **Immunofluorescence analysis of the antidepressant activity of Xiaoyao Pill**
Scientific supervisor Olga Andreyeva, Dr. Sc., Prof.

approved by the order of KNUTD "__"_____2024, №_____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

## EXECUTION SCHEDULE

| № | The name of the stages of the qualification thesis | Terms of performance of stage | Note on performance |
|---|---|---|---|
| 1 | Introduction | From 01 April 2024 to 11 April 2024 | |
| 2 | Chapter 1. Literature review | From 06 April 2024 to 20 April 2024 | |
| 3 | Chapter 2. Object, purpose, and methods of the study | From 21 April 2024 to 30 April 2024 | |
| 4 | Chapter 3. Experimental part | From 01 May 2024 to 10 May 2024 | |
| 5 | Conclusions | From 07 May 2024 to 12 May 2024 | |
| 6 | Draw up a bachelor's thesis (final version) | From 20 May 2024 to 25 May 2024 | |
| 7 | Submission of qualification work to the supervisor for feedback | From 25 May 2024 to 27 May 2024 | |
| 8 | Submission of bachelor's thesis to the department for review (14 days before the defense) | 27 May 2024 | |
| 9 | Checking the bachelor's thesis for signs of plagiarism (10 days before the defense) | 31 May 2024 | |
| 10 | Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense) | 03 June 2024 | |

I am familiar with the task:

Student _____Yan GE

Scientific supervisor _____Olga ANDREYEVA

# SUMMARY

**Yan Ge. Immunofluorescence analysis of the antidepressant activity of Xiaoyao Pill . – Manuscript.**

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2024.

With the continuous development and progress of science and technology, people's material life is satisfied at the same time, the pressure of social life and other aspects also comes immediately, and different people also show different reactions to the high-pressure environment. Being in the high tension and pressure of life rhythm for a long time will have a certain impact on people's physical and mental health. Depression, as a kind of disease, has been widely concerned by the society.

Xiaoyao Pill, as a Chinese medicine prescription for regulating spleen and stomach weakness, has a certain improvement in the treatment of depression. In this paper, laboratory mice were selected as experimental subjects, animal models were prepared, and the depression model mice were treated with Zhizi soybean soup by gavage. After open field experiment, sugar spray experiment and tail suspension experiment, behavioral detection was carried out to evaluate the antidepressant effect of Xiaoyao Pill intervention on the depression model mice.

The expression of c-fos activation in hippocampal brain region was detected by immunofluorescence, and the intervention effect of Xiaoyao Pill on pathological indexes of depression model mice was evaluated by comparing the activation amount of c-fos in hippocampal brain region between different groups. Finally, data analysis is carried out, using the research data obtained from the experiment and the body mass growth rate repeatedly measured by two-factor ANOVA. Therefore, through the detection and verification of the improvement of Xiaoyao pills on depression by biotechnological methods, we can further understand the operation and principle of biotechnological immunofluorescence technology, and provide a theoretical basis for the pharmacological effect of Xiaoyao pills on improving depression.

*Keywords: XiaoYao Pill, Depression, animal model, immunofluorescence technique, Variance analysis.*

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# INTRODUCTION

With the continuous development and progress of science and technology, people's material life is satisfied at the same time, the pressure of social life and other aspects also comes immediately, and different people also show different reactions to the high-pressure environment. Being in the high tension and pressure of life rhythm for a long time will have a certain impact on people's physical and mental health. Depression, as a kind of disease, has been widely concerned by the society. Xiaoyao Pill, as a Chinese medicine prescription for regulating spleen and stomach weakness, has a certain improvement in the treatment of depression. In this paper, laboratory mice were selected as experimental subjects, animal models were prepared, and the depression model mice were treated with Zhizi soybean soup by gavage, and behavioral tests were conducted by open field experiment, sugar spray experiment and tail suspension experiment, so as to evaluate the antidepressant effect of Xiaoyao Pill intervention on the depression model mice. The expression of c-fos activation in hippocampal brain region was detected by immunofluorescence, and the intervention effect of Xiaoyao Pill on pathological indexes of depression model mice was evaluated by comparing the activation amount of c-fos in hippocampal brain region between different groups. Finally, data analysis is carried out, using the research data obtained from the experiment and the body mass growth rate repeatedly measured by two-factor ANOVA. Therefore, through the detection and verification of the improvement of Xiaoyao pills on depression by biotechnological methods, we can further understand the operation and principle of biotechnological immunofluorescence technology, and provide a theoretical basis for the pharmacological effect of Xiaoyao pills on improving depression.

**The relevance of the topic** is Detection and verification of the improvement of Xiaoyao Pill on depression.

**The purpose of the study** is the through the detection and verification of the improvement of Xiaoyao pills on depression by biotechnological methods, we can

further understand the operation and principle of biotechnological immunofluorescence technology, and provide a theoretical basis for the pharmacological effect of Xiaoyao pills on improving depression

**The object of the study** is through the detection and verification of the improvement of Xiaoyao pills on depression by biotechnological methods, we can further understand the operation and principle of biotechnological immunofluorescence technology, and provide a theoretical basis for the pharmacological effect of Xiaoyao pills on improving depression.

