Effect of reserpine on chroninc unpredictable mild stress of rat model.

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Abstract

The objective of the present study was to make comparison between rat models of depression achieved by chronic unpredictable mild stress (CUMS) and reserpine injection from the angle of traditional Chinese medicine syndrome, depict the regularity of syndrome variation of the two models, and preliminarily explain its molecular mechanism. Rat depression models were achieved respectively by CUMS and intraperitoneal reserpine injection. The syndromes of liver stagnation, spleen deficiency and liver stagnation with spleen deficiency were judged by equivalently transforming the related clinical symptoms and into macro-characterization of rats. Elisa was used to measure the content of 5- serotonin (5-HT) in serum and Immunohisto-chemical method was used to measure the content of 5-HT in the hippocampal tissue CA2 district. The regularity of syndrome variation is: Rats in the CUMS group developed liver stagnation syndrome 2 weeks after stress and liver stagnation with spleen deficiency syndrome 6 weeks after stress, while rats in the reserpine injection group developed spleen deficiency syndrome 2 weeks after injection and liver stagnation with spleen deficiency syndrome 4 weeks after injection. Compared with the normal group, rats in CUMS group and reserpine group both presented persistent decrease in brain tissue 5-HT content (P<0.05) and increase in serum and intestine tissue 5-HT content (P<0.05), while degree of increase or decrease differed in the two groups in different period. The model of depression achieved by CUMS develops from liver stagnation syndrome gradually into Liver stagnation and Spleen deficiency syndrome, whose biological basis is that the onset of central 5-HT system dysfunction is faster than the peripheral 5-HT system for the stress conditions; The model of depression achieved by reserpine injection develops from spleen deficiency syndrome gradually into Liver stagnation and Spleen deficiency syndrome, whose biological basis is that the increase of peripheral 5-HT is faster than the decrease of central 5-HT for the effect of reserpine.

Depression is a mood disorder whose main clinic characteristics include lasting low motion, decrease in motivate ability and slowness in function of thinking and recognizing. It is showed in national survey that the suicide rate of people suffering from depression is 20 times higher than that of normal people [1]. Modern medical investigation shows that the biological foundation of depression is related to the lack of 5-HT, a neurotransmitter closely related to human activity and the onset of depression [2], which participates in the regulation of motion, memory, appetite and sexual function. At present, depression is always treated with traditional Chinese and Western medicine integrated method in clinical practice, while the basic research on clinical advantage of traditional Chinese medicine is limited due to a lack of proper animal model combining disease and syndrome. In that case, treatment according to syndrome differentiation has no pathological or physiology basis, thus can't provide objective index for

clinical diagnosis and syndrome discrimination.

Chronic unpredictable mild stress (CUMS) is a currently internationally accepted method to establish a rat model of depression, where animals were exposed to a series of chronic unpredictable mild stresses to imitate a relatively real situation of human life with chronic, low-intensity events. The animals' consumption of food and water decreased after the chronic unpredictable mild stress, reflecting the central symptom of inner-rooted depression: the absent of pleasant sensation [3, 4]. This model is of very high application value since it accurately imitated clinical treatment situation. In the mean time, reserpine injection is also a widely applied method to achieve animal model of depression, which is commonly used in selecting antidepressant drugs with a certain pharmacological effect.

In this paper, we discriminated the TCM syndromes in the two kinds of rat model of depression by equivalently transforming the macroscopic representation of rats into clinical human symptoms and detecting rats' ethology. In the mean time, we tried to describe the difference in syndrome change pattern in the two kinds of model and to preliminarily explain its biological basis through measurement of central and peripheral 5- serotonin (5-HT) contents.

Material and Method

Experimental animals and grouping
A total of 30, 6 week old, male SD rats, SPF class, weighing 200± 10g were provided by Beijing Weitong Lihua Experimental Animal Technology Co., Ltd.(Experimental animal production license: SCXK Beijing 2006-0009). The rats were fed and housed in the animal room of the scientific research center of Beijing University of Chinese Medicine, at an ambient temperature of 20~25°C, under a 12/12h day/night circle. All rats were fed normally for 2 weeks, and then randomly divided into 3 groups: CUMS group (10 rats), reserpine group (10 rats), normal control group (10 rats), among which CUMS group and reserpine group are called the experimental groups.

