

The long-term effect of CBD crystals and CBD oil on depressive-associated rat behaviors

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SUMMARY

Cannabidiol (CBD) is a chemical extracted from cannabis and shown by some studies to alleviate the symptoms of many mental disorders, especially major depressive disorder. Many researchers have explored how acute CBD treatment impacts the attitude of depressive patients, but few researchers have examined how chronic CBD consumption influences the mood of people without depression. To simulate the effect of CBD on people, we used male Wistar Rats as experimental models, divided into three groups: the control group received peanut oil (vehicle), the CBD oil group received CBD oil and vehicle, and the CBD crystal group received CBD crystals and vehicle. We hypothesized that chronic treatments with purified CBD through oral administration would relieve depression-associated behaviors in normal healthy rats under adverse conditions. The CBD oil used in this study was made from crude oil of hemp by molecular distillation, and the CBD crystals were further processed from CBD oil by crystallization. We used forced swimming test and sucrose preference test to assess the characters associated with the diagnosis of depression: despair-like behavior and anhedonia. Furthermore, we used the weight of the rats to assess appetite. A statistical analysis of the experimental data suggested that long-term consumption of CBD could elicit depression associated symptoms in normal rats without depression. The results imply that people should consume CBD-containing products with extreme caution and highlight the need to carefully monitor the use of CBD in health care products.

INTRODUCTION

There are at least 66 kinds of cannabinoids in the cannabis sativa plant. Within those cannabinoids, Δ^9 -tetrahydrocannabinol (THC) is the main psychoactive chemical, while cannabidiol (CBD) is non-psychoactive (1).

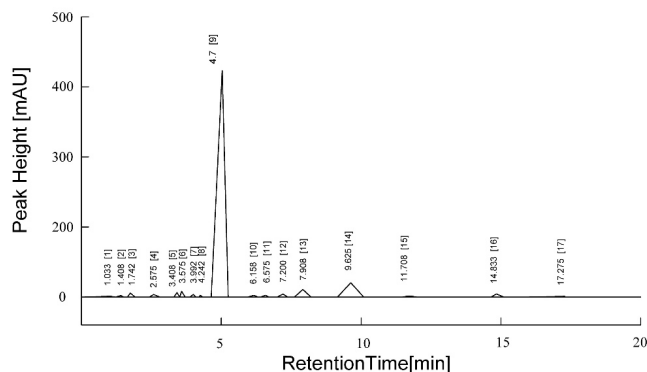
The activation of the endocannabinoid system (ECS) accounts for most of the effects of cannabinoids. The ECS is a highly conserved endocrine network in the process of evolution, and it is a neuroregulatory system that regulates mood, cognition, autonomic nervous system and movement. The ECS is composed of the cannabinoid receptor 1 (CB1)

and cannabinoid receptor 2 (CB2) as well as endogenous ligands (mainly arachidonoyl ethanolamide, 2-arachidonoyl glycerol, and 2-arachidonoyl glyceryl ether) (2). Different from THC, CBD has a low affinity for cannabinoid receptors and acts as an antagonist on both the CB1 and CB2 receptors (3). Clinical and preclinical evidence suggests CBD could ameliorate or reverse some of detrimental effects induced by THC, but the mechanism of this effect is unclear (3). CBD shows promising effects in treating various diseases, including psychosis, substance use disorder, anxiety disorder, cognitive impairment, and epilepsy (4-8). In 2010, the anti-depressant effects of acute CBD administration in mice were first reported by Zanelati *et al.* (9). Many studies have demonstrated the effectiveness of acute CBD preclinical treatment through rodent behavioral tests, including the forced swimming test (FST), sucrose preference test (SPT), tail suspension test (TST), and open field test (OFT), raising the possibility that CBD could be used for treating depression (8). Studies reported that CBD might possess agonist properties at serotonin 1A (5-HT_{1A}) receptors and regulate the brain-derived neurotrophic factor (BDNF). The 5-HT_{1A} receptors and BDNF levels have been consistently related to the neurobiology of depression and the mechanism of antidepressant drugs (9, 10). However, the exact cause for the effects of CBD on the brain is not yet fully understood.

