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Effects of an Chinese Herbal Compound Prescription on Simulated Transport Stress in Mice

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ABSTRACT

Background: In order to alleviate the transport stress reaction of animals, three kinds of chinese herbs with sedative and tranquilizing properties were extracted with ethanol and proportionally composed into a chinese herbal compound prescription. In addition, isorhynchophylline in leaves of *Uncaria rhynchophylla* was isolated and purified for use.

Methods: The enzyme-linked Immunosorbent assay (ELISA) was used to measure the changes in serum stress indicators and hormone levels, three classification blood cell counter and automatic biochemistry analyser was used to measure blood indicators and other biochemical indicators of each group mice before and after transport stress.

Result: The results showed that the blood physiological indexes such as blood glucose (GLU) level, biochemical indexes such as lactate dehydrogenase (LDH) and hormone levels such as adrocorticotropic hormone (ACTH) and other stress indexes were significantly higher in mice after transport than before. The compound agents and isorhynchophylline played a role in regulating the stress indices of mice after the simulating transport processs and have the effect of significantly alleviating the transport stress response in mice, with a view to provide reference for alleviating the adverse effects of transport stress on the animal husbandry industry.

Key words: Chinese herbs, Extracts, Leaves of *Uncaria rhynchophylla*, *Semen Ziziphi Spinosae*, Stress indicators, Transport stress, Valerian.

INTRODUCTION

Transport stress is a kind of defensive reaction caused by a series of changes in physiological and biochemical indexes of an animal due to the continual stimulation of strong comprehensive stressors such as fasting, turbulence, herding and psychological stress produced by the animal itself during the animal transport process. The mechanism of transport stress mainly lies in the activation of SAM (sympathetic-adrenomedullary axis) and its excitation when stress occurs in animals (Drain *et al.,* 2007). This leads to the secretion of catecholamines, including adrenaline and norepinephrine, which can cause increased heart rate, vasoconstriction and raised blood pressure and glucose (Knowles *et al.,* 1997). In severe cases, it can lead to sickness in the animal, as well as secondary infectious diseases and even death due to reduced immune function. Morbidity and mortality caused by transport stress bring significant economic losses to the livestock and poultry industry (Blood *et al.,* 1979). Transport stress often causes changes in physiological, biochemical and hormonal indicators in the animal body (Grigor *et al.,* 2001; Nijdam *et al.,* 2005). In the past, prevention of animal transport stress would often use hypnotic agents of non-benzodiazepine and phenothiazines, which can reduce central nervous system excitation and mental stress, produce a sedative effect and weaken fearfulness, so as to alleviate transport stress. However, these chemical sedatives will not only damage the liver and kidney of livestock, the short-term metabolism is also incomplete, causing drug residues that endanger public safety. It is particularly important to search for green, safe and efficient anti-stress drugs. Compared to chemically synthesized agents, natural products have the advantages of increased safety and lower toxicity and side effects and there is much more research on the use of herbal additives or plant extracts as well as active ingredients from natural products for anti-stress (Sun *et al.,* 2016; Li *et al.,* 2020; Choi *et al.,* 2002). Thus, herbs with tranquilizing and sedative properties that do not easily develop drug resistance and leave minimal unsafe drug residues have become an important direction in research and development and with application prospects (Pao *et al.,* 2012). In this experiment, we selected *Uncaria rhynchophylla* leaves (Fig 1), Valerian (Fig 2) and the traditional Chinese medicine *Semen Ziziphi Spinosae* (Fig 3), which are the

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distinctive local resources of Qian dong nan, Guizhouand these herbal medicines have the characteristics of calming and tranquilizing (Tang *et al.,* 2012) (Cao *et al.,* 2010) (Morazzoni *et al.,*1995). We then prepared a compound agent of these herbs that can alleviate animals the stress of transporting by using alcoholic extracts. Isorhynchophylline, a chemical component in the leaves of *Uncaria rhynchophylla*, also has good sedative and anticonvulsant effects (Kang *et al.,* 2002). We simulated a transport stress model using the compound agent and isorhynchophylline as the anti-transportation stress drug and mice as subjects (Shi *et al.,* 2015). By gavaging the herbal compound agent and isorhynchophylline into mice, the changes in blood physiological, biochemical and hormonal indices of the various groups of mice before and after transport stress were tested, the relief of the effect of animal transport stress was scrutinized to provide reference for the development of animal transport stress drugs in the future.

