Antidepressant-like Effect and Mechanisms of Essential Oils From *Citrus Reticulata* in Reserpine-induced Depression Model Mice

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Research

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Abstract

Citrus reticulata, has been used for various diseases such as cough. According to previous studies, the essential oil of *C. reticulata* (CREOs) have been shown to be effectively alleviate depression-like behaviors in mice. This study is aimed to investigate the antidepressant-like effect of CREOs in the rapid reserpine-induced depression model mice as well as its possible mechanisms. The experiment was conducted in six groups, each with four mice. The essential oil group and the control group were administered by sniffing (1h/d), while the reserpine group and fluoxetine group by intraperitoneal injection. Body weight, forced swimming test (FST) and tail suspension test (TST) were used to assess depressive behavior. The compositions and contents of CREOs were analyzed by GC-MS. The results indicated that reserpine could reduce the weight of mice and prolong the immobility time of FST and TST. Moreover, the level of 5HT-1A, GR and Nissl bodies in the brain tissue were significantly reduced, while the level of BDNF was increased in reserpine-treated mice. The administration of CREOs could effectively inhibit the weight loss and the prolongation of immobility time caused by reserpine. In addition, the treatment of CREOs has also been shown to reverse the changes in Nissl body, 5-HT, GR and BDNF levels. Limonene was the main active component of CREOs and might be related to the reduction of BDNF. By up-regulating the level of BDNF, CREOs could regulate the hyperexcitability of the HPA axis, thereby increasing the level of neurotransmitters and restoring neurons.

1. Introduction

As a common mental disorder, depression is clinically manifested as low interest, decreased energy, low self-identity, inattention, and even repeated idea of suicides [1]. Survey data from the World Health Organization shows that currently more than 350 million people worldwide suffer from depression. As a single-disease mental illness with the highest incidence, depression has become the world's second major disease and is expected to rise to the first place in the global burden of disease by 2030 [2]. The exact pathogenesis underlying the development of depression and antidepressant action have not been totally clarified, but several factors implicated in the etiology of the disease have been reported, such as hypothalamus-pituitary-adrenal (HPA) axis [3, 4], monoamine neurotransmitters [4, 5], cytokines [6, 7], brain-derived neurotrophic factors [6], receptor [8], etc. Most of the present anti-depressants used in clinical have obvious limitations and low remission rate for MDD patients [9, 10]. In addition, the current available anti-depressants are costly and inadequate for number of individuals [11]. Particularly, depression pharmacotherapy has prone to side effects, as treatment process may be accompanied by anxiety, gastrointestinal dysfunction, low alertness, and sexual problems [12, 13, 14]. Therefore, it is of great significance to develop novel antidepressants that are affordable, also have fewer adverse reactions and broader therapeutic effects.

Nowadays, more essential oils from aromatic and medicinal extracts, a part of the human diet, have become effective supplements for the treatment of depression [15, 16]. Citrus reticulata peel is one of the ingredients of traditional Chinese medicine prescriptions such as Liu Yu Tang and Chaihu Shugan San, both of which were used clinically to improve depressive mood caused by different reasons, and antidepressant effects has been showed in CRS-induced depression model in Chaihu Shugan San [17]. According to previous studies, *Citrus reticulata* (CREOs) and the main component limonene could improve depressive behavior, possibly by restoring the expression of hippocampal BDNF in CUMS-induced mice [18], showing that CREOs had antidepressant effects and could prevent

the occurrence of depression. Pharmacological studies have shown CREOs also had a variety of biological activities, such as anti-tumor [19], anti-inflammatory [20, 21], anti-oxidant [20], and anti-bacterial [22].

In this study, the antidepressant-like effects of CREOs in the reserpine-induced depression animal models were studied through the administration method of smell absorption, and other possible mechanisms were explored using biochemical tests. The chemical composition of tangerine essential oil was analyzed by gas chromatography-mass spectrometry technology. Analyze the changes of mouse body weight and behavioral to determine whether CREOs have antidepressant effects. The expression of 5HT-1A, GR, BDNF in mouse brain tissues has been detected by different detection methods to explore their relationship with antidepressant effects.

2. Materials And Methods

2.1 Essential oils and Chemicals

Peel from *Citrus reticulata* fruits was purchased from Chongqing Zhengyuan Trading Co. Ltd, and essential oil was extracted from the volatile compounds of the peels by steam distillation. In brief, fresh peels were dehydrated before being crushed into powder (0.45mm diameter). Next, 50g of crushed powder was placed in a Clevenger-type apparatus for steam distillation 2h [23]. The essential oil above the water layer was collected and a small amount of anhydrous sodium chloride was used to dry it. The CREOs was stored in a refrigerator at 4℃ for subsequent experiments. Peel sample gathered was given a unique registration number (Table 1). And all chemicals used in the study were purchased from Aladdin reagent Database Inc. (Shanghai, China), which were of analytical grade.

Table 1

Latin name, local name, voucher specimen number, and collection time of *Citrus reticulata*.

Latin name	Local name	Voucher number	Collection time	Storage location
Citrus reticulata	Hongju	2020-100A	2020.09	Institute of Natural Medicine & Green Chemistry, School of Chemical Engineering and Light Industry, Guangdong University of Technology

2.2 Gas chromatography-mass spectrometry analyses (GC-MS)

The essential oil from *Citrus reticulate* peel was detected by GC-MS system (GCMS-QP2010PLUS, Shimadzu Co., Japan). The flow rate of carrier gas (Helium) was 0.87 ml/min and the split ratio was 20.0. Follow the steps below to heat up: hold at 90 ℃ for 10 minutes, increase to 250℃ by 5℃/min, and then keep for 8 minutes. As for MS condition, the electron collision energy was 70eV and the temperature of ion source was controlled at 200℃. Under the guidance of the Kovats standard, the retention index (RIs) of each kind of compound was counted against the standard of n-alkanes (C6–C40), and the composition of essential oils was compared and analyzed with reference to NIST chemistry reference books and literature [24–27]. The results are shown in Table 2.

2.3 Animals and Treatment

The experimental animals (weighing about 35g, 5weeks) were SPF-grade healthy male KM mice which were both purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Approval Document: SCXK/2020-0001). The mice were acclimatized to the environment for a week before the experiment. With free eating and moving, the mice

were kept in a pathogen-free animal room where the temperature was maintained at 25℃, and the light and dark cycle was carried out for 12 hours.