Under federal law, CBD has not yet been approved to be added to dietary supplements, food ingredients, or animal food. Cosmetics can contain CBD as long as the THC content is lower than 0.3% (11). Still, because of the wide therapeutic effects of CBD, in the past few years, CBD had seen a surge in popularity and many CBD products have emerged in United States and Europe with widespread use. Health care products, pet products, skincare products, drinks, food, and e-cigarettes containing CBD have been sold in the market, but the market is chaotic, and the quality of the products is mixed (12, 13). According to a study by University of Michigan's Institute for Social Research, in the past year, 38% of high school seniors had used CBD at least one time (14). This popularity has led to healthy people consuming CBD in addition to those using it for potential therapeutic effects.

Although the acute antidepressant properties of CBD have been reported in several preclinical experiments (9), only a few researchers put emphasis on studying the chronic impact of CBD on depressive-associated behaviors in preclinical tests (15). Also, only rodents that had shown depressive-

associated behaviors, not healthy rodents, were included in the few chronic tests (16). In order to assess the potential of CBD in mood improvement and the safety of CBD as a health care product, it is necessary to monitor the impacts of CBD after repeated administration on healthy rodents. Therefore, the aim of the present study is to evaluate how chronic CBD treatment could affect the mood of healthy rats and compare the effects of purified CBD crystals and impure CBD oil in chronic treatments. We hypothesize that chronic treatments with purified CBD through oral administration would relieve depression-associated behaviors in normal healthy rats under adverse conditions and that impure CBD oil would not change the behaviors of rats. Our results from FST indicated that the chronic administration of both CBD crystals and CBD oil elicited depression-associated behaviors compared to the control group. We also observed that other ingredients in CBD oil affected the weight gain of the rats.



	Retention Time [min]	Peak Area [mAU]	Peak Height [mAU]	Area [%]	Height [%]	WOS [min]	Compound Name
1	1.033	35.683	1.208	0.5	0.2	0.79	
2	1.408	34.157	2.604	0.5	0.5	0.18	
3	1.742	140.865	7.015	2.2	1.4	0.19	
4	2.575	141.734	4.543	2.2	0.9	0.67	
5	3.408	138.208	7.900	2.1	1.6	0.24	
6	3.575	127.592	10.062	2.0	2.0	0.21	
7	3.992	60.287	4.561	0.9	0.9	0.29	
8	4.242	42.958	3.272	0.7	0.7	0.25	
9	4.700	4785.036	404.056	73.2	81.3	0.18	Cannabidiol
10	6.158	60.340	2.238	0.9	0.4	0.58	
11	6.575	46.883	2.373	0.7	0.5	0.35	
12	7.200	98.753	4.514	1.5	0.9	0.26	
13	7.908	211.591	12.419	3.2	2.5	0.23	Tetrahydrocannabinol
14	9.625	470.410	24.395	7.2	4.9	0.28	
15	11.708	26.178	1.112	0.4	0.2	0.34	
16	14.833	111.293	4.746	1.7	1.0	0.35	
17	17.275	8.725	0.271	0.1	0.1	0.56	
SUM		6540.693	497.289	100	100		

