Jiaotai Wan exerts antidepressant effects through regulating gut microbiota in rat model of chronic unpredictable mild stress

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Research Article

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Abstract

This study used 16S rDNA high-throughput sequencing to determine the effects of Jiaotai Wan on gut microflora of rats experiencing chronic unpredictable mild stress. We randomly divided 135 healthy male rats into nine groups. All groups except the control received chronic unpredictable mild stress. Rats were weighed before being subjected to sucrose preference and open field tests. Rat fecal samples were used for 16s rDNA high-throughput sequencing; the results allowed us to determine gut microfloral structure and changes. The stress treatment decreased body weight, sucrose preference, and performance in open field tests from control levels. Beta diversity of gut microflora differed across rat groups. Jiaotai Wan increased Lachnospiraceae, *Bacteroides*, and *Akkermansia* abundance, while decreasing Ruminococcaceae abundance. Therefore, Jiaotai Wan may exert antidepressant effects through regulating microbial abundance in the gut.

Introduction

Depression is a mental illness characterized by persistent negative mood, cognitive disorders, and impaired social function (Gururajan et al. 2019). Suicide and self-mutilation associated with depression affect both the lives of patients and their families; Elevated levels of depression in a population generates a sense of societal unrest.(Gerhard et al. 2016). However, available drugs cannot meet the treatment needs of all patients with depression, and novel drug options should be explored (Ghanean et al. 2018).

An increasing number of researchers have proposed that gut microflora influences other elements of human physiology (including the brain), especially in mental diseases (Moya et al. 2016). A study in rats found that depression alters the structure of gut microflora (Yu et al. 2017). Therefore, the pathogenesis of depression may be related to gut microflora conditions.

Jiaotai Wan (JTW) is a well-known prescription from traditional Chinese medicine (TCM) that has been used to treat depression. The formula comprises *Coptidis Rhizoma* (CR) and *Cinnamomum cassia* (CC). Jiaotai Wan has an obvious curative effect and high safety as a medical treatment of depression, especially when symptoms are mild. Xiang et al. (2020) suggested that this antidepressant effect occurs through JTW increasing production of the A1R protein and its downstream protein ERK1/2 in the prefrontal cortex and hippocampus. In addition, we recently demonstrated that JTW's antidepressant effect could stem from its regulation of amino acid metabolism, glycerophospholipid metabolism, and energy metabolism (Jiao et al. 2020).

Thus far, few studies have used 16S rDNA high-throughput sequencing to examine how depression alters gut microflora and investigate the effect of JTW on these changes. Therefore, in this study, we used this technique to determine the effects of JTW on gut microflora in rats subjected to chronic unpredictable mild stress (CUMS), an animal model of depression. Our results provide experimental evidence for the treatment of depression with JTW.

Materials And Methods

Grouping and care of experimental animals

A total of 135 healthy male Wistar rats (Beijing Vital River Laboratory Animal Technology) weighing 140–160 g were raised in the animal room of the Tianjin Experimental Animal Center, under the following conditions: 24–26 °C, 40–60% humidity; 12-h light/dark cycle. Rats were randomly split into nine groups (n = 15): control (C), model (M), fluoxetine (FLX, 7.5 mg/kg), JTW high dose (JTW.H, 3 g/kg), JTW low dose (JTW.L, 1.5 g/kg), CR high dose (CR.H, 2.73 g/kg), CR low dose (CR.L, 1.36 g/kg), CC high dose (CC.H, 0.27 g/kg), and CC low dose (CC.L, 0.14 g/kg). All rats except for the control would need to be subjected to the stress treatment. The M group that was only subjected to stress treatment but no drugs. Common feed and water were provided ad libitum. All animal experiments were approved by the Ethical Committee for Animal Experimentation of Tianjin University of Traditional Chinese Medicine (May 9, 2019, TCM-LAEC2019066) and were conducted in compliance with university's Guidelines for Animal Experimentation. Investigators were not blinded during the study.

Determination of sample size

Pharmacological experiment methodology and related literature were used to demonstrate that statistical requirements are met when the sample size per group is ≥6 (Gu et al. 2020; Yang et al. 2020; Yu et al. 2015). To improve accuracy, we selected 15 rats per group for the experiment. Rat body weight and sucrose preference test (SPT) results were analyzed according to data from individual subjects at each time point. Some animals were eliminated because of death or model failure (see Supplementary Material Table 1). Animal feces were used to analyze gut microflora. Eight animals were for the gut microflora test, exceeding the minimum sample size of six.

Pharmaceutical preparation

A 10:1 ratio of CR (400 g) and CC (40 g) was used to prepare coarse (20 mesh) powders. For extraction, CR was treated with water under reflux three times, while CC and JTW were treated with water three times using double extraction. The amount of water added was 10, 8, and 8 times, respectively. Extraction duration for CR, CC, and JTW were 70, 80, and 40 min, respectively; solutions were then filtered. Each of the three extracts were separately centrifuged at 2,000 rpm for 20 min, and supernatants were mixed.

Extractive CR, CC, and JTW solutions were concentrated in a water bath, then dried at 60°C and pulverized into a dry powder. Paste rate was calculated as dry powder mass/decoction piece mass × 100%. The extract was stored in a refrigerator at 4°C until further use. When needed, the extract was dissolved and diluted to a solution with ultrapure water according to the paste rate.