After being adaptively fed, the experiment was conducted in six group (4head/group), including Control group, Reserpine model group, Fluoxetine group, and CREOs light, medium, and high concentration group (the daily inhalation volume was respectively 25μL/head, 50μL/head, 100μL/head), of which the blank control group was not processed for modeling, and the rest were all used to establish reserpine depression models. The control group and CREOs group sniffed the physiological saline containing 1% Tween80 and the corresponding concentration of tangerine essential oil for one hour every day. Reserpine group (6mg/kg/day) and fluoxetine group (20mg/kg/day) were injected intraperitoneally. On the fifth day, after administration, reserpine was injected to establish a depression model. On the sixth day, half an hour after the administration of the control group and the CREOs group, the fluoxetine group was intraperitoneally injected with 2 mg/mL fluoxetine hydrochloride and placed for half an hour for the following behavioral testing to evaluate the depression-like symptoms of the mice. After 24 hours, the mice were dissected and their brains were taken for testing and analysis.

2.4 Body weight

Obvious weight change was a criterion for diagnosis of depression [1]. The effects of reserpine and essential oils on the weight of the mice were measured by an electronic balance before the administration every day. The weight data of mice were recorded for analyzing.

2.5 Tail suspension test (TST)

Using the method established by Steru et al. [28], mice were subjected to TST in a dark environment with low background noise to evaluate the antidepressant activity of essential oils. After one hour of acclimatization, the experiment was carried out. Each mouse was respectively suspended by its tail (1 cm from the end) using a clip in a 25×25×30cm box for 6 minutes, and the head was kept at a distance of 5 cm from the bottom. During the 6 minutes test period, the time that the mice gave up escaping and remained still was recorded. In order to eliminate interference, the mice were isolated auditorily and visually.

2.6 Forced swimming test (FST)

FST is an effective and extensive experiment for evaluating depression-like behaviors in mice. The experiment was referred to the experiment of Porsolt et al. [29]. During the experiment, the mice were individually placed in a glass cylinder (diameter 14cm, height 20cm, water height 10cm). The temperature of the water was controlled at 25 ± 1 ℃ and mice were allowed to swim for six minutes. Record the total time of immobility for the last 4 minutes. The immobility was defined as the mouse has greatly reduced activity in the water and stopped struggling, except for the duration of the small movements which are necessary to keeps the head above the water [29]. After the experiment, the animals were dried with a towel before being returned to the cages.

2.7 Brain collection and detection

When the mice were sacrificed, 4% paraformaldehyde was added to save the whole brain tissues. The fixed tissues were made into 4μm paraffin sections for Nissl staining and immunohistochemistry experiments to observe the number of neurons and the expression of 5HT-1A and GR in the brain tissues. The mice brain homogenate were frozen and then Western blot and RT-qPCR were performed to detect the expression of mouse 5HT-1A and BDNF.

Nissl Staining: The sections were deparaffinized and stained with Toluidine Blue for 5 minutes. After differentiation, the sections were placed in xylene for 10 minutes and then sealed with neutral resin. Being observed with a microscope inspection and images were collected, then the number of normal nerve cells was counted for analysis.

Immunohistochemistry (IHC) Staining: The tissue sections were sequentially deparaffinized in xylene, absolute ethanol and different concentrations of alcohol. After completed, the slices were placed in citric acid (pH 6.0) antigen retrieval buffer for microwave retrieval of antigens for 30 minutes, and then placed in 3% hydrogen peroxide for blocking. When the previous steps were completed, 3% of BSA dropwise was added to seal the tissue for 30 minutes before diluted primary antibodies (5HT-1A, GR) were added to be incubated overnight in a refrigerator at 4°C. On the second day, the secondary antibody was added for incubation, while DAB and hematoxylin were used for dyeing. After mounting the slide, observe under a microscope and the positive cells were analyzed with Image pro plus (IPP) 7.0.

Western blot (WB) analysis: After the brain tissue was lysed to extract protein, BCA protein detection kit was used to measure protein concentration. Then the protein was separated and transferred to PVDF membrane by SDS-PAGE electrophoresis. Being blocked for 30 minutes with skim milk, primary antibodies (5HT-1A, BDNF) were added to incubate overnight at 4°C, then corresponding secondary antibodies were added. ECL chemiluminescence kit was applied to observe the protein, and Alpha software was used to process the optical density value of the specific band.

Real Time PCR (RT-qPCR): Total RNA from mouse's brain tissue was extracted by Trizol extraction method, and Nanodrop 2000 was used to detect RNA concentration and purity. Then Servicebio®RT First Strand cDNA Synthesis Kit (Servicebio, Wuhan, China) was used to synthesize cDNA by reverse transcription. The mRNA expression was measured using 2×SYBR Green qPCR Master Mix (High ROX) (Servicebio, Wuhan, China), and the relative expression of 5HT-1A and BDNF genes at the mRNA level was calculated and analyzed by the 2-△△CT method. The primer sequence was as follows.

Gen	Forward primer	Reverse Primer
5-HT1AR	CCAACTATCTCATCGGCTCCTT	CTGACCCAGAGTCCACTTGTTG
BDNF	TATTAGCGAGTGGGTCACAGCG	TACGATTGGGTAGTTCGGCATT

2.8 Statistical analysis

The histograms were drawn by GraphPad Prism 8 and the experimental data were represented as means ± SD. SPSS 21.0 system was used for statistical analysis and ANOVA was used to determine statistical significance. Difference is statistically significant when $P < 0.05$.

3. Results

3.1 Chemical composition analysis of CREOs

The essential oil compositions of CREOs are enumerated in Table 2 and the chromatograms of the compounds of CREOs are shown in Fig. 1. 42 different compounds were detected totally. With 57.98% of the total essential oil compounds, limonene has become the most abundant compound in CREOs, followed by Linalool, γ-Terpinene, Terpinolene, 1-Nonanol, (S)-β-bisabolene, β-Caryophyllene and Decanal. Among the top ten compounds, the content of limonene (57.98%) is greatest, which has been considered to be the most important active component of the herb's anti-depressant effects [16, 30]. Encouragingly, linalool (9.63%), another major component of essential oils, has also been shown to have antidepressant activity in previous experiments [31, 32]. Moreover, although presented at a lower proportion, β-Caryophyllene, which is identified in CREOs, has been found it to significantly improve depression-like effects [33, 34]. These findings demonstrate the potential of CREOs as an effective antidepressant.

Table 2

Retention index (*RI*) and relative content (%) of each compound identified from *Citrus reticulata* essential oils.