**The subject of the study** is SPF male healthy C57BL/6J mice and SPF male healthy CD-1 mice.

**Research methods:** open field test, Sugar spray experiment, tail suspension test.

**The scientific novelty:** c-fos immunofluorescence detection in hippocampus.

**The practical significance of the results obtained** is through this experimental design, it provides more possibilities for the study of neural mechanism.

# CHAPTER 1
# LITERATURE REVIEW

## 1.1 Overview of Depression

Depression is a common chronic mental illness. Depression is mainly depressed mood, loss of interest, lack of energy, self-negative decadence as clinical manifestations, there are also some early symptoms such as slow response, slow thinking, memory decline. Depression often affects the patient's life, work and relationships. In traditional Chinese medicine, depression does not have a corresponding medical name, which belongs to the category of "depression". According to the Huangdi Neijing, the word "depression" appears in it and contains a large number of descriptions of "sadness, sadness, unhappiness" and other depression-related emotions. According to records, the therapeutic factors of depression are multi-factorial, such as genetic constitution, zang-fu dysfunction, and other diseases [1]. According to the WHO survey in 2022, the number of people suffering from depression in China accounts for 4.2% of the total population.

The classification of diseases according to the 10th edition of the International Classification of Diseases shows that depressive episodes can be classified as mild, moderate or severe based on the number of symptoms and clinical severity [2]. At present, depression has become a globally recognized and highly concerned disease.

## 1.2 Research progress of depression in China

In China, the incidence of depression is gradually increasing. Although the domestic research progress on depression started late, it has been paid more and more attention in recent years. At present, domestic research progress on depression mainly includes the following aspects:

1. Diagnosis and evaluation: Domestic researchers have made certain progress in the diagnosis and evaluation of depression, including the use of various clinical

assessment tools and questionnaires to identify patients with depression, in order to investigate the correlation between different clinical manifestations and characteristics.

2. Treatment methods: Research on the treatment of depression is also deepening in China. In addition to traditional drug therapy, non-drug therapy such as psychotherapy and physical therapy have also been paid attention to and achieved certain curative effects. For example, Qianghuihe [3] et al. proposed the method of soothing liver and regulating Qi as one of the methods to treat depression, and used Bupleurum Shugan powder as the main prescription.

3. Etiology research: Domestic researchers are also exploring the etiology of depression, including genetic factors, environmental factors, neurobiological mechanisms and other aspects of the study. These studies contribute to a better understanding of how depression occurs and more effective strategies for treatment and prevention. The research progress of depression in China in 2023 has made a great breakthrough.

### 1.3 Research progress of depression in foreign countries

It is reported that more than 19 million adults in the United States suffer from depression each year, with direct or indirect costs exceeding $30 billion [4], and the incidence of depression is about 3% to 5%. Compared with the research progress of depression in China, the research in the field of depression in foreign countries started earlier.

In recent years, some important progress has been made in the study of depression abroad, mainly reflected in the following aspects:

Biological mechanism research: through neuroimaging, genetics and other means, a better understanding of the biological basis of depression, such as abnormal brain function, neurotransmitter imbalance, genetic susceptibility and so on. This provides a basis for finding new therapeutic targets.

Diagnosis and assessment methods: More accurate and comprehensive depression assessment tools, such as the Structured Diagnostic Interview Scale, have

been developed to help more accurately diagnose and assess the severity of depressive symptoms.

Therapeutic innovation: In addition to traditional drug therapy, some emerging therapeutic methods such as transcranial magnetic stimulation and deep brain stimulation have also achieved certain curative effects. The combination of psychotherapy and drug therapy has been emphasized.

Prevention and intervention: Through early identification and intervention of high-risk groups, as well as community health education, efforts are made to reduce the incidence and delay of depression.

Large-scale cohort studies: Some countries have carried out large-scale population follow-up surveys, which provide important data support for in-depth understanding of the epidemiological characteristics and influencing factors of depression. Under the modern medical model, the treatment of this disease is mainly dominated by psychotherapy, drug therapy and physical therapy. Especially antidepressant drugs, such as Duloxetine is a new drug in recent years, can block 5-hydroxytryptamine, adrenaline reuptake reaction, improve the content of 5-hydroxytryptamine in the central nervous system, can reduce the anxiety caused by depression, physical symptoms at the same time, the disease treatment effect increased to 60% to 80%. However, they will also face adverse reactions such as nausea, vomiting, diarrhea, constipation, anorexia, and insomnia, which will also reduce the patient's medication compliance, prolong the treatment cycle, and reduce the treatment effect [5,6].