MATERIALS AND METHODS

Main reagents and instruments

Anhydrous ethanol (AR), dichloromethane (AR), ethyl acetate (AR) Tianjin Fuyu Fine Chemical Co. Mouse adrenocorticotropic hormone (ACTH), cortisol (CORT), corticosterone (CORTISOL), phosphocreatine kinase (CTK), lactate dehydrogenase (LDH) ELISAL detection kit, were purchased from Shanghai Tong wei Industrial Co. Macroporous resin D101: Tian jin Yun kai Resin Technology Co. *Semen Ziziphi Spinosae* was purchased from local Yi xin tang Pharmacy. Valerian and *Uncaria rhynchophylla* leaves were purchased from jian he gui zhou Province. JJ224BC electronic molecular balance, precision 0.0001g, Guangzhou Jing bo Electronics Co. Evela N-1001 rotary evaporator, Shanghai Aiming Instrument Co. Analytical Instrument Co. TM5 enzyme marker, Beijing PUYA General Instrument Co. KG300DE ultrasonic cleaning instrument, Kunshan Shumei Ultrasonic Instrument Co. 580 Blood Glucose Tester, Yu yue Medical Instrument Co. Three classification blood cell counter. Shanghai Jimmy Pet Products Co. Automatic Biochemistry Analyser, Hitachi Diagnostic Products (Shanghai) Co.

Test animals and ethics statement

One month old, healthy, male and female Kunming breed mice (each sex half), body mass 18-22 g, purchased from Changsha Tian qin Biotechnology Co. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Changsha Tian qin Biotechnology Co., I promise that the study was performed according to the international, national and institutional rules considering animal experiments, that was approved by the Ethics Committee of School life sciences, Guizhou Education University.

Preparation of herbal extract and isorhynchophylline

1 kg of commercially available *Semen Ziziphi Spinosae* was crushed 1-2 times with a pulveriser, soaked in 5 L of ethanol 3 times, 72 hr. each time, with continuously stirring during soaking, then extracted and filtered. The filtrate was combined and concentrated under reduced pressure to obtain the alcohol extract of *Semen Ziziphi Spinosae*. Valerian (1 kg) purchased from Jian he County, Guizhou, was cut into short pieces, crushed 1-2 times with a pulveriser, soaked in 5L ethanol 3 times for 72 hr. each time, with continuous stirring during soaking, filtered, combined the

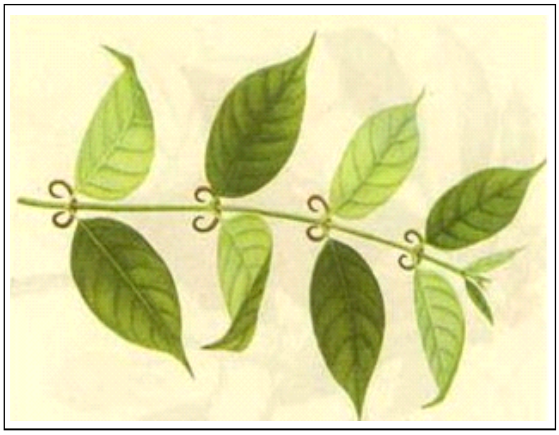

Fig 1: *Uncaria rhynchophylla* leaves.



Fig 2: *Valeriana officinalis* L.