Drug, Administration approach and Reagent
Reserpine injecta was purchased from Guangdong Bangmin Pharmaceutical Co., Ltd., specifications: 1ml:1mg, 10 branches in each package, stored in shading, airtight environment at room temperature.

Rats in reserpine group were given intraperitoneal injection of reserpine 4mg/kg/d [5]. The injection was given to 8-week-old rats, and lasted for 8 weeks.

Main reagent: the rat's 5-HT Elisa kit were provided by American RD company; Mouse anti-rat 5-HT monoclonal antibody was provided by Santa Cruz Company (USA), the secondary antibody was biotin labeled-anti-mouse IgG, provided by gene technology (Shanghai) Co., Ltd.;

immunohistochemistry detection kit was purchased from gene technology (Shanghai) Co., ltd. The analysis software Image-Pro Plus6.0 was adopted.

Establishment of rat model of depression by CUMS
The stress methods of chronic unpredictable mild stresses include: no food or water for 24h, 30 degrees tilted cage for 8h or overnight, wet housing for 8h or overnight, 4°C ice-water swim for 5min, 45°C heat for 5min, restriction for 2h, tail clamp for 1min(placing a hemostatic clamp 1cm away from the root of the rat's tail until the rat cries), electrical stimulation at 36V for 1min every 30s for 30 shocks a time. 3 kinds of stress were chosen to be carried out daily by a random number table. The stress was given to 8-week-old rats, and lasted for 8 weeks.

Body weight determination and sugar consumption test
Rats in every group were weighed every 2 weeks from 6 weeks of age. Sucrose consumption test was carried out every two weeks before the stress. Fasting and water deprivation lasted for 12 h before the test. During the experiment, the rats in the sham operated were also kept in solitary cages. Place 2 bottles with 2% sucrose water vial (bottle of 100ml) in each cage, and then change to a bottle of 2% sucrose water and another bottler of pure water(bottle of 100ml) per cage 12h later. 2h later, remove all the bottles and weigh the rats [6], then calculate the consumption of sucrose.

Determination of ethology
Open-field test: test device is composed of the reaction tank and recording and analysis system. The rat open-field reaction tank is 35cm high, with a square bottom and a side length of 100cm. The inner bottom surface was black, divided into a total of 25 small boxes sizing 4cm*4cm. There is a digital camera just 2m above the open field, whose vision can cover the whole internal field. During the test, the rats were placed in the center of the clean open tank, recorded by the recording and analysis system for small animal ethology (Smart1.0, Panlab) for 3 min a time. Then calculate the frequency when the rat cross a small box and total distance of activity. In order to avoid the animal adaptability to the open-field, the test was carried out every 2 weeks since 8 weeks of age.

Acquisitions of macroscopic characterization and discrimination of syndrome
According to Chinese *guiding principle of clinical research on new drugs of traditional Chinese (2002 Edition)*, we transformed clinical symptoms of depression [7] equivalently into rat macroscopic representation, so as to realize the consistency of clinical syndrome and syndrome on rats.

The specific correspondence of Liver stagnation syndrome is: depressive mood and distension, stuffiness and scurrying pain in chest, hypochondrium or lower abdo-

men correspond to the decrease in both total distance of activity and the frequency of small-box crossing in rat open-field test and intensification of grab resistance. The clinical diagnostic criteria include these symptoms, so it is with the discrimination of syndrome in rats.

The specific correspondence of spleen deficiency syndrome is: poor appetite, abdominal distention after eating or in the afternoon and hypodynamia, abnormal stools (pond, rotten, pond after hard, change between pond and hard) correspond to delayed increase of body weight, reduction in sucrose consumption, decrease in total distance of activity and the frequency of small-box crossing in the open-field test, and abnormal stools (pond, rotten, pond after hard, change between pond and hard). The clinical diagnostic criteria require at least 2 of the symptoms above, so it is with the discrimination of syndrome in rats.