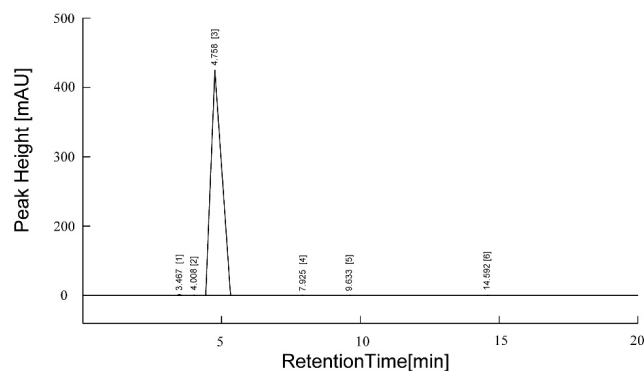
Figure 1. The composition of the CBD oil. The chromatogram and composition of the CBD oil were determined by high-performance liquid chromatography. The result of the chromatography showed that there were 17 kinds of chemicals presented in the CBD oil. The retention time for CBD was 4.700 mins, while the retention time for THC was 7.908 mins. The content of each substance is equal to the percentage of each peak area. The CBD content was 73.2%, and the THC content was 3.2%.

RESULTS

This study compared the influence of oral intragastric administration of CBD crystals and CBD oil on 6-8 week-old male Wistar rats once a day for 21 days at a dose of 30mg/kg body weight. The CBD oil was made from crude oil of hemp by molecular distillation, and the CBD crystals were further processed from CBD oil by crystallization. Chromatography showed that there were 17 kinds of chemicals presented in the CBD oil, with CBD and THC comprising 73.2%, and 3.2%, respectively (**Figure 1**). The result of the chromatography showed that after crystallization, there were only six substances present in the CBD crystals, and the percentage of CBD was 98.8% and THC was 0.2% (**Figure 2**).

Forced swimming test (FST) is used to model hopelessness and helplessness of the rats by putting them in extreme conditions. Rodents are placed into large graduated cylinders filled with water. In the beginning, rodents display active movements trying to escape. As time goes on, rodents tend to be less active and be more motionless. The rodents only exhibit minimal movement essential to keep their head above water. Immobility of the rodents is regarded as an indicator of despair (17).

In the forced swimming test, both CBD crystal and CBD oil treatments significantly shortened the active periods of the rats before they showed immobility as compared to the vehicle treatment ($p = 0.00019$ and $p = 0.00021$, respectively).



	Retention Time [min]	Peak Area [mAU]	Peak Height [mAU]	Area [%]	Height [%]	WOS [min]	Compound Name
1	3.467	25.705	1.892	0.5	0.5	0.18	
2	4.008	10.495	0.992	0.2	0.2	0.17	
3	4.758	4683.473	405.755	98.8	98.9	0.19	Cannabidiol
4	7.925	7.957	0.639	0.2	0.2	0.19	tetrahydrocannabinol
5	9.633	7.405	0.463	0.2	0.1	0.26	
6	14.592	7.565	0.378	0.2	0.1	0.33	
SUM		4742.601	410.120	100	100		

Figure 2. The composition of the CBD crystals. The chromatogram and composition of the CBD crystals were determined by high-performance liquid chromatography. The result of the chromatography showed that after crystallization, there were only six substances present in the CBD crystals. The retention time for CBD was 4.758 mins, while the retention time for THC was 7.925 mins. The content of each substance is equal to the percentage of each peak area. The CBD content was 98.8%, and the THC content was only 0.2%.