No.	Compounds [i]	RI [ii]	Exp.RI	Ref.	Relative content (%)
					Citrus reticulata
1	Limonene	1022	881	a	57.98
2	γ-Terpinene	1043	889	a	7.63
3	1-Nonanol	1154	900	b	2.40
4	Terpinolene	1083	915	b	2.41
5	Linalool	1086	932	a	9.63
6	trans-p-Mentha-2,8-dienol		976	-	1.42
7	(+)-limonene oxide		986	-	1.12
8	LEO		994	-	0.56
9	Citronellal	1133	1009	b	0.80
10	Terpinen-4-ol		1073	-	0.16
11	Decanal	1186	1004	b	1.69
12	Octyl acetate	1191	1010	b	0.43
13	2,3-expoxycitral		1033	-	0.33
14	2-methoxy-4-methyl-1-propan-2-ylbenzene		1042	-	0.76
15	L(-)-Carvone		1060	-	0.19
16	Linalyl acetate		1067	-	0.28
17	5-Isopropyl-2-methylphenol		1150	-	0.22
18	(+/-)-δ-elemene		1170	-	0.19
19	α-copaene		1211	-	0.74
20	β-cubebene		1228	-	0.14
21	(-)-β-elemene		1231	-	1.07
22	Lauryl aldehyde		1245	-	0.20
23	β-Caryophyllene		1254	-	1.81
24	β-cubebene		1265	-	0.12

i Compound listed in the order of elution from methyl silicone capillary column

ii Retention indices (RIs) relative to n-alkanes (C6-C40) on the same methyl silicone capillary column.

a b c d

No.	Compounds [i]	RI [ii]	Exp.RI	Ref.	Relative content (%)
					Citrus reticulata
25	α-bergamotene	1432	1270	d	0.99
26	α-Caryophyllene		1291	-	0.17
27	cis-β-farnesene	1446	1294	a	0.15
28	β-cubebene		1313	-	0.79
29	(+)-Valencene		1322	-	0.47
30	(Z)-α-bisabolene		1330	-	0.16
31	(S)-β-bisabolene		1333	-	1.90
32	(+)-DELTA-CADINENE		1347	-	0.27
33	γ-Elemene	1439	1374	b	0.42
34	Caryophyllene Oxide	1570	1399	c	0.29
35	Spathulenol		1437	-	0.20
					0.09
36	2-pentyl-2-Nonenal		1506	-	0.09
37	Spathulenol		1509	-	0.99
38	Nootkatone		1549	-	0.21
39	((+)-ledol		1617	-	0.20
40	D,L-tetrahydroactinidiolide		1620	-	0.10
41	methyl 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]cyclopropane-1-carboxylate		1693	-	0.18
42	2-Dodecen-1-yl succinic anhydride/ □□□□□□□□		1722	-	0.14
	Total identified	**-**	**-**	**-**	**100%**
	Total monoterpenoids				70.33
	Monoterpene hydrocarbons				68.2
	Oxygenated monoterpenes				2.31
	Total sesquiterpenoids				9.68
	Sesquiterpene hydrocarbons				8.97

i Compound listed in the order of elution from methyl silicone capillary column

ii Retention indices (RIs) relative to n-alkanes (C6-C40) on the same methyl silicone capillary column.

a b c d

No.	Compounds [i]	RI [ii]	Exp.RI	Ref.	Relative content (%)
					Citrus reticulata
	Oxygenated sesquiterpenes				0.71

i Compound listed in the order of elution from methyl silicone capillary column

ii Retention indices (RIs) relative to n-alkanes (C6-C40) on the same methyl silicone capillary column.

a b c d

3.2 Effect of CREOs on the Relieving of Depression in Reserpine-Treated Mice.

The antidepressant effect of CREOs was tested by reserpine depression model, which was performed one day before the behavioral test which has the advantage of being fast and effective [35].

As illustrated in Fig. 2-A, the body weight change rate of reserpine-treated mice decreased significantly compared with the control group, indicating that the weight of the mice lost severely. This reduction is significantly resisted by the inhalation of CREOs. Fluoxetine treatment don't show resistance to weight loss, which may be related to the gastrointestinal side effects of fluoxetine, resulting in reduced intake of mice. In addition, CREOs-M and CREOs-H are more effective in preventing and inhibiting weight loss in mice than the control group, indicating that inhalation of CREOs had a positive regulatory effect on mice.

The comparison of FST immobility time is showed in Fig. 2-B. After reserpine treatment, the mice's desire to escape decreased and the immobility time is significantly prolonged ($P < 0.05$). CREOs and fluoxetine can shorten the immobility time of mice, and CREOs-H has the best improvement effect in the CREOs group. These results proves that CREOs had antidepressant effects.

As depicted in Fig. 2-C, CREOs treatment can improve immobility of mice in TST. The immobility time of reserpine-induced mice is significantly longer than that of control mice ($p < 0.01$). It is encouraging that both fluoxetine and CREOs-H group shows an excellent improvement effect on immobility time ($P < 0.01$). CREOs-M also has a significant therapeutic effect ($P < 0.05$). Generally, CREOs could effectively alleviate depression-related behaviors.

3.4 CREOs suppressed neuronal damage induced by reserpine-treatment in brain

Nissl staining is a common method to assess changes in neurons, and it can detect Nissl bodies in neurons. The staining results (Fig. 3-A) show that the control group has plenty of neuronal cells and arranged neatly, while the Nissl substance in the reserpine-induced mouse neuronal cells is missing, accompanied by blurred cell boundaries and disordered arrangement. CREOs treatment can change the consequences of damage caused by reserpine through reducing neuronal loss and reversing neuronal damage, and the effect is better than fluoxetine. In particular, compared with reserpine group, neuronal cells in the hippocampus and hypothalamus of the CREOs-H group arranges more neatly and densely, and Nissl body recovers better.

The statistics of normal neurons in different parts of the mouse brain is shown in Fig. 3-B. After reserpine treatment, the number of neurons in each tissue of the mice is significantly less than that of the control group (cerebral cortex, $P < 0.05$; hippocampus, $P < 0.05$). Among them, CREOs-M treatment on cortex neurons ($P < 0.05$) and CREOs-H treatment on hypothalamic neurons ($P < 0.01$) both have significant recovery effects, which are better than fluoxetine treatment. However, the reduction effect is seen in the treatment of hippocampal neurons with low concentrations of essential oils. In the hypothalamus, each concentration of essential oil has similar effect on neuron recovery.