Fig 3: *Semen ziziphi spinosae.*

filtrate, then concentrated under reduced pressure to produce alcohol extract of Valerian. Leaves of *Uncaria rhynchophylla* was purchased from Guizhou Jian he County. It was sun-dried and then crushed powder was put into 95% ethanol of ten times its amount, immerse 3 times, each time 72 hr, immersed in constant stirring so that it can be immersed fully. Then put soaked solution under reduced pressure filtration; when concentrated to half the its volume, perform petroleum ether extraction to de-esterand then continue to be concentrated under reduced pressure to get the leaves of *Uncaria rhynchophylla* extracts. Add 30% ethanol ultrasonication 50 times the mass of the concentrate to dissolve the extract completely, the initial extract on the sample solution is made. Using macroporous resin D101 as adsorbent material, to 0.5%-1.5% per minute of the volume of the column speed of the column adsorption, adsorption is completed with 30% of ethanol for elution, chlorophyll in the 30% of the ethanol elution is adsorbed by the macroporous resinand a large number of *Uncaria rhynchophylla* alkaloids was eluted down so as to remove the chlorophyll in the leaves of *Uncaria rhynchophylla*. The eluent was diluted to 5-10 times with waterand then reapplied to the macroporous resin for adsorption of *Uncaria rhynchophylla* alkaloids. The adsorption was carried out on the column at the speed of 2%-3% of the column volume per minuteand then the elution was carried out with 95% of ethanoland the total alkaloids of *Uncaria rhynchophylla* leaves of were obtained after the concentration. The crude extract of the total alkaloids was dissolved in 1% hydrochloric acid and then filtered and the obtained filtrate was adjusted to pH 9-10 with 1 M ammonia and then extracted with chloroform, repeated for more than four times. The chloroform layers were combined and the solvent was removed to obtain the total alkaloids extract under reduced pressure. The extract was fully dissolved with dichloromethane: methanol (10:1) mixed solution, then mixed with (60-80 mesh) silica gel for dry sampling, then (300-400 mesh) silica gel for column chromatography and then eluted with (dichloromethane: ethyl acetate=5:1'→15:1) for gradient elution; modified bismuth potassium iodide was used as the chromogenic agent of thin-layer chromatography. We have obtained the crude product of isorhynchophylline. Then we obtained the pure product of isorhynchophylline through 2~3 times of methanol recrystallisation. Thin-layer chromatography of the product showed consistent Rf values with standard sample of isorhynchophylline, white powder, modified potassium iodide solution coloration, positive reaction. ^{1}H NMR (600 MHz, $CDCl_3$), δ: 8.14 (s, 1H), 7.45 (s, 1H), 7.19 (t, J = 7.5 Hz, 1H), 7.04 (t, J= 6.1 Hz, 1H), 6.87 (d , J = 7.4 Hz,1H), 3.72 (s, 3H), 3.58 (s, 3H), 2.51 (m, 2H), 2.40 (m, 2H). 2H), 2.40 (m, 2H), 2.07 (m, 2H) 1.25-1.54 (m, 2H), 0.82 (3H, t, J= 7.1 Hz) and the data of 1H NMR are in agreement with the reports (Haginiwa *et al*., 1973), this can be identified as isorhynchophylline.

Preparation of drug samples for testing

The above extracts were composed according to the mass ratio percentage: 30% of *Semen Ziziphi Spinosae* extract, 40% of valerian extract and 30% of the total alkaloids of *Uncaria rhynchophylla* leaves. For this experiment, the compound was divided into compound agent high dose group (300 mg/kg), medium dose group (200 mg/kg), low dose group (100 mg/kg) and isorhizobium group (20 mg/kg), 1% DMSO was added to aid in solubilization and the extract was configured according to the above composition as 6.66 g/L, 13.2 g/L and 20 g/L low, medium and high doses respectively and isorhynchophylline was configured as 1.332 g/L.

Test animals group management and simulated transport stress.

120 mice weighing 18-22 g were divided into six groups: blank control group (no stress, no drug administration), blank stress group (stress only, no drug administration), isorhynchophylline group and drug stress groups (low, medium and high dose), with ten female and ten males in each group. One hour before applying the stress, the mice in the stress group were administered 0.3 mL of the prepared compound agents and isorhynchophylline aqueous solution *via* gavage, while the blank group was administered 1% aqueous solution of DMSO *via* gavage. Then the blank stress group and the drug stress group were placed on a shaking table at 180-190 times/min at a temperature of 22~25°C for 8 hours to simulate transport stress.

Measurement of blood glucose and collection and determination of serum samples in mice

After the stress, blood was taken from the tail of mice in each groupand blood glucose (GLU) was measured using a glucometer. In each group, blood was taken from the eyeballs of 10 randomly chosen mice (five from males and five from females) into 1.5 mL EP centrifuge tubes and placed for 30 min. Then centrifugation was carried out with a speed of 3,000 r/min for 5 min and serum was transferred to 1.5 mL EP centrifuge tubes with a pipette gun. The serum hormone index of the subject mice was tested by applying the ELISAL kit to detect the adrenocorticotropic hormone (ACTH), Cortisol (CORT), corticosterone (CORTISOL) and phosphocreatine kinase (CTK), lactate dehydrogenase (LDH)and the biochemical analyzer was used to measure ALP (alkaline phosphatase), UREA (urea nitrogen), CREA (creatinine), ALT (alanine aminotransferase), AST (glutamate aminotrans ferase), TP (total protein), ALB (albumin), CHOL (cholesterol), TG (triglyceride). The remaining 10 mice in each group had their blood taken from their eyeballs in single-use 2 mL vacuum blood collection tubes and the changes in the hemophysiological indexes of WBC (total white blood cell count), GRAN (granulocyte count), LYM (lymphocyte count), HGB (total hemoglobin content), RBC (red blood cell count) and PLT (platelet count) were carried out using a triple-classification hemocytometer.