### 1.4 Advances in the modernization of Chinese medicine and its compounds

In recent years, great progress has been made in the modernization of TCM, which is mainly reflected in the following aspects: Drug composition research, through modern scientific and technological means, the active ingredients in TCM are extracted, separated, purified and structural identification, revealing the material basis of their efficacy and speeding up the modernization of TCM; Pharmacological

research, to study the pharmacological mechanism of Chinese medicine, to explore the way and influence of Chinese medicine on the human body, is conducive to better understand the efficacy of Chinese medicine; Quality control research, the establishment of more scientific Chinese medicine quality control standards and evaluation system, including fingerprint, chemical composition analysis and other technical means to ensure the quality and safety of Chinese medicine; Clinical research, through clinical trials to verify the efficacy and safety of traditional Chinese medicine in the treatment of various diseases, to promote the application and promotion of traditional Chinese medicine in modern medicine; Drug development: Using the active ingredients and pharmacodynamic mechanisms of Chinese medicine to develop new drugs or Chinese medicine compounds to provide new treatment options for the treatment of diseases. In general, the research progress of the modernization of traditional Chinese medicine provides more scientific support for the development and application of traditional Chinese medicine, and also provides more possibilities for the development of integrated Chinese and Western medicine.

### 1.5 Research progress of Xiaoyao pills

At present, there is a commonly used Chinese medicine prescription called Xiaoyao pill, which is a pill form of Xiaoyao powder, mainly composed of various Chinese herbs such as poria, white art, Xiangfu, licorice and so on. According to the analysis of the components of Xiaoyao Pill, it is concluded that Xiaoyao Pill has the functions of harmonizing the spleen and stomach, invigorating the spleen and appetizing the appetite, indigestion and abdominal distension caused by the weakness of the spleen and stomach. Therefore, Xiaoyao pill can be applied to the disorder symptoms caused by spleen and stomach qi deficiency, such as loss of appetite, abdominal distension, fatigue and so on. Through investigation and research, it was found that scientists mainly focused on paeoniflorin, saikosaponin, ferulic acid, poria, atractylol alcohol, glycyrrhizic acid and menthol and other ingredients [7,8]. However, the pharmacological research of Xiaoyao Pill is still dominated by gynecological

diseases, such as female mammary hyperplasia, premenstrual syndrome, menstrual disorders, polycystic ovary syndrome and so on. At present, the relevant therapies for depression in China mainly include Western medicine therapy and Chinese medicine therapy, and most patients diagnosed with depression can be treated by traditional Chinese medicine preparation Xiaoyao Pill. In recent years, some studies have begun to isolate and identify the active ingredients in Xiaoyao pills, such as baicalin and angelicin. These studies are helpful to further understand the pharmacodynamic substances of Xiaoyao pills and their mechanism of action

**Conclusions to chapter 1**

1. In general, the study of depression abroad presents the characteristics of multi-discipline and innovative methods, which provides an important basis for better prevention and treatment of depression.
2. In general, Xiaoyao Pill, as a traditional Chinese medicine compound, although it has a certain history and effect in clinical application, it still needs more research to verify and improve in terms of pharmacology and medicinal substances.

# CHAPTER 2
# OBJECT, PURPOSE AND METHODS OF THE STUDY

## 2.1 Research progress of animal modeling

Animal modeling is generally used in medical research to establish animal experimental objects and related materials with human disease simulation performance [9]. Through animal modeling, targeted scientific research, efficient understanding of human related diseases, treatment and prevention measures. Animal modeling has played an important role in disease research and drug research, becoming the most critical step before drugs are applied to humans. Animal modeling continues to develop in modern medical research, obtaining new techniques and means.

According to different disease types, it can be divided into spontaneous disease model and exogenous disease model. And the laboratory usually chooses the individual is moderate, the price is reasonable cheap animal as the model object. Animal modeling experiments have also provided important references for the development of vaccines, antibiotics, and a deeper understanding of human disease processes. For example, Albert Sabin used monkeys to develop a polio vaccine in the 1930 s. [10]

## 2.2 Research progress of biotechnological detection methods for Xiaoyao pills

The research progress of biotechnological detection methods of Xiaoyao pills mainly includes the following aspects:

*Fingerprint analysis:* The fingerprint of Xiaoyao pills was established by high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC-MS) and other technologies for quality control and component analysis.

*Component separation and identification:* The active components in Xiaoyao pills were separated by chromatographic separation techniques (such as HPLC, supercritical fluid chromatography, etc.). The structures of the isolated components were identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS).

*Biological activity evaluation:* Cell experiment and animal experiment were used to evaluate the biological activity of Xiaoyao pills and its components, such as anti-depression and anti-anxiety effects. To develop in vitro enzyme activity, receptor binding and other biological analysis methods, screening and evaluation of active ingredients.

*Quantitative analysis method:* A high-sensitivity quantitative analysis method based on HPLC-MS/MS and UPLC-Q-TOF-MS was established for the quantitative detection of active ingredients in Xiaoyao pills.

*Standardization research:* According to the above research results, the quality standards and detection methods of Xiaoyao pills were formulated to provide scientific basis for its clinical application and industrialization.

### 2.3 Overview of immunofluorescence techniques

Immunofluorescence technology is an immunolabeling technology in which antibodies or antigens are chemically combined with fluorescein under conditions that do not affect their immunological properties, and then antibodies or antigens labeled with fluorescein are used as reagents to react with corresponding antigens or antibodies under specific conditions, and then fluorescence microscopy is used to observe specific immunofluorescence phenomena [11]. Immunofluorescence technology has the characteristics of fast, reliable, high sensitivity and strong adaptability, and is widely used in medical diagnosis, pharmaceutical diagnosis and clinical rapid diagnosis.