The specific correspondence of Liver stagnation and Spleen deficiency syndrome is: distending pain in gastral cavity or lateral thorax necessary, poor appetite, loose stools as main symptoms correspond intensification of grab resistance, delayed increase of body weight and reduction in sucrose preference, loose stool; depressive mood with deep sigh, borborygmus and flatus, diarrhea after abdominal pain as secondary symptoms correspond decrease in total distance of activity and the frequency of small-box crossing in the open-field test, increase in frequency of stool when the rat's tail is pulled. The clinical diagnostic criteria require at least 3 of the main symptoms (distending pain in gastral cavity or lateral thorax necessary) or 2 of the main symptoms (distending pain in gastral cavity or lateral thorax necessary) and 1 of the secondary symptoms, so it is with the discrimination of syndrome in rats.

Materials processing

Get orbital blood
Get 2 ml orbital blood in the 3 groups respectively at the time before stress, 2 weeks, 4 weeks and 6 weeks after stress.

Get breakpoint material
Select 20 rats randomly from each group to get material 4 weeks after stress and the other rats material was got 8 weeks after stress. Sampling process: Anesthetize the rats with 10% chloral hydrate, abdominal blood 5ml, and then directly remove the entire brain and intestine on the ice quickly, place the material in 4% paraformaldehyde to fix for 48h, imbedded in paraffin, the cross section is 3.80mm after bregma, 5.20mm on the ear line.

Determination of 5-HT content in serum by Elisa and expressions in brain tissues by Immunohistochemistry
Balance the kit to room temperature, remove the required reaction plate, add 10ul of standard product and 10ul of

serum in the corresponding reaction plate hole, add 40ul Anti Rat 5-HT Biotin and 40ul Anti Rat 5-HT POD to each hole, mix gently for 30 seconds, and then seal the plate hole, keep the plate in room temperature for 45 minutes, reject the liquid in the plate, wash the plate with washing liquid (350ul washing liquid per hole), and remove all the water (in a thick stack of absorbent paper); repeat the washing 5 times, then add 100ul color liquid to each hole, mix gently for 10 seconds, room temperature incubation for 20 minutes; add 100ul terminated liquid to each hole, mix gently for 30 seconds. Read the OD value intrinsic 450nm within 30 min. Draw a standard curve with the OD value as the vertical and standard concentration as the abscissa, we can find the concentration of the serum sample on the standard curve according to its OD value.

Sections were preheated, dewaxed, gradient dehydrated in alcohol, microwave repaired for 6 min, oxidated in 3% (v/v) hydrogen peroxide at room temperature for 15 min, and rinsed 3 times (5 min each) with phosphate buffered saline (PBS). Sections were then incubated overnight at 4° C with 5-HT primary antibody using a wet box. Sections was dropped by secondary antibody signed by horse radish peroxidase and incubated at room temperature for 1 h, colored with diaminobenzidine (DAB), re-stained with hematoxylin, dehydrated with alcohol without coverslip and sealed. Negative controls were also performed where sections were incubated in PBS instead of primary antibodies. Sections were analyzed using the Image-Pro Plus 6.0 image analysis software. One brain slice from each rat was chosen for analysis. Five randomly selected fields of view were captured under a ×200 optical microscope and the analysis index was average optical density (OD).

Statistical methods
Analysis of data was performed using SPSS17.0 statistical software. Data were expressed as mean ±standard deviation, using ANOVA process and LSD method. A $P<0.05$ was considered statistically significant.

Results

Results of weight measurement
Weight of rats in the three groups had no significant difference before intervention and 2 weeks after intervention ($P>0.05$); 4 weeks after intervention, weight of rats in reserpine group was significantly lower than that of the normal group and CUMS group ($P<0.05$), which remains lower till 8 weeks after intervention ($P<0.05$); weight of rats in CUMS group began to show significant inferiority to that of normal group 6 weeks after intervention ($P<0.05$), which remains lower till 8 weeks after intervention ($P<0.05$). (see Table 1).