Statistical analysis.

The experimental data were collated using EXCLE softwareand the analysis of variance (ANOVA) and significance test were performed on the experimental data using Matlap software. The data were expressed as Mean±Standard Error (Mean±S.E). When $P<0.01$, it is considered to have a highly significant difference; when $P<0.05$, it is considered to have a significant difference; and when $P>0.05$, there is no significant difference.

RESULTS AND DISCUSSION

The data on the changes of blood physiological indexes, hormone level indexes, biochemical indexes of the mice in each group, measured and calculated by different methods, are as follows:

Table 1 shows that the concentrations of hormone levels ACTH, CORT and Cortisol in the blank stress group of mice were significantly higher than those in the blank control group ($P<0.05$) and there was no significant change in the levels of CORT and Cortisol in the stress group compared with that of the blank control group, the high dose group (ACTH) levels were highly significant ($P<0.01$) lower than those in the blank control and blank stress groups.

Table 2 shows that blank stress group shows that there was a highly significant ($P<0.01$) increase in blood physiological indexes GLU, WBC and GRAN than blank control group, in which the levels of WBC and GRAN in the drug stress group were highly significantly ($P<0.01$) lower than those in the blank stress group and the levels of GLU in the medium-dose group and high-dose compound group were significantly lower than that in ($P<0.05$) the blank stress group. There was no significant difference in HGB, RBC, PLT and LYM between the blank control, blank stress and drug stress groups and the blank stress group.

Table 3 shows the levels of UREA and LDH in the blank stress group were significantly ($P<0.05$) higher than those in the blank control group. The TG level of blank stress group was highly significantly ($P<0.01$) lower than blank control group, the UREA level of high dose group was significantly ($P<0.05$) lower than blank stress group and blank control group and the LDH level of high dose group was significantly ($P<0.05$) lower than blank stress group. The level of TG in the drug group was highly significantly higher ($P<0.01$) than the blank stress group.

When stress occurs in animals, the neuroendocrine response is enhanced, triggering an increase in hypothalamic-pituitary-adrenocortical hormones. CORT, ACTH, Cortisol levels increase during stress (Hartung, 2003; Nwe *et al.,*1996). The results of this experiment showed that CORT, ACTH and Cortisol showed significant ($P<0.05$) elevation after simulated transport stress in mice with the results reported in the above study (Hartung, 2003; Nwe *et al.,*1996), CORT and Cortisol levels in the drug group did not show significant change. Compared to the blank group, ACTH in the high-dose group was extremely significantly lower than the blank control group and the blank stress group. It indicates that this compound agent and isorhynchophylline have certain effects on regulating hormone levels in transport stress mice and the high dose group of the compound has a stronger role in regulating ACTH than the isorhynchophylline group.

Intense stress can disrupt the balance of animal metabolism and experiments have shown that transport stress causes an increase in blood glucose levels in cattle (Sartorelli *et al*., 1992). In the present study, GLU was

Table 1: The information of hormones indicators in each group.

Indicators unit	Blank control	Blank stress	Isorhynchophylline	Low-dose	Medium-dose	High dose
CORT (ng/mL)	7.35^{Aa}±0.48	8.03^{Ab}±0.38	7.65^{Aa}±0.41	7.74^{Aa}±0.27	7.63^{Aa}±0.26	7.66^{Aa}±0.61
ACTH (ng/mL)	94.49^{Aa}±3.97	109.16^{Ab}±9.42	94.11^{Aa}±2.85	101.2^{Ab}±5.22	102.91^{Ab}±4.50	84.11^{ABa}b±1.85
Cortisol (ng/mL)	71.65^{Aa}±9.27	84.53^{Ab}±13.07	76.85^{Aa}±6.32	76.99^{Aa}±8.26	73.20^{Aa}±11.31	71.20^{Aa}±9.31

Note: The difference uppercase in the superscript at the same row of data indicate a highly significant difference (P<0.01), the difference the lowercase indicate in the superscript at the same row of data indicate a significant difference (P<0.05) and the exact same letters indicates a non-significant difference P>0.05.