Immunofluorescence technology includes the following aspects: indirect immunofluorescence technology, direct immunofluorescence technology, time-resolved fluorescence immunoassay, etc. [12].

Indirect immunofluorescence technique (IFA) is one of the most widely used immunofluorescence techniques. Indirect immunofluorescence technique is a method to detect antigen-antibody reaction. It consists of the following steps: fixing the antigen to be measured on a slide or plastic plate; Add samples to be tested, such as the patient's serum; If the sample contains antibodies corresponding to the antigen, they will bind

to the fixed antigen; A secondary antibody labeled with a fluorescent substance is added, which can bind to the antibody that binds to the first step; Observation through a fluorescence microscope, if the fluorescence signal appears, it indicates that the sample contains the corresponding antibody. This indirect method has higher sensitivity and wider application than the direct labeling method. It is widely used in disease diagnosis, immunology research and other fields. Indirect immunofluorescence techniques are also used to detect parasites. Scientist Chen Jingqing [13] compared IFA method of pneumofluke with enzyme-labeled adsorption test and indirect hemagglutination test.

Direct immunofluorescence techniques (DFA) are the earliest fluorescentanti-body techniques. In direct immunofluorescence, an antibody labeled with a fluorescent dye binds directly to the target molecule to form an antigen-antibody complex. In this way, when the sample is excited, the fluorescent dye will emit a fluorescence signal, so that the location of the target molecule in the sample can be observed. The direct immunofluorescence technique is relatively simple and is suitable for situations where the molecule to be detected matches a known antibody. It has a wide range of applications in biomedical research and clinical diagnosis, such as detection of cell surface markers, detection of protein expression levels. At the same time, DIF has also been applied to skin autoimmune bullous diseases [14].Direct immunofluorescence technology can be used as a medical test tool to diagnose and distinguish diseases from other lesions. In cases with clinical and histological diagnostic dilemmas, DIF can improve the diagnosis rate.

Time-resolvedfluoroimmunoassay (TRFIA) is a commonly used immunoassay technique that is particularly suitable for the detection of low concentrations of biomolecules [19], such as proteins or hormones. The technique combines the principles of fluorescent labeling and time-resolved measurement. In TRFIA, the target molecule in the sample to be tested binds to specific antibodies to form an immune complex. These antibodies are often labeled with a fluorescent substance, such as europium or praseodymium, that emits a long-lived fluorescent signal. When

measurements are made, the light signal in the sample is excited, producing fluorescence. Then, the emission time of fluorescence signal is measured by time-resolved fluorescence measuring instrument. Due to the long-lived fluorescence signal of the marker, it can be accurately measured under the influence of other background light signals in the sample, thus improving the sensitivity and accuracy of the detection. Time-resolved fluorescence immunoassay is widely used in the fields of medical diagnosis, biological research and drug development, and is especially suitable for the detection requirements requiring high sensitivity and accuracy. There are also examples of applications in food safety testing. For example, the detection of deadly microorganisms, biotoxins and harmful metals in food [15].

**Conclusions to chapter 2**

1. In general, the research of these biotechnological detection methods is helpful to understand the chemical composition, pharmacological activity and quality control of Xiaoyao pills, which lays a foundation for the further development and application of Xiaoyao pills.

# CHAPTER 3
# EXPERIMENTAL PART

## 3.1 Research method

### 3.1.1 Experimental animals and grouping

SPF male healthy C57BL/6J mice, body weight 18-22 g, 30 mice; SPF grade male healthy CD-1 mice, body weight 30 ~ 35g, 10 mice. They were negative control group, depression model group and Xiaoyao pill treatment group. After entering the laboratory, the mice adapted to the environment for a week, ate and drank freely, kept the temperature 21±1℃, 12 h/12 h light and dark cycle, day and night were reversed (the light was turned on at 20:00 and the light was turned off at 8:00); Daily line grip parallel operation, eliminate the influence of human operation. All animal experiments are conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

All behavioral tests were conducted in the dark phase of mice, and the time was fixed from 09:00am to 17:00pm. After the modeling, mice in XYW intervention group were given intragastric administration (1.365 $g \cdot kg^{-1}$) for 14 days, and the other groups were given the same volume of normal saline. The experimental flow chart is shown below (Fig. 3.1).