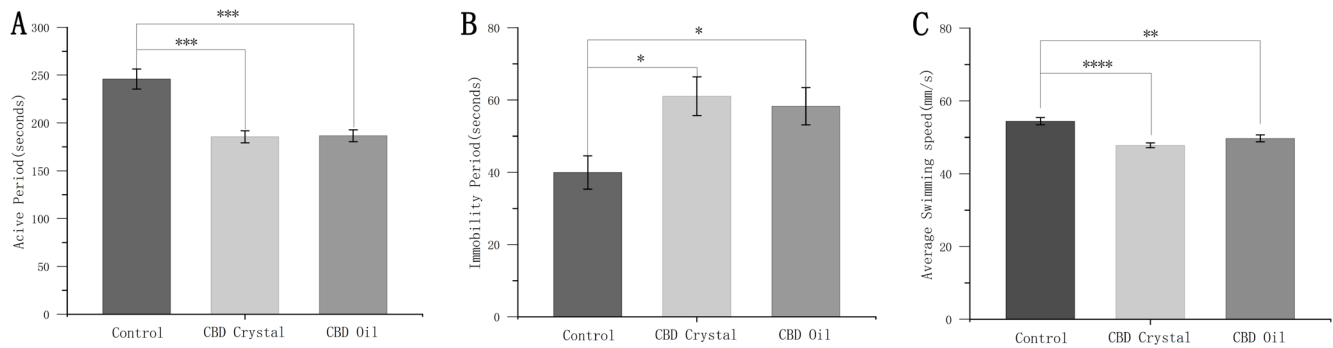


Figure 3. Effect of chronic administration of CBD on forced swimming test. CBD shortens the active periods (A), increases the immobility periods (B), and slows down the average swimming speeds of rats (C) in the forced swimming test regardless of the purity of CBD. The active periods before showing immobility, the immobility periods, and the average swimming speeds were recorded or calculated for three groups. The means were calculated. Data are presented as means, and error bars represent standard error of the mean ($n = 10$). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

However, no significant difference was observed between the CBD crystal and CBD oil groups ($p = 0.93$, **Figure 3A**). Additionally, both CBD crystal and CBD oil treatments significantly increased the immobility periods as compared to the vehicle treatment ($p = 0.011$ and $p = 0.016$ respectively). However, no significant difference is observed between CBD crystal and CBD oil groups ($p = 0.82$, **Figure 3B**).

Both CBD crystal and CBD oil treatments significantly slowed down the average swimming speeds of the rats as compared to the vehicle treatment ($p = 0.00005$ and $p = 0.0043$, respectively). However, no significant difference was observed between the CBD crystal and CBD oil groups ($p = 0.13$, **Figure 3C**). Overall, compared with rats only treated with vehicle, rats treated with CBD crystals and rats treated with CBD oil spent less time swimming and were more immobile.

The sucrose preference test (SPT) is used to measure whether rats show anhedonia-like behavior. Anhedonia is the inability to experience pleasure from normally pleasurable activities and is a core symptom of depression in humans (18). This test assesses the animals' preference for sweet-tasting sucrose solution relative to plain water. Studies have shown that sucrose solution with 1-2% (wt/vol) would be the optimal concentration to distinguish whether or not animals have anhedonia syndromes (19).

Rats in the same treatment group ($n = 10$ /group) were placed into a single cage and the preference for sucrose against water was measured. The consumption of CBD, either oil or crystal, decreased the sucrose preference as compared to the control group (**Figure 4**). The sucrose preference of the control group, the CBD crystal group, and the CBD oil group were respectively 73.57%, 67.74%, and 57.29%. Also, compared with the CBD crystal group, the CBD oil group showed even less sucrose preference. The total volumes of water consumed by the control group, the CBD crystal group, and the CBD oil group were respectively 439 mL, 589 mL, and 391 mL (**Figure 5**).

Animals' weight can be used to assess appetite and food consumption, which can reflect the influence of CBD. Rats were weighed on the first and seventh day after purchase,

and then every four days after that. Intra-gastric administration of CBD began on the tenth day. At the initial time point, the mean weight of the rats in the control group, CBD crystal group, or CBD oil groups was respectively 287.7, 283.7, and 284.2 grams. On the seventh day, the mean weights of the rats in the control group, CBD crystal group, or CBD oil groups were respectively 314.7, 317, and 314.4 grams. There were no significant differences in mean weight between the groups, during these two weighing sessions before CBD intra-gastric administration. To rule out the effect of original weight, the ratio of daily weight to original weight of each group was calculated (**Figure 6**). After 21 days of chronic treatments, the mean weight ratios of the control group and the CBD crystal group showed significant differences compared with that of the CBD oil group ($p = 0.0039$ and $p = 0.03$, respectively). The CBD oil group had a significantly lower body weight compared to the vehicle control and CBD crystal groups.