Table 1. *Comparison of body weight of rats in each group (unit: g)*

Groups	Before inter-vention (n=40)	2 weeks after intervention (n=40)	4 weeks after intervention (n=40)	6 weeks after inter-vention (n=20)	8 weeks after inter-vention (n=20)
control group	378.28±24.48	431.48±31.21	495.01±25.43	548.09±33.84	588.17±45.33
CUMS group	375.65±25.10	420.01±34.25	469.22±32.98▲	493.93±30.78★▲	507.21±44.02★▲
reserpine group	380.56±21.20	403.67±29.22	419.03±31.58★	421.04±39.67★	420.19±40.88★

★*compared with normal group,P<0.05;*▲*compared with reserpine group,P<0.05*

Table 2. *Comparison of consumption of 1% sucrose water in each group (unit: ml)*

Groups	Before intervention (n=40)	2 weeks after intervention (n=40)	4 weeks after intervention (n=40)	6 weeks after intervention (n=20)	8 weeks after intervention (n=20)
control group	90.83±3.87	84.40±6.91	87.16±11.74	85.32±14.73	88.07±10.93
CUMS group	89.01±2.89	79.98±9.36▲	67.89±10.22★▲	47.71±13.14★▲	28.44±8.89★
reserpine group	89.91±4.56	63.16±7.77★	46.06±12.15★	24.95±10.57★	20.28±11.98★

★*compared with normal group, P<0.05;* ▲*compared with reserpine group, P<0.05*

Table 3. *Comparison of frequency of small-box crossing (n=10, unit: /3min)*

Groups	Before intervention (n=40)	4 weeks after intervention (n=40)	8 weeks after intervention (n=20)
control group	71.83±6.68	73.50±7.34	69.83±6.34
CUMS group	76.08±6.34	17.17±2.98★	6.75±2.38★
reserpine group	77.25±7.09	17.17±1.61★	8.08±3.96★

★*compared with normal group, P<0.05;* ▲*compared with reserpine group，P<0.05*

Table 4. *Comparison of total distance of rats activity (n=10, unit: mm/3min)*

Groups	Before intervention (n=40)	4 weeks after intervention (n=40)	8 weeks after intervention (n=20)
control group	1635.21±130.87	1208.27±120.42	883.15±74.07
CUMS group	1611.25±105.51	371.82±26.95★	238.01±33.77★
reserpine group	1575.56±109.46	347.98±19.25★	305.79±20.68★

★*compared with normal group, P<0.05;*▲*compared with reserpine group, P<0.05*

Results of sucrose consumption test

Sucrose consumption of rats in the three groups had no significant difference before intervention and 2 weeks after intervention (P>0.05); 2 weeks after intervention, sucrose consumption of rats in reserpine group was significantly lower than that of the normal group and CUMS group (P<0.05), which remains lower till 6 weeks after intervention (P<0.05); sucrose consumption of rats in CUMS group began to show significant inferiority to that of normal group 4 weeks after intervention (P<0.05), which remains lower till 6 weeks after intervention (P<0.05); 8 weeks after intervention, sucrose consumption of rats in CUMS group and reserpine group showed no significant difference (P>0.05) although still lower than that of normal group (P<0.05). (see Table 2)

Determination of ethology

In the open-field test, there was no significant difference among groups before intervention (P>0.05); since 4 weeks after intervention, the total distance of activity and the frequency of small-box crossing group of rats in CUMS group and reserpine group began to be lower than that of rats in normal group (P< 0.05), but the CUMS group and reserpine group showed no significant difference (P>0.05). (see Tables 3, 4)

Syndrome discrimination results
Discrimination of syndromes in rats was based on the criteria mentioned in 1.10 (see table 5). Rats in CUMS group presented Liver stagnation syndrome 2 weeks after stress (10 weeks old) and Liver stagnation and Spleen deficiency syndrome 6 weeks after stress (14 weeks old), while rats in reserpine group presented Spleen deficiency syndrome 2 weeks after injection (10 weeks old) and Liver stagnation and Spleen deficiency syndrome 4 weeks after stress (12 weeks old).