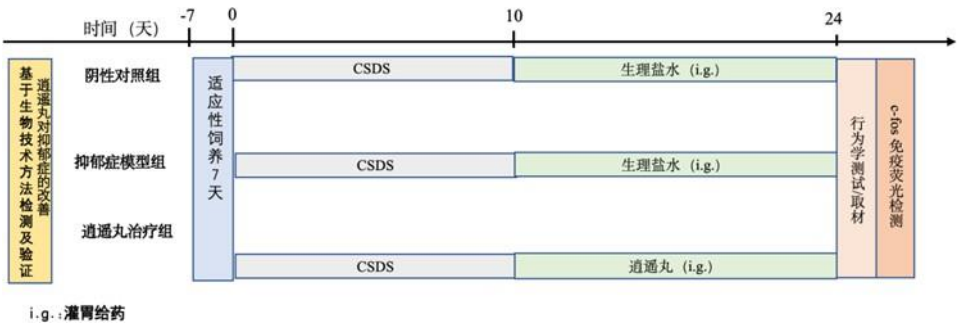


Figure 3.1 – **Experimental flow chart**

### 3.2 Behavioral indicators detection

#### 3.2.1 Open field experiment

OFT is carried out in an open field box with a length of 50 cm, a width of 50 cm and a depth of 50 cm. The bottom of the open field box is evenly divided into 9 squares, the middle area of the open field box is set as the central area, and the rest area is the peripheral area [20]. The bottom and sides of the open field box are black. The experiment was carried out in a quiet and weak red light environment. The mice were placed in the central zone at the beginning, and the activities of the mice within 6 minutes were observed and recorded, as well as the total distance of the mice, the residence time of the central zone, the movement distance of the central zone and the number of times of the central zone. In the process of behavioral experiment operation, the experimental mice should be placed in the laboratory one hour in advance so that they can adapt to the experimental environment in advance to reduce the impact on behavior. While keeping the experimental environment quiet, the mice were gently caught according to the laboratory specifications and kept away from the test box. After the experiment, the bottom of the box was sprayed with 75% ethanol and wiped with gauze to remove the odor secreted by the animal, and then the next animal experiment was carried out. In order to avoid the odor of the previous animal's movement track to interfere with the next test mouse [16].

#### 3.2.2 Sugar spray experiment

A 10% sucrose solution was sprayed on the back hair of mice. The sucrose solution soiled the back hair and caused grooming behavior, and the cameras recorded the mice's activity for six minutes. The experimental indicators to be recorded during the experiment are the incubation period of grooming, the time of grooming, the number of grooming and the proportion of grooming time to the total time.

### 3.2.3 Tail suspension test

Attach the desensitization tape to 1/3 of the distal end of the tail tip of the mouse, and fix it in the tail box, keep the mouse in an upright state with the head facing the camera, about 30 cm from the ground. The Smart3.0 animal behavior analysis system was used to record the behavior state of mice within 6 minutes in real time, and the tail suspension time was counted. The immobile time of mice stopped struggling and was in an inverted overhanging state, which was used as the criterion to judge their behavior desperation. The tail immobilization time and incubation period were collected and analyzed.

### 3.3 Tissue sampling

The tissue samples were collected after the last behavior test. The mice were anesthetized with pentobarbital sodium, the animals were given cardiac perfusion, the thorax was cut from both sides of the forelimb, the heart was lifted to find the active vessel, and a blunt needle was used to enter the right atrium from the left ventricle of the heart oblique to the upper left direction of 45 degrees, and the needle was fixed with an aortic clamp after entering the active vessel to prevent it from falling off. The right atrial ear of the mice was cut, and the blood of the whole body was quickly rinsed with pre-cooled 1X PBS. After the color of the blood became pale, the blood was irrigated with 4% PFA fixed solution. After the whole body was stiff like a plate, the brain was decapitated with a mouse decapitator and the complete brain tissue was extracted.

### 3.4 c-fos immunofluorescence detection in hippocampus

1. According to the brain atlas, continuous coronal frozen sections were conducted at -20℃ with a section thickness of 40μm.Two mouse brain tissues were selected for each group, and 4 slices were reserved for each mouse. The brain slices of the target region were placed on a slide (2 brain slices were placed on each slide) and immersed in a wet box filled with 0.01M PBS (pH7.4) and gauze.

2. Four more brain slices from the normal control group were selected as negative control.

3. 0.01MPBS was washed, 10 min×3

4. Goat serum was added and incubated at room temperature for 2 h, then the solution was sucked out and discarded.

5. Add monoclonal antibody and dilute with a blocking solution (goat serum blocking solution) at 4 ℃ overnight; (The primary antibody can be recycled and reused, and the negative control is not added to the primary antibody, only the sealing liquid is added).

6. Rewarm at room temperature for 1h;

7. PBST wash, 10 min × 3; PBST: 990 ml PBS+10ml Triton

8. Add secondary antibody, dilute with a blocking solution, and incubate for 2h (avoid light at the beginning of this step);

9. PBST washing,10 min × 3

10. Mounting: under the condition of avoid light, mount the brain piece neatly on the slide, filter paper to absorb moisture, natural drying, about 20 min.

11. Sealing plate: Add a drop of anti-fluorescence quenching sealing solution in the center of the slide, cover the cover glass, and seal a circle of nail polish around the cover glass. The sealed film was stored at 4 degrees Celsius away from light for fluorescence microscopic observation.