3.5 Immunohistochemical staining to detect the effect of CREOs on 5HT-1A and GR in brain

5HT-1A and GR are important factors in evaluating the treatment of depression. We assessed the positive expression levels of 5HT-1A and GR in the cerebral cortex, hippocampus and hypothalamus of mice by immunohistochemistry to reveal the role of CREOs in the treatment of depression (Fig. 4).

Figures A_1 and A_2 are the detection of 5HT-1A. The control group has more positive cells in the three areas, with clear cell outline and uniform cytoplasmic staining. 5HT-1A protein in the reserpine group is significantly less than that in the control group, some cells in the hippocampus appear vacuoles and some cells are markedly atrophy. After the administration of CREOs, positive cells increase and the cells are arranged neatly and clearly. However, CREOs-M further damages the arrangement of cells and makes them disorderly. Through using the method of integrated optical density analysis, we can see that compared with the control group, the expression of 5HT-1A in the three areas treated by reserpine is reduced and there is significant difference in the cortex ($P < 0.01$). In the cortex, the level of 5HT-1A in CREOs and fluoxetine group increases to varying degrees (fluoxetine, $P < 0.01$; CREOs-M, $P < 0.05$) compared with reserpine group. It is worth mentioning that in the hippocampus, CREOs-H has a better recovery effect on 5HT-1A levels than fluoxetine and the overall therapeutic effect of CREOs-H approached fluoxetine. CREOs-H treatment can reverse the 5HT-1A reduction induced by the reserpine procedure, but there are general therapeutic effects of low-medium concentration essential oils in the hippocampus and hypothalamus.

Figures B_1 and B_2 show GR detection. The number of GRs in the reserpine group was significantly less than that in the control group and the cells were not arranged tightly and the staining became lighter. Some cells in the hippocampus also appeared vacuoles or atrophy. After sniffing CREOs, GR-positive cells recovered to varying degrees, and the cells were arranged neatly and clearly. However, the recovery effect of CREOs-L is not obvious. Analysis of the comprehensive optical density found that the GR levels of the reserpine group in the three areas were significantly reduced. However, the GR levels of CREOs-M, CREOs-H and fluoxetine groups all increased to varying degrees. Among them, the treatment of CREOs-M on the hippocampus increased significantly ($P < 0.05$).

3.6 Western Blot detected the effect of CREOs on 5HT-1A and BDNF in brain homogenate

In order to further explore the potential mechanism of CREOs on depression-like behaviors, Western Blot was used to detect the protein expression of 5HT-1A and BDNF in brain tissue homogenate.

We detected that the expression of 5HT-1A increased and the expression of BDNF decreased significantly after reserpine treatment ($P < 0.01$). Compared with the reserpine group, CREOs and fluoxetine treatments could reduce the increase in 5HT-1A expression to varying degrees, and CREOs had a significant down-regulation effect ($P <$

0.01). Regarding the expression of BDNF, the tangerine medium and low concentration group can significantly inhibit down-regulation ($P < 0.01$), which is better than the therapeutic effect of CREOs-H and fluoxetine.

3.7 RT-qPCR detected the effect of CREOs on 5HT-1A and BDNF protein gene expression in mouse brain homogenate

Next, the effect of CREO on 5HT-1A and BDNF in the brain of reserpine-treated mice was further analyzed. RT-qPCR was used to analyze the expression of 5HT-1A and BDNF at the mRNA level.

It can be seen from Fig. 6 that the relative expression of 5HT-1A mRNA was decreased in the brain tissue of the reserpine group, while the relative expression of BDNF mRNA was significantly increased ($P < 0.05$). This corresponded to the results of the Western Blot test, indicating that the effect of reserpine can affect the mRNA expression of 5HT-1A and BDNF in mice, but the results at the mRNA level were contrary to the results of receptor expression. CREOs treatment could effectively reduce the expression of BDNF mRNA and reverse the damage of reserpine treatment, and it had shown obvious improvement effects in both CREOs-L and CREOs-H groups ($P < 0.01$). At the same time, 5HT-1A mRNA was found to be improved in the treatment of fluoxetine and CREOs group, and there was a significant difference in the CREOs-M group ($P < 0.05$).

4. Discussion

With the increasing number of stress factors in today's society, depression has become one of the main causes of disability in the world, and the recurrence rate is high [1, 2]. Drug treatment often brings a series of adverse reactions. The search for safe and effective new drugs has always been a hot topic. The reserpine model is a commonly used and effective depression model which can be used for behavioral tests such as forced swimming tests, tail suspension tests, and open field tests [35]. With the merits of simple operation, animal-friendly, and high efficiency, this model played an important role in the evaluation and development of antidepressant drugs [35]. This experiment analyzed the chemical composition of CREOs and explored its role in the depression model induced by reserpine.

Weight loss is an important indicator of depression performance [1], and the immobility of forced swimming test and tail suspension test is a behavioral desperate performance, which can be used to evaluate the depressive behavior of mice [36]. Our experiments show that the weight of mice treated with reserpine has decreased significantly. What's more, the immobility time of FST and TST in the experiment has been significantly prolonged. These results indicated that the reserpine depression model is successful and effective, which is in line with previous studies [28, 29, 38]. Fluoxetine is a clinically recognized and effective antidepressant which has a significant antidepressant effect according to previous studies [39]. In this experiment, it is used as a positive control to compare and verify the antidepressant effect of tangerine. By analyzing the body weight changes and behavioral tests of each group, it can be seen that CREOs treatment can prevent weight loss and reduce the immobility time of FST and TST in reserpine-treated mice. Among them, the therapeutic effect of CREOs-H optimal. In general, CREOs can at least improve depression-like behaviors in mouse depression models induced by reserpine.

Nissl bodies are composed of rough endoplasmic reticulum and free ribosomes interspersed in it. Basophilic particles are presented and the shape and number of Nissl bodies are different in different neurons [40]. They are

related to the synthesis of structural proteins needed to renew organelles, the enzymes needed to synthesize neurotransmitters, and the neuromodulators of peptides. Previous research has shown that neuron damage could cause the sensitivity of Nissl body to decrease, dissolve or disappear [41]. Therefore, the shape and number of Nissl body are often used to identify neurons and their pathophysiological changes. Study has found that the total number of neurons in the hippocampus of patients with depression was reduced by 20–35% compared with the control group [42], and injection of reserpine could cause neuronal damage [43]. Our research shows that the treatment of CREOs can reverse the nerve damage caused by reserpine treatment. The number of neurons in the CREOs treatment group is close to that of the control group, indicating that CREOs treatment cause less damage to neurons. In addition, the results demonstrate that CREOs treatment can protect the morphology of brain tissue neurons, and have relatively good treatment safety.