Expression of 5-HT in brain tissue
4 weeks after intervention, the 5-HT content in intestinal tissue of rats in CUMS group was lower than that of rats in normal group and reserpine group (P<0.05), while reserpine group showed inferiority compared with normal group but the difference was not statistically significant (P>0.05); 8 weeks after intervention, the 5-HT contents in intestinal tissue of rats in experimental groups were significantly lower compared with normal group (P<0.05), while CUMS group and reserpine group showed no significant difference (P>0.05). (see Table 6, the results of photos in Fig. 1).

A 4 weeks after intervention in control group; B 4 weeks after intervention in CUMS group; C 4 weeks after intervention in reserpine group; D 8 weeks after intervention Group; E 8 weeks after intervention in CUMS group; F 8 weeks after intervention in reserpine group.

Content of 5-HT in serum
Before intervention, no significant difference was shownd among groups (P>0.05); 2 weeks after intervention, the content of 5-HT in serum of rats in the CUMS group was higher than that of rats in the normal group (P<0.05), but lower than that of rats in the reserpine group (P<0.05), which remained till 6 weeks after intervention; 8 weeksafter intervention, content of 5-HT in serum of rats in the CUMS group was significantly higher than that of mats in normal group (P<0.05), but not significantly different from that of rats in reserpine group (P>0.05). (see Table 7)

Average optical density of 5-HT in small intestine tissue
4 weeks after intervention, the contents of 5-HT in small intestine tissue of rats in experimental groups were higher

*Table 5. Results of rats syndrome discrimination at different time (n=10)*

Groups	Before intervention (n=40)	2 weeks after intervention (n=40)	4 weeks after intervention (n=40)	6 weeks after intervention (n=20)	8 weeks after intervention (n=20)
control group	—	—	—	—	—
CUMS group	—	20%Liver stagnation syndrome	60%Liver stagnation syndrome	80% Liver stagnation syndrome 20% Liver stagnation and Spleen deficiency syndrome	30%Liver stagnation syndrome 70% Liver stagnation and Spleen deficiency syndrome
reserpine group	—	70%Spleen deficiency syndrome	60% Liver stagnation and Spleen deficiency syndrome 40% Spleen deficiency syndrome	90% Liver stagnationand Spleen deficiency syndrome 10% Spleen deficiency syndrome	100% Liver stagnation and Spleen deficiency syndrome

*"-" indicates no syndrome of Liver stagnation or Spleen deficiency.*

Table 6. Average optical density of 5-HT in CA2 of rat hippocampus

Groups	4 weeks after intervention (n=40)	8 weeks after intervention (n=20)
control group	430.57±26.09	497.49±149.56
CUMS group	308.33±35.86★▲	196.78±44.10★
reserpine group	401.17±40.58	209.98±50.22★