### 3.5 Data Processing

GraphPadPrism9.0 was used for data statistics, and normality test and variance homogeneity test were required for each group of data before analysis. The data were normally distributed and homogeneity of variance was satisfied. One-way analysis of variance was used for comparison among multiple groups, and Tukey test [17]was used for further pound-wise comparison. Two-factor ANOVA was used for the growth rate of body mass that needed to be measured repeatedly. Qualitative data was expressed as percentage (%), quantitative data was expressed as mean ± standard error, and the significance level was set as $P < 0.05$ [18]. Data that deviated two or more standard

deviations from the mean were considered outliers and were excluded for statistical purposes, as were mice with abnormal behavior.

(1) Preparation of depression model mice

All the animals were divided into three groups: normal blank control group, depression model group and Xiaoyao pill intervention group. CSDS modeling was performed for 10 days in the depression model group and Xiaoyao pill intervention group.

(2) Behavioral evaluation of Xiaoyao Pill intervention in depressed mice

The mice were treated with Xiaoyao pill by gavage, and the behavior of the mice model was detected by open field test, sugar spray test and tail suspension test to evaluate the antidepressant effect of Xiaoyao pill intervention on depression model mice (Fig. 3.2-3.4).

Open field experiment results:

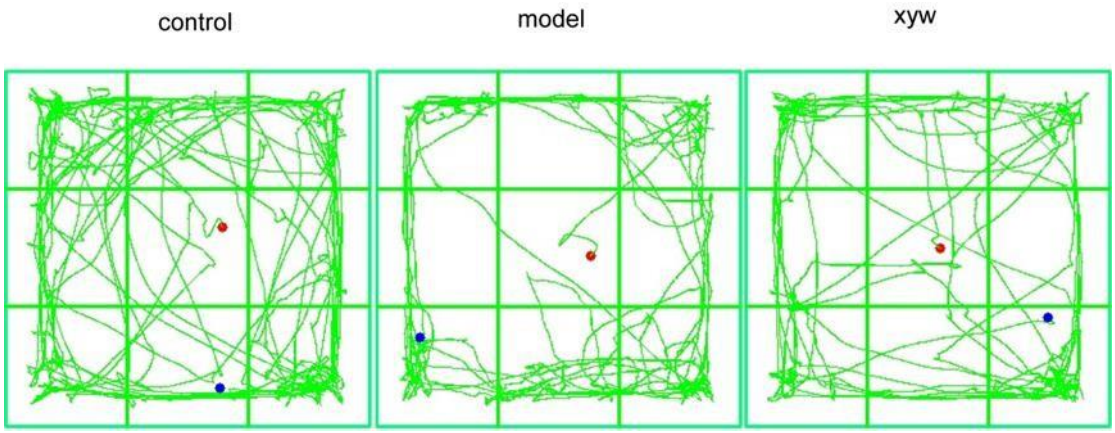


Figure 3.2 – **Open field box activity**

Note: control group was normal control group, model group was model group, xyw was Xiaoyao pill intervention group.

The results showed that: in the comparison of the total distance of open field experiment, compared with the blank control group, the total distance of depression model group was slightly less than that of the blank group, while the total distance of Xiaoyao pill intervention group was slightly less than that of the blank group and the depression model group. According to the data of entry times in the central area of open field, the number of times in the depression model group was significantly lower than that in the blank control group, which could indicate that the exercise motivation

of depressed mice in this area was reduced, which was consistent with the clinical situation of depression.

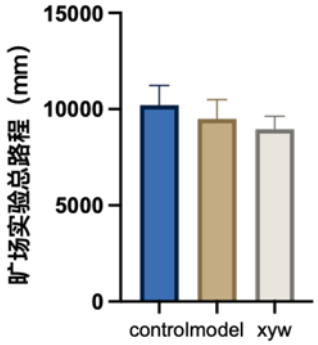


Figure 3.3 – **Comparison chart of total distance traveled**

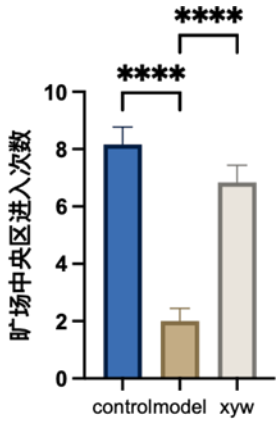


Figure 3.4 – **Comparison of the number of times of entry in the central area of the mine**

At the same time, the data of Xiaoyao Pill intervention group showed that the frequency of entering the center of the open field was significantly higher than that of the depressed mice group, and there was a comparative difference between the two groups, and it was close to the blank control group, indicating that Xiaoyao pill can significantly increase the autonomic movement of mice and improve the depressive state of mice (Fig. 3.5).

Sugar spray experiment results:

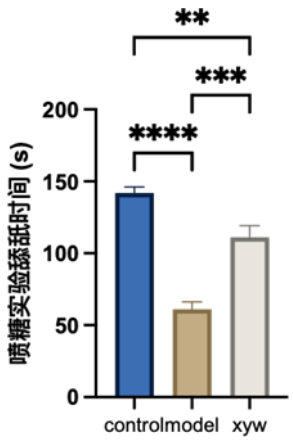


Figure 3.5 – **Licking time comparison chart**

The results showed that: Compared with the blank control group, the licking time of the depressed mouse model was significantly lower than that of the blank control group, indicating that the mice's love of sugar was reduced, indicating the disappearance of pleasure, which could indicate the emergence of depressive symptoms in mice. The results of the Xiaoyao pill intervention group were significantly higher than those of the depression model group, indicating that after the intervention of Xiaoyao pill, the mice's love of sugar increased, basically similar to the results of the blank group, indicating that Xiaoyao pill can improve the symptoms of depression in mice(Fig. 3.6).