In depression-related patients and some animal experiments, it has been usually observed that symptoms of depression were accompanied by disorders of glucocorticoid secretion. Therefore, the hypothalamic-pituitary-adrenal (HPA) axis dysfunction has been studied by evaluating GR and MR levels in the past work [44]. Hypothalamic-pituitary-adrenal axis (HPAA) disorder is an important finding in the pathophysiology of depression. This disorder is thought to be due to the central glucocorticoid receptor (GR) level and the chronic glucocorticoid (GC) release or changes in function, leading to the receptor positively or negatively regulate the expression of glucocorticoid-responsive genes [45]. Excessive stress stimulus may activate GR through cortisol, so that GR stimulates the hippocampus to issue negative feedback commands, resulting in HPA axis imbalance, showing excitement [46]. As shown before, antidepressants could improve the GR-mediated inhibition of corticosteroids by increasing the expression of GR on the HPA axis, thereby reducing cortisol levels in different regions [47]. The results of this study found that reserpine caused a significant reduction in GR expression in the brain, indicating the negative feedback regulation of the HPA axis was impaired and the HPA axis was hyperactive, which is in line with previous studies [48]. In addition, our research shows that treatment with CREOs and fluoxetine could increase the expression of GR, indicating that CREOs had antidepressant effects by regulating neuroendocrine. The HPA axis may be a mechanism of CREOs, but the difference in our data is not significant and need to be further explored.

The monoamine hypothesis was considered by many researchers as the mechanism of action of depressive drugs. The hypothesis believes that depression is caused by insufficient activity of monoaminergic neurons [4]. Previous study has shown that patients with major depressive episodes had lower serotonin transporter binding potential in the midbrain and amygdala, compared with non-depressed individuals [49]. Although the complexity of the emotional state cannot be attributed to the imbalance of a single neurotransmitter, it is recognized that 5-HT is significantly involved in depression [4, 5, 50], and 5HT-1A receptors are considered to treat mental illness, Especially a potential target for depression [50, 51].Studies have found that the antidepressant effect of lemon oil whose main component is limonene was closely related to the 5-HT energy pathway, especially through the 5-HT 1A receptor pathway [52]. In this study, immunohistochemical methods were used to detect the content of 5-HT 1A in different areas of the brain. It is found that fluoxetine and CREOs can increase the content of 5-HT 1A in the brains of reserpine-induced depression mice, but there are differences in dosage and location differences. Insignificant effects are shown in the treatment of low-concentration essential oils and in the treatment of hippocampus and hypothalamus. Studies have found that excessive activity of the HPA axis might damage hippocampal monoaminergic neurons, resulting in a decrease in monoamines [18], and 5-HT1A receptor agonist suppressed

stress-induced activation of the HPA axis as measured [53]. It is speculated that CREOs may increase 5-HT1A in the cerebral cortex through the action of the HPA axis, thereby increasing neuronal activity.

BDNF is a neurotrophic factor that can stimulate neurogenesis and regulate synaptic plasticity [6]. Widespread in the brain, especially in the hippocampus and cerebral cortex, BDNF could promote the survival of dopaminergic, GABAergic and serotonergic neurons [54]. It is also involved in regulating the activities of HPA axis [55, 56]. Antidepressant drugs can increase the expression of BDNF in the brain of mice, such as selective serotonin reuptake inhibitors (SSRI) and norepinephrine reuptake inhibitors (NARI) [57]. Of course, there are also antidepressants that have different effects on the mRNA and protein levels of BDNF [58]. The regulating effect of these drugs on depression might be related to neurotrophic activity and benefited from long-term chronic regulation [59]. Study has shown that the loss of BDNF in the hippocampus could induce neuronal apoptosis and ultimately led to depression [58]. This experimental data supports the study. WB staining shows that the expression of BDNF protein in reserpine-depression mice is decreased. Further study on BDNF mRNA by RT-qPCR is found that BDNF mRNA level expression and protein level expression have an opposite expression trend, and there is a negative feedback relationship. The treatment of CREOs and fluoxetine can significantly increase the BDNF protein and reduce the expression of BDNF mRNA, which further confirms this negative feedback effect. Interestingly, the changes in the 5HT-1A protein in the brain homogenate detected by WB have different and opposite results from the changes in the 5HT-1A protein in different regions of the brain detected by immunohistochemistry. This may be related to the location of 5HT-1A receptors, which have opposite effects on depression regulation. For example, the post-synaptic 5HT-1A receptor is thought to cause anxiety and depression effects. On the contrary, it is believed that the activation of 5-HT1 autoreceptor will inhibit the activity of serotonergic neurons and release serotonin, thereby reducing the marginal zone and producing anti-anxiety and anti-depressant effects [60]. Similarly, the use of RT-qPCR to detect the mRNA expression of 5HT-1A also showed a different trend from the expression of 5HT-1A protein. Therefore, we speculate that there is a brain-derived neurotrophin-neurotransmitter interaction in at least a certain area of the brain, which makes BDNF and 5HT-1A feedback regulation in protein and mRNA. The disorder of this mechanism can be regulated by CREOs, while fluoxetine is invalid for it.

Hyperactive HPA axis is one of the main abnormal phenomena found in depression, and the treatment of depression promotes the production of BDNF [61, 62]. Therefore, the decreased expression of BDNF in reserpine animals may indicate abnormal changes in HPA axis and monoaminergic circuit function. Previous studies also found that limonene also restored CUMS-induced depressive behavior, HPA axis hyperactivity, and decreased levels of monoamine neurotransmitters by down-regulating hippocampal BDNF and its signaling pathways [18]. Our experimental data also meets this conclusion, which provides a reference for the antidepressant mechanism of CREOs, indicating that CREOs may improve the expression of GR, BDNF and 5HT-1A through the connection of the HPA axis.

5. Conclusions

In conclusion, inhalation of CREOs can significantly reduce the depressive behavior of reserpine mice, inhibit the weight loss of mice, and shorten the immobility time. Limonene is an important component of CREOs, and it is likely to be the antidepressant active component of CREOs. CREOs may regulate the expression of GR in the HPA axis in the brain, and regulate the expression of BDNF and 5HT-1A, thereby restoring reserpine-induced depression-like behavior. Therefore, we believe that CREOs may be potential materials for the development of depression drugs, which can provide a reference for the development of new drugs for depression.