★*compared with normal group, P<0.05;* ▲*compared with reserpine group, P<0.05*
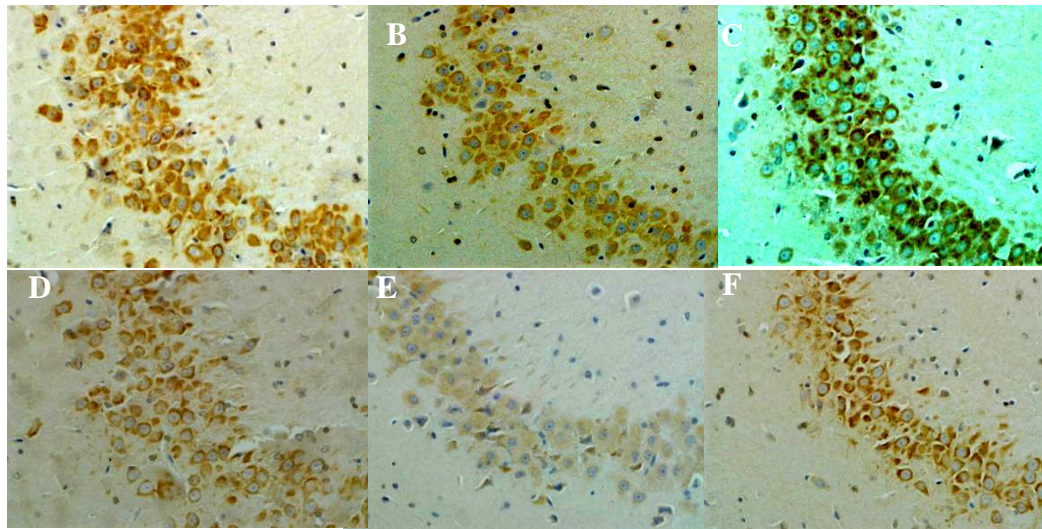
Figure 1. *Expression of 5-HT in hippocampal CA2 area of rats in the two groups (immunohistochemical staining x200) A: 4 weeks after intervention in control group；B: 4 weeks after intervention in CUMS group；C: 4 weeks after intervention in reserpine group；D: 8 weeks after intervention in control group；E: 8 weeks after intervention in CUMS group；F: 8 weeks after intervention in reserpine group.*
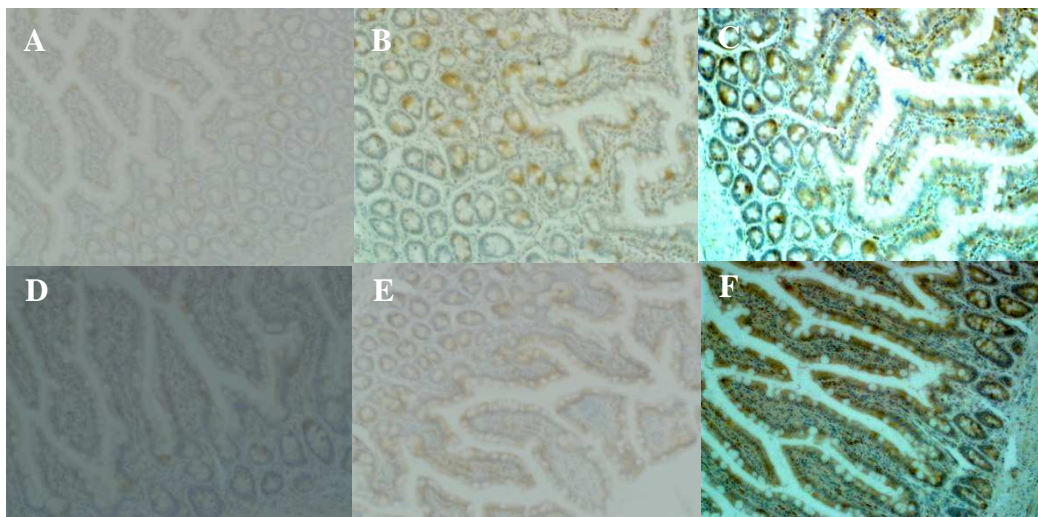
Figure 2. *Expression of 5-HT in small intestine tissue of rats in the two groups (immunohistochemical staining, x200) A: 4 weeks after intervention in control group；B: 4 weeks after intervention in CUMS group；C: 4 weeks after intervention in reserpine group；D: 8 weeks after intervention in control group；E: 8 weeks after intervention in CUMS group；F: 8 weeks after intervention in reserpine group.*

Table 7. *Ccomparison of content of 5-HT in serum among groups*

Groups	Before intervention (n=40)	2 weeks after intervention (n=40)	4 weeks after intervention (n=40)	6 weeks after Intervention (n=20)	8 weeks after Intervention (n=20)
control group	118.97±18.10	120.00±26.47	125.06±19.69	113.51±26.17	119.88±25.11
CUMS group	126.38±26.97	140.52±24.60★▲	159.59±35.64★▲	196.50±40.36★▲	218.92±21.04★
reserpine group	122.39±17.85	160.03±30.67★	203.52±44.28★	216.77±30.72★	221.39±19.83★

★compared with normal group, P<0.05; ▲compared with reserpine group, P<0.05

Groups	4 weeks after intervention (n=40)	8 weeks after intervention (n=20)
control group	68.22±20.36	86.66±37.25
CUMS group	234.10±41.59★▲	317.51±153.20★▲
reserpine group	336.22±73.46★	468.89±139.71★

★compared with normal group, $P<0.05$; ▲compared with reserpine group, $P<0.05$

than those of rats in the normal group ($P<0.05$), and content of 5-HT in small intestine tissue of rats in reserpine group was significantly higher than that of rats in CUMS group ($P<0.05$); 8 weeks after intervention, the content of 5-HT in small intestine tissue of rats in experimental groups continued to increase compared with that of rats in normal group ($P<0.05$), while the CUMS group and reserpine group showed no significant difference ($P>0.05$). (see Table 8, the results of photos in Fig. 2).