Table 2: The information of blood indicators in each group.

Indicators unit	Blank control	Blank stress	Isorhynchophylline	Low-dose	Medium-dose	High dose
GLU (mmol/L)	4.63^{Aa}±0.65	6.63^{Bb}±0.79	6.32^{Bb}±0.78	6.63^{Bb}±0.79	5.34^{Ab}±0.53	5.31^{Ab}±0.87
WBC ($10^9/L^{-1}$)	2.36^{Aa}±0.94	5.00^{Bb}±0.94	2.73^{Aa}±0.65	2.8^{Aa}±0.53	3.35^{Ab}±0.21	2.5^{Aa}±1.04
GRAN ($10^9/L^{-1}$)	1.36^{Aa}±0.38	3.39^{Bb}±0.32	1.67^{Aa}±0.85	1.38^{Aa}±0.19	1.57^{Aa}±0.70	1.85^{Aa}±0.40
LYM ($10^9/L^{-1}$)	0.325^{Aa}±0.19	0.49^{Aa}±0.03	0.55^{Aa}±0.35	0.40^{Aa}±0.23	0.39^{Aa}±0.06	0.74^{Aa}±0.27
HGB (g/L^{-1})	158.23^{Aa}±40.61	177.23^{Ab}±21.99	177.23^{Ab}±21.99	183.1^{Ab}±6.24	171.0^{Ab}±5.3	158^{Aa}±13.43
RBC ($10^{12}/L^{-1}$)	7.52^{Aa}±0.9	7.61^{Aa}±21.99	7.22^{Aa}±0.45	7.31^{Aa}±0.25	7.04^{Aa}±1.04	6.9^{Aa}±0.74
PLT ($10^9/L^{-1}$)	543^{Aa}±228.74	613^{Ab}±90.66	621^{Ab}±228.74	447^{Ac}±68.29	583^{Aa}±98.04	476^{Ac}±96.19

Note: The difference uppercase in the superscript at the same row of data indicate a highly significant difference (P<0.01), the difference the lowercase indicate in the superscript at the same row of data indicate a significant difference (P<0.05) and the exact same letters indicates a non-significant difference P>0.05.

Table 3: The information of biochemical indicators in each group.

Indicators unit	Blank control	Blank stress	Isorhynchophylline	Low-dose	Medium-dose	High dose
ALT (U/L)	36.82^{Aa}±7.a15	47.25^{Ab}±6.2	42.34^{Aa}±5.6	41.59^{Aa}±9.5	45.30^{Aa}±3.91	43.01^{Ab}±12.88
AST (U/L)	106.88^{Aa}±23.5	120.64 ^{Ab}±9.15	140.26^{Ba}±26.05	135.88^{Aa}±23.5	143.88^{Aa}±17.6	126.78^{Ab}±11.5
TP (g/L)	52.34^{Aa}±4.56	56.02 ^{Ab}±12.2	55.15^{Aa}±3.17	56.67^{Aa}±4.92	56.75^{Aa}±4.15	57.12^{Ab}±5.85
ALB (g/L)	33.14^{Aa}±3.20	36.28 ^{Aa}±5.10	35.95^{Aa}±2.75	34.6^{Aa}±3.73	34.93^{Aa}±2.30	34.35^{Aa}±3.57
UREA (mol/L)	6.54^{Aa}±0.52	8.18 ^{Ab}±0.36	7.73^{Aa}±1.7	6.13^{Aa}±1.65	5.93^{Aa}±1.98	5.12^{ABab}±2.10
CREA (μmol/L)	8.25^{Aa}±0.36	9 .0^{Ab}±1.2	7.18^{Aa}±2.81	10.66^{Aa}±1.3	11.5^{Aa}±1.3	10^{Aa}±0.3
CHOL (mol/L)	2.08^{Aa}±0.34	2.55^{Aa}±0.24	2.78^{Aa}±0.2	2.88^{Aa}±0.21	2.76^{Aa}±0.28	2.3^{Aa}±0.32
LDH (U/L)	465^{Aa}±80.18	594^{Ab}±68.15	574^{Aa}±60.18	721^{ABab}±90.18	591.86^{Ab}±127.1	509.86^{Aa}±149.1
TG (mol/L)	1.45^{Aa}±0.24	0.8^{Bb}±0.028	1.08^{Aa}±0.28	1.00^{Ab} ±0.1	1.06^{Ab}±0.19	0.93^{Ab}±0.23
CTK (U/L)	113.51^{Aa}±3.42	121.32^{Ab}±5.38	109.70^{Aa}±1.67	111.78^{Aa}±2.7	110.46^{Aa}±3.13	110.01^{Ab}±2.08