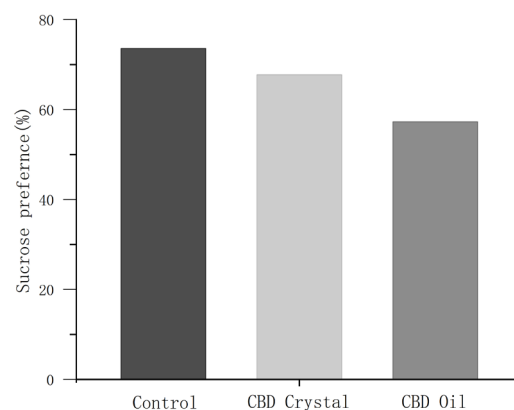


Figure 4. CBD decreases the sucrose preference of the group. As 10 rats were placed in the same cage and the sucrose preference was not individually recorded, therefore there are no statistics. The sucrose preference of the control group, CBD crystal group, and CBD oil group are respectively 73.57 %, 67.74 %, and 57.2 9%. Both the CBD crystal group and CBD oil group showed lower sucrose preference than the control group. The sucrose preference is calculated using the equation: sucrose solution intake/(sucrose solution intake + tap water intake). There were 10 rats in each cage.

There was no significant difference in the mean weight ratio between the CBD crystal group and the control group. These data indicated that the impurities in the CBD oil affected the weight gain of the rats compared with CBD crystals.

DISCUSSION

In the present study, we hypothesized that that chronic administration of purified CBD products would be able to relieve depression-associated behaviors in normal healthy rats under adverse conditions, but the results from FST did not confirm this hypothesis. On the contrary, the chronic administration of both CBD crystals and CBD oil elicited depression-associated behaviors compared to the control group.

The consumption of CBD shortened the active periods during swimming for rats in the CBD crystal group and CBD oil group as compared to the control group ($p < 0.001$, **Figure 3A**). An active period accounts for the rats' persistence when they are trying to escape and is defined as the period between when a rat is first put into water and when the rat starts to show immobility. The shortened active period implied that under adverse conditions, rats revealed less persistence when treated with CBD crystals, or CBD oil. This result indicates that after taking CBD products for a long period, the rats had behavioral patterns associated with anxiety and pessimism under adverse conditions.

Compared to the control group, repeated CBD treatments using CBD crystals and CBD oil increased the immobility period for normal rats ($p = 0.011$ and $p = 0.016$, respectively; **Figure 3B**). As the passive immobility period is considered representative of behavioral hopelessness and helplessness in human depression, rats that exhibit more immobility are thought to have behavioral patterns associated with depression (20). The results of the tests showed that male Wistar rats treated with CBD crystals, or CBD oil demonstrated more behavioral patterns associated with depression than rats

treated only with vehicle. This result demonstrates potential of CBD consumption to elicit depression-associated symptoms in normal people.

The consumption of CBD slowed down the average swimming speed, a measure of activity level, of rats in the CBD crystal and CBD oil groups as compared to the control group (**Figure 3C**). The swimming speed differences induced by the CBD crystal group and the CBD oil group were statistically significant compared with the control group, as both p values are less than 0.01. These results showed that the consumption of CBD reduced the active level of the rats.

The consumption of CBD also decreased sucrose preference. Lower sugar preference is thought to reflect the anhedonia condition present in human depression. The CBD crystal group and the CBD oil group had lower sucrose preferences than the control group. Although the lower sucrose preference may suggest that the rats treated with CBD exhibited anhedonia-like behavior, but we could not draw any strong conclusions for this SPT experiment, because the sucrose preference was not individually recorded.