A 4 weeks after intervention in control group; B 4 weeks after intervention in CUMS group; C 4 weeks after intervention in reserpine group; D 8 weeks after intervention in control group; E 8 weeks after intervention in CUMS group; F 8 weeks after intervention in reserpine group.

Discussion

Seen from the result of weight measurement, sugar consumption test and determination of ethology, both CUMS group and reserpine group presented depression symptom, while there is a difference in the determination of TCM syndrome and the micro physicochemical index of 5-HT level. This is an embodiment of traditional Chinese medicine theory "same disease with different syndrome" in animal models.

Specifically, rats in CUMS group developed from liver stagnation syndrome into liver stagnation and spleen deficiency syndrome, whose time window was 14-16 weeks of age, that is, 6-8 weeks after stress; while rats in reserpine group developed from spleen deficiency syndrome into liver stagnation and spleen deficiency syndrome, whose time window was 12-16 weeks of age, that is, 4-8 weeks after injection of reserpine. This result reflects that there is obvious difference between stress and medicine interventions in the mechanism of causing depression. It is reported in the literature that the biological basis of depression is the lack of 5-HT, a monoamine neurotransmitter closely related to human activity and the onset of depression [2], which participates in the regulation of motion, memory, appetite and sexual function. Due to the existence of blood-brain barrier, it is very difficult for peripheral 5-HT to enter the central nerve system, thus the central and peripheral nervous 5-HT can be considered as two independent system of different functions [7].

When the function of 5-HT system is in disorder, the failure to adapt to stress can lead to the onset of symptoms of depression and anxiety [8]. Brunswick [9] discovered by the determination of the content of 5-HT in brain tissue of rats model of depression achieved by stress that the content of 5-HT in brain tissue of rats with depression was significantly lower than that of the normal control group, suggesting that a reduction in 5-HT can lead to depression, and the experimental results consistent; but the study also showed that, which is consistent with results of our experiment. In the meantime, research showed that both acute and chronic stress can increase the release of peripheral 5-HT [10, 11], which may be the reason why the content of 5-HT in serum and intestinal tissue of rats in CUMS group continued to rise after stress in this experiment. This indicates that the both central and peripheral 5-HT had a target for function of adapting to stress. Seen from the TCM perspective, rats in CUMS group presented Liver stagnation syndrome first and then developed into liver stagnation and spleen deficiency syndrome, showing that in stress conditions, the dysfunction of central 5-HT system is faster than that of peripheral 5-HT system.

Reserpine reduces sympathetic nerve tension, leading to the domination of parasympathetic nerve. As a result, smooth muscle 5-HT excited $5-HT_2$ receptors in gastrointestinal smooth muscle or $5-HT_4$ receptor in ganglion cell, causing contraction of gastrointestinal smooth muscle, so that gastrointestinal tension increased, and peristalsis is accelerated. In the meantime, the consumption of brain catecholamine and 5-HT storage reaches a level to inhibit the central nervous system [12]. Result of our experiment shows that the content of 5-HT in serum and intestinal tissue increased while that in cerebral tissue decreased continuously in rats in reserpine group, which is consistent with the reported literature. Therefore, considering the TCM theory, we can draw the conclusion that peripheral effect of reserpine results in Spleen deficiency syndrome in rats while central effect of reserpine leads to the occurrence of Liver stagnation syndrome. However, the experimental results showed that Spleen deficiency syndrome appeared earlier than Liver stagnation syndrome, namely the peripheral effect of increasing 5-HT content is faster than central effect of reducing 5-HT content, which may be related to the fact that reserpine need to pass through the blood-brain barrier to perform its central effect.

Acknowledgments

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