Tail suspension test results:

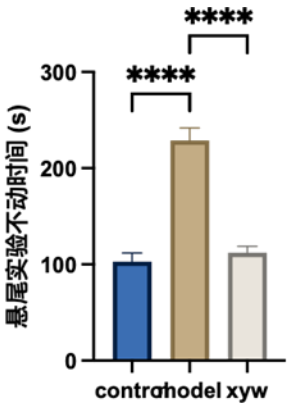


Figure 3.6 – **Comparison diagram of stationary time of tail suspension experiment**

The results showed that: compared with the blank control group, the suspension time of the depressed mouse model was significantly longer, indicating that the mice appeared intermittent immobility for a longer time, and the behavior of despair for a longer time, indicating the emergence of depression symptoms. However, the duration of tail suspension in the Xiaoyao pill intervention group was significantly lower than that in the model group, which was basically the same as that in the blank group, indicating that the depression state could be significantly shortened after the intervention of Xiaoyao Pill.

(3) Biotechnological method detection of Xiaoyao pill in the intervention of depressed mice

The expression of c-fos activation in hippocampal brain region was detected by immunofluorescence, and the intervention effect of Xiaoyao Pill on pathological indexes of depression model mice was evaluated by comparing the activation amount of c-fos in hippocampal brain region between different groups (Fig. 3.7-3.8).

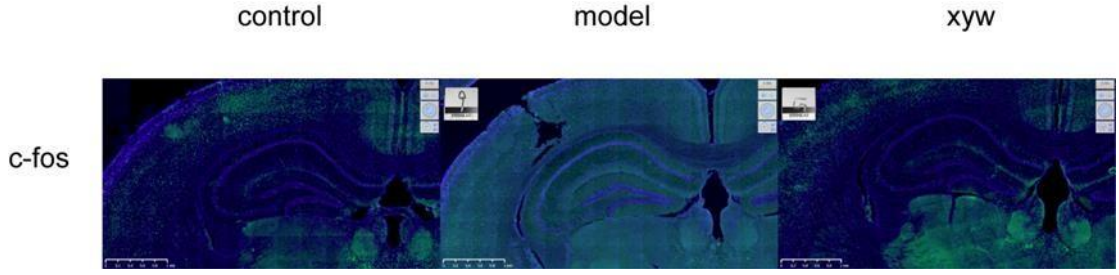


Figure 3.7 – c**-fos activation diagram of hippocampus**

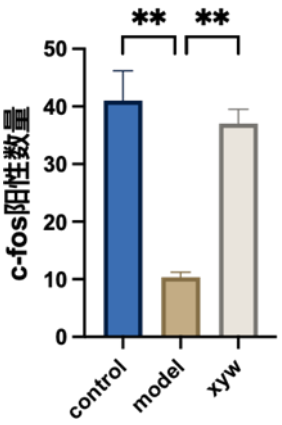


Figure 3.8 – **Comparison of the number of c-fos positives in the hippocampus**

The results showed that there were differences in the number of c-fos activations in hippocampal regions among different groups. Compared with the blank control group, the content of c-fos in the depression model group was significantly reduced, while that in the Xiaoyao pills intervention group was significantly higher than that in the depression model group, indicating that the activation amount of c-fos in the hippocampus of different groups would change after the intervention of the depression group and Xiaoyao pills, reflecting the correlation between c-fos and depression from a microscopic perspective. It also shows that the index of c-fos can be used as a reference index of depression.

**Conclusions to chapter 3**

1. The intervention of Xiaoyao Pill can significantly shorten the depression state, reduce the fear and despair of mice, and thus improve depression.
2. C-fos is a marker that can reflect neuronal activity and can react quickly to external stimuli, so as to be expressed in the brain. Through the activity of different neurons, the mice respond to different emotional stimuli.

## CONCLUSIONS

1. Animal model preparation and behavioral index discussion. First, learn to prepare animal models of disease, and divide all animals into three groups: normal control group, model group, Xiaoyao pill intervention group. Behavioral tests of mouse models were conducted to verify the preparation of animal models through open field experiment, sugar spray experiment and tail suspension experiment, which can reflect animal behavior indicators. The results show that: First of all, in the open field experiment, the number of times of entering from the central area of the open field shows that the number of times of entering from the central area of the depression model group is significantly lower than that of the blank control group, indicating that the exercise motivation of depressed mice in this area is reduced, which is in line with the clinical situation of depression. In general, the amount of time a mouse spends in the central and peripheral areas can indicate whether it is mentally or depressed. Mice with lower or normal depression tended to spend more time in the central area of the open field, while mice with higher depression tended to spend more time in the surrounding area. Secondly, in the sugar spray experiment, compared with the blank control group, the licking time of the depression mouse model was significantly lower than that of the blank control group, indicating that the mice's love of sugar was reduced, and the stimulation of sweetness was reduced, indicating the disappearance of pleasure and indicating the emergence of depressive symptoms. The sugar water preference test is a sensitive study of neural responses that can reflect depression or anxiety in mice. Finally, the results of tail suspension experiment showed that the time of tail suspension of mice with depression was significantly longer, indicating that the time of intermittent immobility of mice was longer, and the time of behavioral despair was longer, indicating the emergence of depressive symptoms. The mice were in a certain space, more fearful, in a state of despair, a state of despair consistent with depression. From the above behavioral indicators of mice, it can be proved that the model of depression in mice is successful.