After chronic treatments, both the CBD oil group and CBD crystal group had apparently shorter active periods, significantly longer immobility periods, and obviously slower swimming speeds compared to the control group (**Figure 3**). These results showed that the consumption of CBD elicited depression-associated behaviors in the rats. However, some

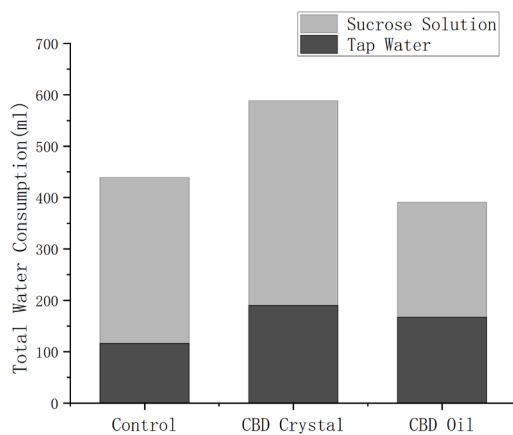


Figure 5. Total water consumption for the three groups. The total volumes of water consumed by the control group, the CBD crystal group, and the CBD oil group were respectively 439 ml, 589 ml, and 391 ml. There were 10 rats in each group.

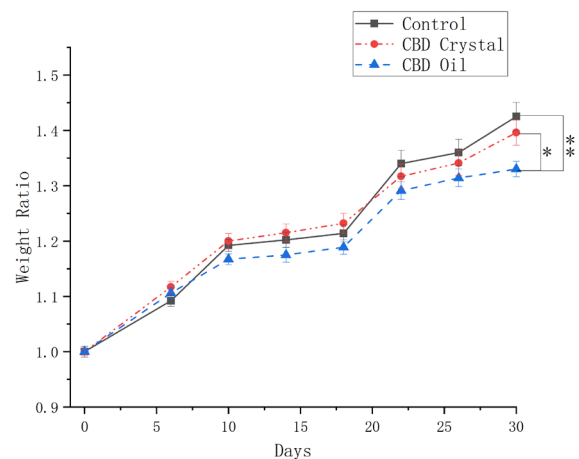


Figure 6. Change in the weight ratio of daily weight to original weight of rats for a month. The impurities in the CBD product had an effect on the weight of the rats. The weight of the rats was measured, and the weight ratio of daily weight to original weight of each group is shown on the Y-axis. The number of days from the beginning of feeding is indicated on the X-axis. At the start of the experiment, the weight ratios showed no significant difference among the groups. At the end of the experiment, after three weeks of the intragastric administration, the mean weight ratio of the control and the CBD crystal groups was significantly greater than that of the CBD oil group. However, the weight ratio of the control group showed no significant difference with that of the CBD crystal group throughout the experiment. Data are given as mean ratio, and error bars represent standard error of the mean ratio ($n = 10$). * indicates $p < 0.05$, ** indicates $p < 0.01$. There is no significant difference between the points without asterisks plotted for the same day.

studies have reported that CBD showed an antidepressant-like property, as acute CBD administration (30 mg/kg body weight) significantly reduced the immobility time compared to the vehicle group, without changing locomotor activity in the open field test or altering hippocampal BDNF levels (9-10). Even though there are some minor differences in methodology, such as total swimming period, it is unlikely that the difference in results between acute and chronic administration of CBD could be explained by these factors. The treatment time period of our study is very short, so future studies could treat normal healthy rats for longer periods of time and examine more behavioral and physical indicators of depression-like symptoms.

Only a few long-term studies conducted using depressive rat models showed that CBD has an antidepressant-like property. Campos *et al.* claimed that CBD administration (30 mg/kg of body weight) for 14 days prevented the anxiogenic-like effect of chronic unpredictable stress (CUS) in wild type mice in the novelty suppressed feeding test and elevated plus maze (21). Linge *et al.* reported that chronic CBD treatment provided both fast-acting and sustained antidepressant-like effects for an olfactory bulbectomy mouse model of depression (OBX) in open field and sucrose preference tests (16). That these two studies present results that are different from our study's findings may be related to animal species, behavior tests, or the health conditions of animal models. Both of the chronic tests used animal models, CUS and OBX, which displayed depressive behaviors before the CBD treatment, while our long-term experiment used healthy rats as subjects.