Declarations

Availability of data and materials

Please contact corresponding author for data requests.

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Contributions

LL constructed the firing model, MT, SZ and NS performed the experiments. YA, XX and BR analyzed the data. LZ, TH and XZ wrote the manuscript. All authors discussed the results and revised and approved the manuscript.

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Ethics Declarations

Ethics approval and consent to participate

The use of mice had been reviewed and approved by the Animal Experiment Ethics Committee of Guangdong University of Technology (Guangzhou, China). All laboratory procedures and animal care were performed under the guidance of the National Institutes of Health Laboratory Animal Care and Use Guidelines (7th edition, USA).

Consent for publication

Not applicable

Competing interests

All authors declare no conflict of interests.

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Figures

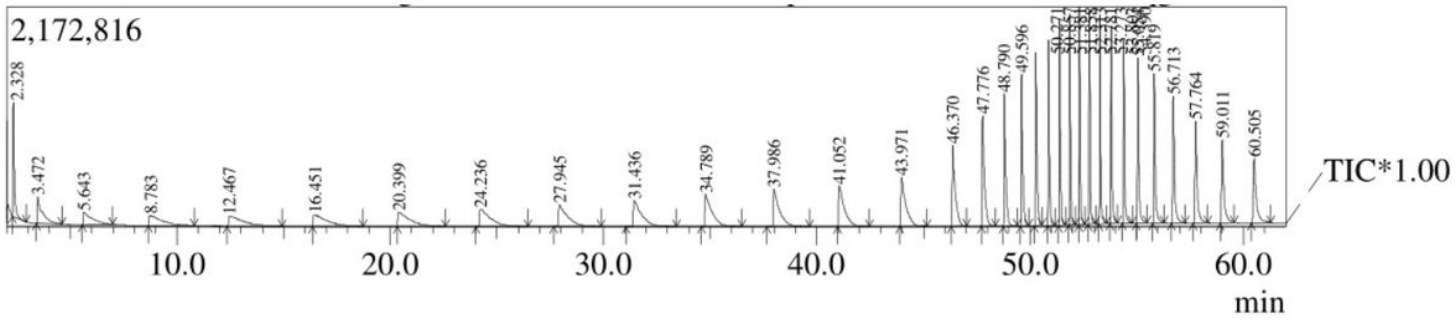

Figure 1

GC-MS total ion chromatogram of tangerine essential oil

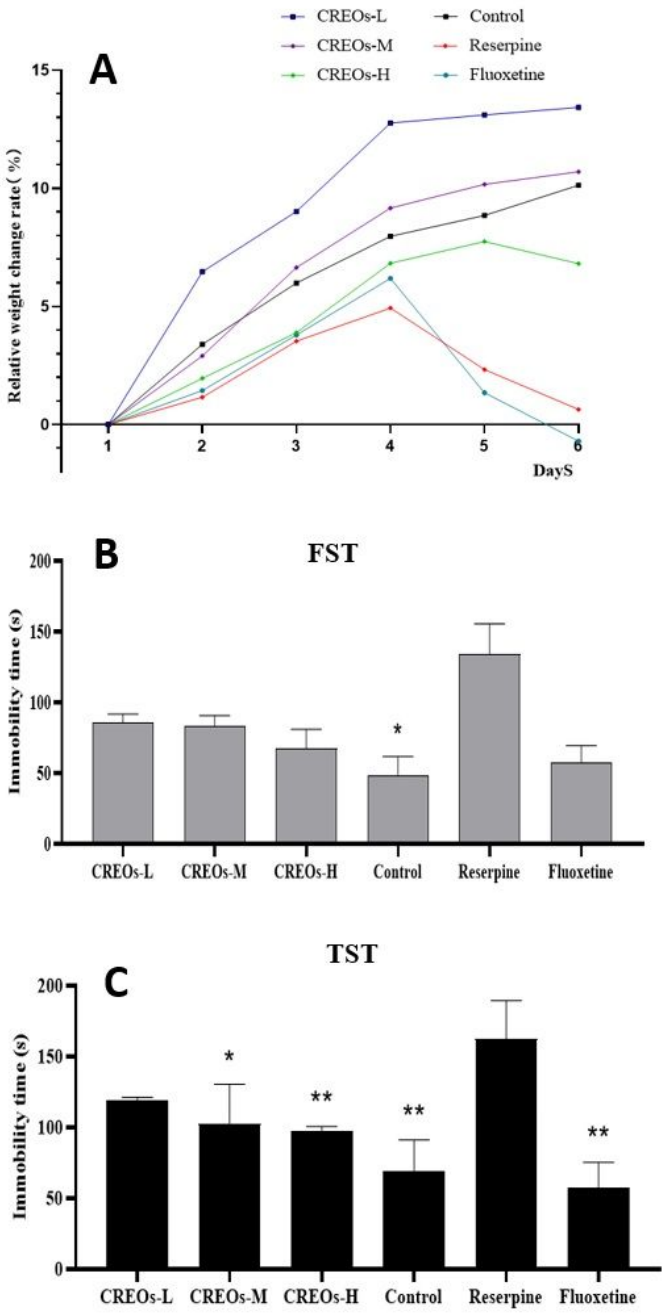

Figure 2

Mouse body weight change rate and behavioral test results. A)Change rate of body weight in mice (%, n=4, $\bar{x} \pm s$). B) Fixed time on tail suspension in mice (n=4, $\bar{x} \pm s$). The significance of differences from the model group is at *P<0.05.C) Fixed time on tail suspension in mice (n=4, $\bar{x} \pm s$). The significance of differences from the model group is at *P<0.05 and **P<0.01.