Note: The difference uppercase in the superscript at the same row of data indicate a highly significant difference (P<0.01), the difference the lowercase indicate in the superscript at the same row of data indicate a significant difference (*P*<0.05) and the exact same letters indicates a non-significant difference *P*>0.05.

highly significantly (*P*<0.01) elevated in the blank stress group versus the blank control group, which is consistent with the reports (Kannan *et al.,* 2000; Sartorelli *et al*., 1992).

There was no significant change in the GLU level in the isorhizobium group and low-dose group compared with the blank stress group, but in the high dose group was extremely significantly (*P*<0.01) lower than that of the blank stress group, suggesting that the compound group had a certain regulatory effect on the blood glucose level of the mice after stress. Transportation stress may impair the immune system of the body due to the body's attempt to defend itself against the adverse effects of the external environment, leading to the elevation of WBC. The level of WBC in the blank stress group was highly significantly higher (*P*<0.01) than that in the blank control group, which is consistent with the results of the paper (Lan *et al.,* 2023). There was no significant change in the level of WBC in drug group and blank control group, the level of GRAN will be elevated due to transport stress and the level of RBC will be significantly reduced, the result showed that the level of GRAN in blank stress group was significantly higher (*P*<0.01) than that of blank control group which is in agreement with the patent documentation (Wang *et al.,* 2015) and there was no significant change in the level of GRAN in drug group and blank control group. The drug group did not have obvious regulatory effect found in HGB, RBC, PLT, LYM, but the results still indicate that both the compound and isorhynchophylline have a certain role in alleviating the physiological indexes of transport stress in miceand the effect on the regulation of blood glucose is stronger in the compound agent group than in the isorhynchophylline group.

Blood biochemical indicators are important indicators reflecting the physiological state of the animal body, in order to resist external stress stimuli, certain enzymes or other biochemical indicators in the blood will undergo large changes, so that the body reaches regulatory capacity and thus achieve homeostatic regulation. Stress will lead to an increase in blood serum CTK and LDH activity (Green wood *et al.,* 1992). The UREA levels in horses show a significant increase after 4 hours of transport (Wang *et al.,* 2015). The levels of LDH, UREA in the blank stress group of the experiments were significantly higher than that of the blank control group and TG levels in the blank stress group were lower than that of the blank control group, which is consistent with the results reported in the paper above (Lu *et al.,* 2016). Except for the low-dose and medium-dose LDH levels in the drug stress group, the levels of LDH in the drug group were significantly lower than the blank stress group and the levels of TG in the drug group were higher than the blank stress group but lower than blank control group, which indicated that the drug group was effective in alleviating the stress biochemical indices of LDH and UREA. CTK indexes were not consistent with the paper which reported an increase after transport Stress (Li *et al.,* 2017), however, high dose group was still significantly lower than the blank stress group after stress. This inconsistency is probably due to the differences in the time of transport that led to adaptation of transport, causing CTK levels to not increase significantly. Overall, the drug still played a significant role in regulating the stress indices such as LDH, UREA and TG in mice after transport stress.

The key stress evaluation indexes of hormone level, biochemical indexes and blood physiological indexes of transport stress in the experiment were tested by using the mouse transport stress model reported in the paperand the results showed that the herbs compound agent with sedative and tranquilizing effects and isorhynchophylline had a significant regulating effect on most of the key stress evaluation indexes of the test mice. Isorhynchophylline's effect on the regulation of key indicators of stress was not as outstanding as that of the traditional Chinese medicine compound agent, probably due to the complex composition of the compound agent having a synergistic anti-stress effect. The mechanism of the anti-transport stress needs to be further researched.

CONCLUSION

In summary, the three kinds of herbal extracts with sedative and tranquilizing components preparated compound agent and isorhynchophylline have shown different degrees of regulation for mice key stress indicators before and after

transport stress. The anti-transport stress effect of the compound agent is better than solitary isorhynchophylline group in general. The source of the components of this compound agent is sufficient, the extracts preparated compound agent is expected for further improvement, so as to alleviate the adverse effects caused by transport stress in the animal husbandry industry.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

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