Similar to our findings, another study using normal healthy Lister-hooded rats showed that chronic administration of CBD produced an anxiogenic-like effect (22). The results of that study further showed that chronic administration of CBD significantly decreased BDNF expression in the hippocampus and frontal cortex (22). The neurotrophic hypothesis of depression suggests that BDNF is reduced in depression and increased after antidepressant treatments. The decrease in BDNF level could represent depression in rats. These results suggest that chronic administration of CBD has the potential of eliciting depressive-like behaviors in healthy rats, but this speculation needs to be thoroughly tested in the future.

Other ingredients in CBD oil affected the weight gain of the rats. At the end of the experiments, there were apparent differences between the mean weight ratio of the oil group and those of the control group and the CBD crystal group ($p = 0.0039$ and $p = 0.03$, respectively) (Figure 6). The presence of other cannabinoids in the CBD oil could be the cause for the slow weight gain rate. This suggests that the effect of CBD purity on human health should also be taken into account in licensing for market sales (12-13, 23).

Many researchers have already studied the effects of CBD on the human body. For healthy people, some studies used the dose of 10 mg/day or 3 mg/kg body weight, which is a much smaller dose than the dose in this study (24-25).

For patients with psychotic symptoms, the therapeutic doses were 600-1500 mg/day (26-29). This higher therapeutic dose is more comparable to the dose of 30 mg/kg used in this study. Although a lower dose maybe yields less distinct behavioral effects, but the effect of a long-time administration should be further studied. Therefore, additional long-term multi-dose studies are needed to determine the optimal dose for healthy individuals.

For humans, acute and chronic CBD administrations may have different effects. The mechanism of action underlying the effects of CBD seems to be complex. However, chronic CBD studies on humans are still scarce. Our study provides evidence for the possibility that CBD could elicit depression when consumed by normal people for a long period. Our experiments also highlight the need for carefully monitoring CBD dose and its effects when used in health care and medicine.

METHODS

CBD preparation

In this study, crude hemp oil (supplied by Beijing Beihua Engineering Co., China) was used as the raw material. Using an operating temperature of around 110°C and an operating pressure (absolute) of approximately 40 Pa, the crude oil went through the first distillation step to remove the volatile components, comprising about 15-20% of the total weight. The residue from the first distillation went through a second distillation at the operating temperature of approximately 160°C and operating pressure of approximately 5Pa. The distillate was the middle product, which was called CBD oil. In order to obtain a CBD product of higher purity, the CBD oil was further crystallized. First, CBD oil was heated to approximately 60°C. As the CBD oil cooled down slowly, the CBD started to crystallize out. Under the final temperature of around 5°C for several days, CBD crystals could be obtained after filtering, washing, and drying. The composition of the CBD oil and the CBD crystals were determined by high performance liquid chromatography with C18 column (Shimadzu, Japan).

Animals

Male Wistar rats weighing 270-300 g were used as the experimental subjects. Thirty male Wistar rats (between six and eight weeks old) were purchased from the Medical Discovery Leader Company (Beijing, China). Rats were housed in stainless steel cages (535x390x200 mm), with ten rats per cage under a 12h-12h dark, light cycle (dawn at 7 am). Humidity maintained between 20% and 40%, and room temperature was between 20–23°C (30). Food and water was provided ad libitum. Rats were allowed to acclimate to the living environment for nine days prior to the beginning of the test. Rats were weighed on the first day and the seventh day after purchase, and then they were weighed every four days.