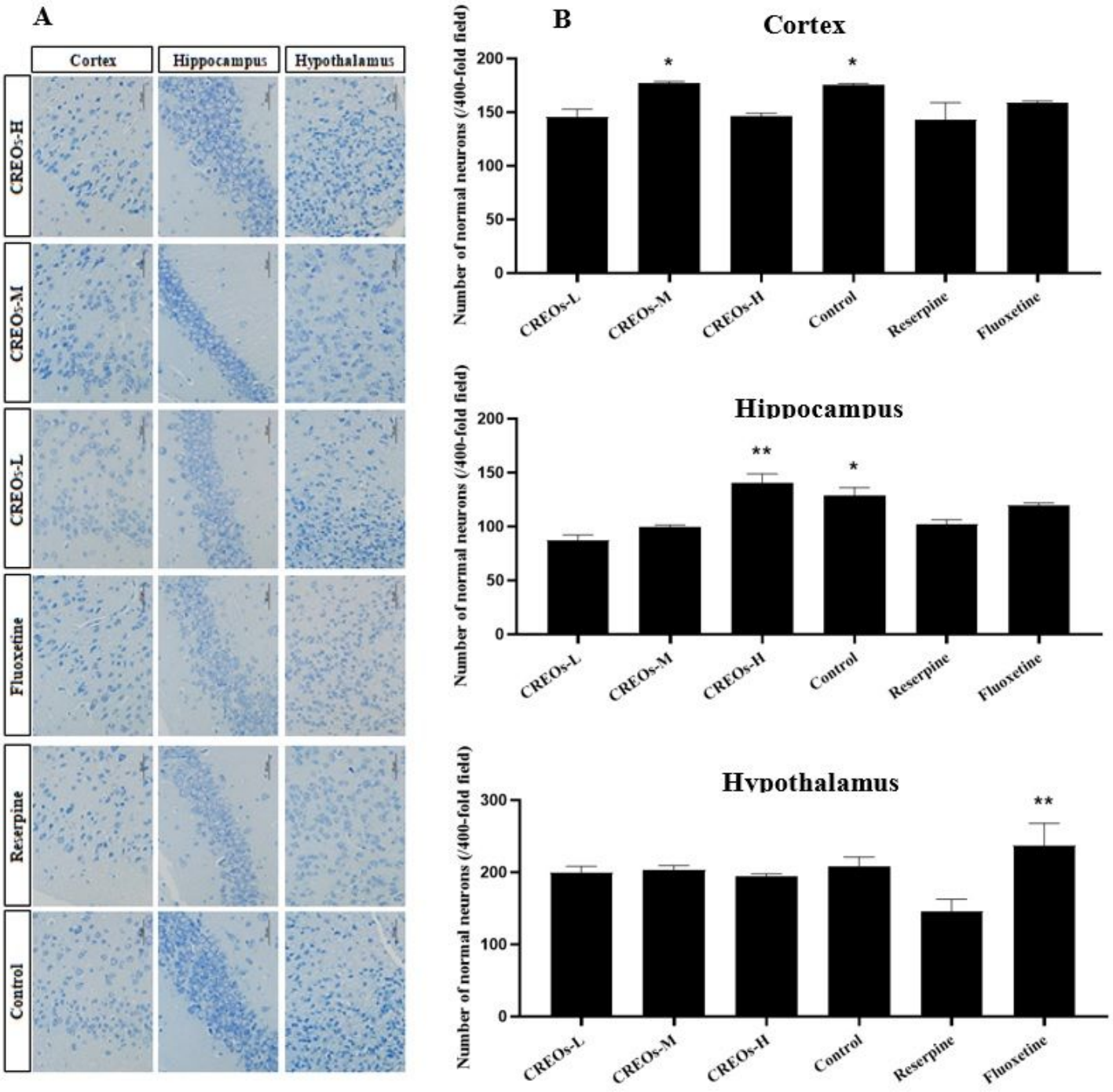

Figure 3

Effects of tangerine essential oil on neuronal damage induced by reserpine. Nissl staining (A) and normal neuronal counts (B) in cortex, hippocampus and hypothalamus of each group, scale bar = 100μm (n=4, $\bar{x} \pm s$). The significance of differences from the model group is at *P<0.05 and **P<0.01.