2. Behavioral evaluation of Xiaoyao Pill intervention in depressed mice. The mice with depression were treated with compound Xiaoyao pill by intragastric administration, and the results of anti-depression intervention with Xiaoyao pill were compared by open field experiment, sugar spray experiment and tail suspension experiment. The results of open field experiment showed that the frequency of entering the center of open field in the Xiaoyao Pill intervention group was significantly higher than that in the depression mouse group, with a relatively different frequency, and was close to the blank control group, indicating that Xiaoyao Pill can significantly increase the autonomous movement of mice, change the original behavior of depressed mice like to move in the surrounding area, and prefer to stay in the center of open field differently. Therefore, Xiaoyao pills can improve the depressive state of mice. The results of the sugar spray experiment showed that the results of Xiaoyao Pill intervention group were significantly higher than those of the model group, indicating that after the intervention of Xiaoyao pill, the mice's preference for sugar was increased, basically not much different from that of the blank group, indicating that Xiaoyao pill can improve depressive symptoms. The sugar spray experiment reflected the mice's stress response, as well as their interest in sweets and ability to experience pleasure from sugar. After modeling, the mice's pleasure disappeared, the ability to experience happiness decreased, and the indicators were improved after the intervention of Xiaoyao Pill, which can reflect the effect of Xiaoyao pill in the intervention of depression. The results of the tail suspension experiment showed that the tail suspension time of Xiaoyao Pill intervention group was significantly lower than that of the model group, which was basically the same as that of the blank group, indicating that the intervention of Xiaoyao Pill can significantly shorten the depression state, reduce the fear and despair of mice, and thus improve depression. Xiaoyao powder composition: licorice (slightly scorched red) half two (15 g), angelica (seedling, file, stir-fry), Poria (peel) white, white peony medicine, white art, Bupleurum (seedling), one or two (30g each). It is a conciliatory agent that can ease the liver and relieve depression, thereby improving the symptoms of depression. From

the perspective of animal behavior indexes, it can be found that different animal behavior indexes are improved after the intervention of Xiaoyao Pill, which can reflect the effectiveness of Xiaoyao Pill.

3. Immunofluorescence was used to detect the expression of c-fos activation in the hippocampal brain region, and the comparison results of the activation amount of c-fos in the hippocampal brain region between different groups were discussed. The expression of c-fos activation in hippocampal brain region was detected by immunofluorescence, and the intervention effect of Xiaoyao Pill on pathological indexes of depression model mice was evaluated by comparing the activation amount of c-fos in hippocampal brain region between different groups. This experimental step can first master the basic experimental operation immunofluorescence detection technology in the biotechnology major, and master the experimental operation of biotechnology. Secondly, from the point of view of micro-index, evaluate and verify the effectiveness of Xiaoyao pill intervention on depression. The results show: Firstly, there were differences in the content of c-fos in the hippocampal brain region between different groups. The content of c-fos in the depression group decreased, and the content of c-fos in the Xiaoyao pills intervention group could increase the content of c-fos, indicating that the activated number of C-Fos in the hippocampal brain region between different groups would change after the intervention of the depression group and Xiaoyao pills. The correlation between c-fos and depression is reflected from the microscopic perspective, and the index of c-fos can be used as a reference index of depression.

4. This experiment has the following purposes: First, as a biological major, learn to prepare animal models of diseases, which can lay a foundation for the preparation of other disease models in the future. Secondly, I can master the basic experimental operation immunofluorescence detection technology in biotechnology majors, and master the experimental operation of biotechnology. These two purposes can learn the basic operation of biotechnology professional and meet the basic requirements. Thirdly, through the intervention of compound Xiaoyao Pill, from the

perspective of animal behavior indicators and the expression of c-fos activation in hippocampus, the improvement of depression was verified. The results showed that: First, the open field experiment, sugar spray experiment and tail suspension experiment were conducted to verify the success of the animal model. Secondly, open field experiment, sugar spray experiment, tail suspension experiment and the expression of c-fos activation in hippocampal brain region showed that Xiaoyao Pill can improve depression. The purpose of the experiment is basically completed. Although the mouse model experiment is the basic carrier for the study of diseases and drugs, it may have certain limitations due to many conditions, and it can only reflect behavioral indicators, but cannot directly detect emotions. The results need to be further validated before they can be used in clinical trials. It is expected that this experimental design will provide more possibilities for the study of neural mechanisms in the future.

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