CBD administration

Thirty mg/kg of purified CBD crystals or CBD oil dissolved

in peanut oil (vehicle) as a dose (8). CBD crystals, CBD oil, and vehicle solution were prepared immediately before use. The solution was prepared together for thirty rats at a ratio 3:1 (mg solute / mL vehicle). Thirty Wistar male rats were randomly separated into three groups (10 rats per group). The three groups were fed with CBD crystal solution, CBD oil solution, or only peanut oil (vehicle), through intragastric administration using a 10 mL syringe (Jiangsu Zhiyu Medical Equipment Co. Ltd.) and a 10 cm gavage needle (HL-GWQ-16, Beijing Heli Science and Technology Development Co. Ltd.). The rats consumed these solutions at about 10 o'clock every morning once a day for 21 days. The volume of the solution which a rat consumed was calculated according to the weight of the rat.

Forced swimming test

The forced swimming test began the day after the rats had finished their 21 days of intragastric gavage, between 10 am and 3 pm in an experimental room. Rats were placed in a cylinder (45 x 20 x 30 cm) and water at a temperature of 24°C ± 2°C was added. The water was so deep that the rat could not reach the bottom with its feet or tail. The rat was immersed in the cylinder for six minutes. The total immobility time was measured based on how long it took the rat to stay afloat without a struggle and keep its head above water using only a minimum amount of movement (31). The test also monitored the active period before showing immobility, the amount of time between when the rat was first placed into the cylinder and when despair-like behaviors started. Swimming was a non-stationary activity and the swimming time was calculated by subtracting the total immobility time from the total test period. At the beginning of each trail, rats were initially placed in the same position to avoid environmental effects. When the experiment was over, the animal was dried with a towel. Between test animals, cylinders were cleaned and water was replaced to prevent the effects of water pollution (21)

The whole experiment was recorded by a video analysis system (Model number: ZS-001, Beijing Zhongshi Dichuang Technology Development Co., Ltd.). This avoided the subjective error and interference to experimental rodents by manual observation. Thus, the objectivity and reliability of the experimental results could be ensured. It could display indicators in various ways: exporting a trajectory diagram, trajectory coordinates, and average swimming speed. The behavior of the rats was recorded during the experiment and the experimenter could play back the recording for manual calibration. The active period before showing immobility, the immobility period, and the swimming distance were recorded. Swimming speed was calculated by dividing the distance by the swimming time.

Sucrose preference test

The sucrose preference test began one day after the forced swimming test's last day. Sucrose water was prepared by dissolving sucrose in pure water to form a 1% weight/volume sucrose solution. Forty-eight hours before the actual

experiment, the mice were given adaptive training, which reduced their anxiety about the sucrose solution (18). Rats in the same treatment group were placed in the same cage (10 in each cage) to adapt to the SPT condition by placing sucrose and water bottles in the cages at the same time. Water or food was still available during the adaptive training and the actual experimental period. On the test day, each group was given two identical 500 mL bottles: one contained sucrose solution (1%) and one contained drinking water. Over the next 24 hours, the animals were given the opportunity to drink at their will (18). To minimize the influence of water bottles' positions, the positions of the two bottles were turned every 8 hours. The volumes of the sucrose solution, drinking water, and total liquid consumption were recorded before and after the experiment. The ratio of sucrose solution intake to total liquid intake is defined as the sucrose preference. The following formula was used to determine sucrose preference: sucrose preference = sucrose solution intake / (sucrose solution intake + water intake) × 100%.

Statistical analysis

For statistical analysis, the mean and standard deviation of the data was calculated. The sample fulfilled the following criteria: first, the sample was randomly selected from the population and randomly assigned to three different groups; second, although the sample did not reach the standard threshold (sample size ≥ 30), there were no major outliers or skewness in the three groups. Thus, the distribution of the sample could be assumed as approximately normal distribution. The data of the forced swimming test and the data of the weight ratios for the three groups were analyzed using one-way ANOVA. If the calculated *p*-value was less than 0.05, the difference was considered statistically significant between the two groups.

Received: August 4, 2020

Accepted: December 12, 2020

Published: December 18, 2020

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