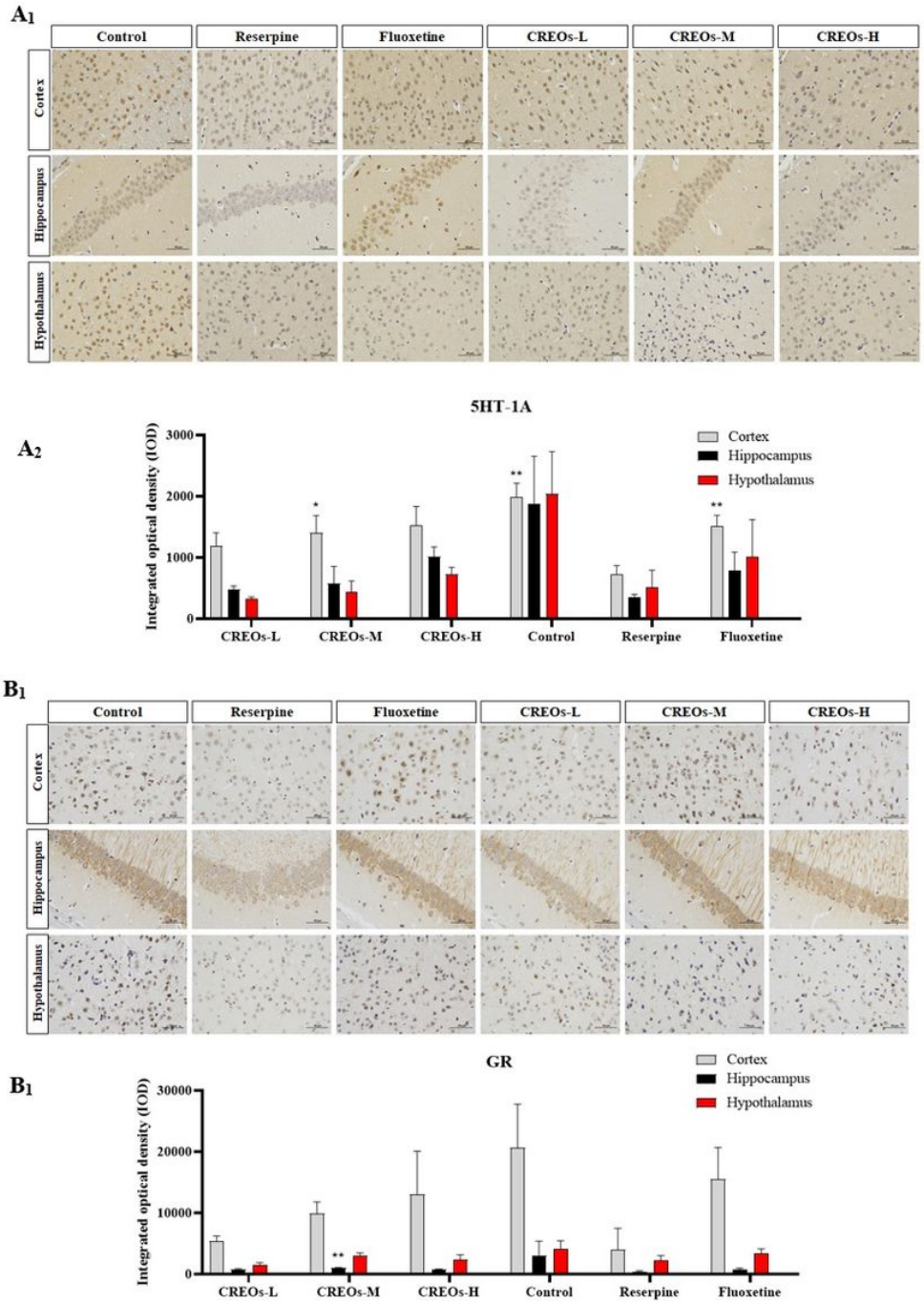

Figure 4

Effects of 5HT-1A and GR in brain tissues treated with CREOs by Immunohistochemistry. (A1, A2) The image of 5HT-1A immunostaining in three areas of the brain (the ratio is) and densitometric analysis of 5HT-1A expression (n=4, $\bar{x} \pm s$). (B1, B2) Diagram of GR immunostaining in the three regions of the brain (the ratio is) and densitometric analysis of GR expression. (n=4, $\bar{x} \pm s$). The significance of the difference from the model group was *P<0.05 and **P<0.01.

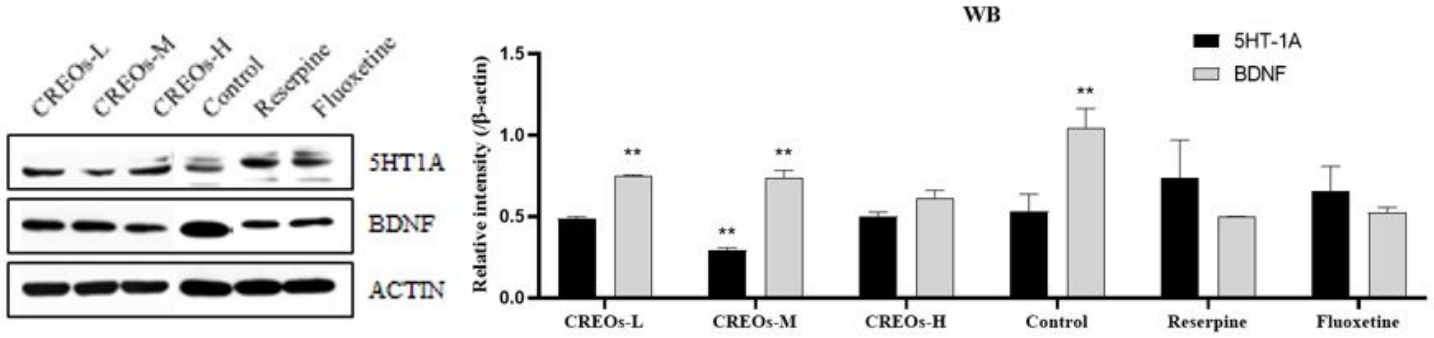

Figure 5

Effect of CREOs on 5HT-1A and BDNF level detected by Western blot in brain homogenate after reserpine treatment in mice. Western blot analysis detected a protein band of 5HT-1A and BDNF of expected size (left). The 5HT-1A and BDNF protein levels in different groups were expressed as a ratio to that of corresponding β-actin in brain homogenate (right). The significance of differences from the model group is at *P<0.05 and **P<0.01.

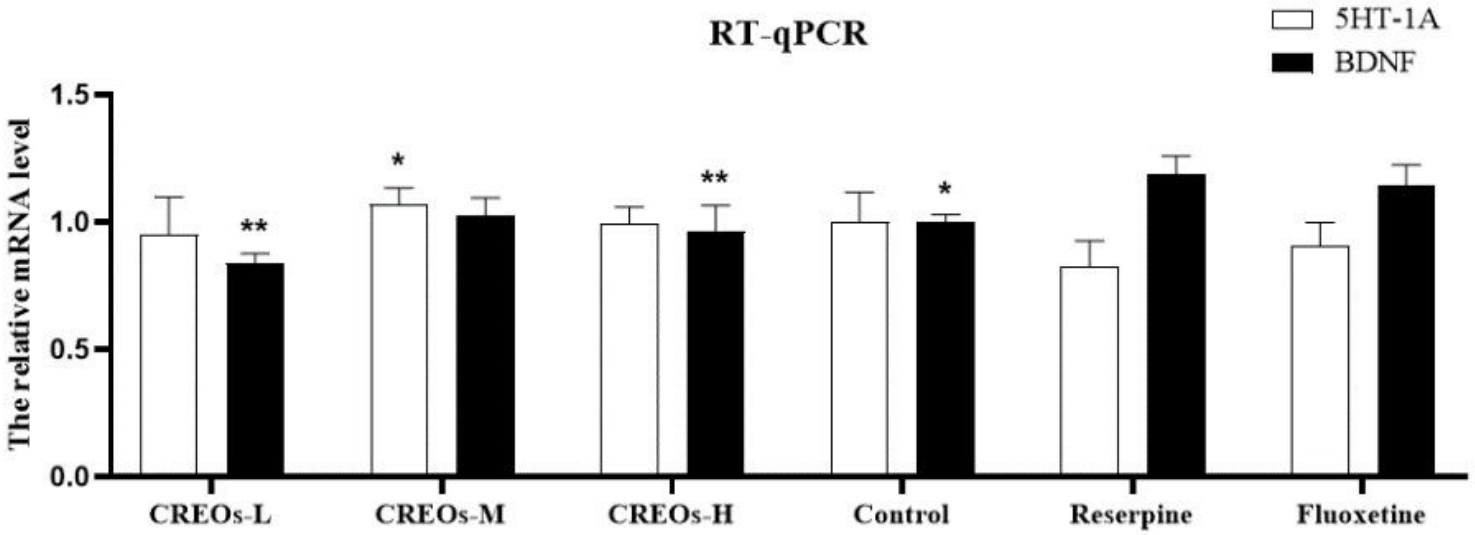

Figure 6

Relative expression of mouse 5HT-1A and BDNF protein gene by RT- qPCR (n=4, x ± s). The significance of differences from the model group is at *P<0.05 and **P<0.01.