Fluoxetine was purchased from Eli Lilly Suzhou Pharmaceutical (catalog number: 6663 B), while CR and CC were purchased from Bozhou Chinese and Western Medicine, Anhui Province, China. The two herbal ingredients were identified by Professor Tianxiang Li from the School of Chinese Medicine of Tianjin

University of Traditional Chinese Medicine. See supplementary materials for details on CR, CC, and JTW preparation.

Rat model of CUMS

This study used the CUMS rat model of depression (Liu et al. 2020). Feed and drinking water were supplied ad libitum before the experiment, and 1% sucrose water training was conducted before the experiment 24 hours. All groups except group C received the CUMS process. After 21 d of stimulation, rats were intragastrically administered the pharmaceutical treatments at a weight of 1.0 mL/100 g (the C and M groups were given the same amount of distilled water). Subsequently, CUMS continued for another 14 d (total 35 d of CUMS).

Rats in the C group were supplied with feed and water ad libitum (except for 24 h of water deprivation before 1% sucrose consumption), and did not receive any CUMS. Stressful stimulators involved swimming for 5 min in 4°C water, reversal of day and night (dark for 9:00–21:00, light for 21:00–9:00 on the next day), tail clamping for 2 min, oscillating for 2 min, and food/water deprivation for 24 h. The stimulus was randomized daily for animals in each group, and each stressor was used no more than six times total.

Behavioral test

Body weight

We measured the body weight of each rat before the experiment (day 0), as well as on days 7, 14, 21, 28, and 35 of CUMS.

Sucrose preference test

The SPT is mainly used to measure lack of pleasure in rats. Before CUMS treatment began, subjects were trained to adjust to 1% sugar consumption. For 24 h, two water bottles were placed side by side in each cage, both containing 200 mL of 1% sugar water. Subsequently, sugar water in one bottle was replaced with distilled water and again left for 24 h. Then, rats were food and water deprived for 24 h, before being provided with a bottle each of 1% sugar solution and distilled water. After 1 h, volumes of the remaining sugar water and distilled water were measured. Sucrose preference rate was calculated using the following equation: sucrose preference rate (%) = sucrose intake/(sucrose intake + water intake) × 100%.

Open field test

An open field test (OFT) was performed as follows: rats were lifted by grasping one third from the tip of the base, then placed in the middle of an open field box for 2 min. An animal behavior video analysis system was used to record subject autonomous activity for 5 min. The number of times rats crossed the horizontal grid (by more than three claws) and stood upright (both forelimbs off the ground by 1 cm) in 4 min were counted. Overall OFT scores per rat were determined, and between-group differences were

compared. The open field box was 50 cm × 50 cm × 40 cm with black inwalls and a floor that was separated using white lines into 25 same-sized squares. All rats were tested on days 0, 21, and 35. The experimental area was washed with 70% ethanol after each rat completed its session.

Sample collection and preparation

Rats were sacrificed at the end of behavioral tests to dissect their rectums. Fresh feces were collected using sterile cotton swabs and deposited into tubes. These tubes were then placed in an eppendorf tube. Fecal samples were frozen in liquid nitrogen and stored at -80°C until use.

DNA extraction and PCR amplification

Genomic DNA was extracted from fecal samples with the CTAB or SDS method. The following primers were used to PCR-amplify the V3-V4 variable region of the 16S rRNA gene: 338 F(ACTCCTACGGGAGGCAGCAG) and 806 R(GGACTACNNGGGTWTCTAAT). Amplicons were detected using 2% agarose gel electrophoresis.

Bacterial diversity analysis

After clustering all valid sequences of samples in Uparse (Haas et al. 2011), representative operational taxonomic units (OTUs) were selected. The Mothur method and the SSUrRNA database of SILVA132 were used to annotate species, genus, family, order, class, and phylum (Wang et al. 2007; Edgar, 2013).

The Chao 1, Simpson, and observed species indices were calculated in Qiime version 1.9.1. R version 2.15.3 was used to draw the rarefaction curve, rank abundance curve, and species accumulation boxplot. Tukey's test in R was used to analyze inter-group differences in the alpha diversity index.Unifrac distance was calculated in Qiime. R was used to draw the PCoA diagram and analyze between-group differences in the beta diversity index with Tukey's test. The Linear discriminant analysis-effect size (LEfSe) tool in Qiime was used to determine significant differences in relative species abundance between groups.

Statistical analysis

Data are expressed as means ± standard deviation ($\bar{\chi}$ ± SD). Statistical analyses of data were performed in SPSS 19.0 (IBM Corp, New York, NY). Changes in body weight and SPT were assessed for significance using repeated measures ANOVA and a test for sphericity. The Greenhouse-Geisser correction was used for violation of sphericity. Changes in upright times were assessed for significance using one-way ANOVA. Changes in horizontal movements were assessed for significance using the Kruskal-Wallis H test. Gut microflora composition was investigated with LEfSe. Statistical significance was set at $p < 0.05$.

Results

JTW attenuated CUMS-induced depressive-like behaviors in rats

To observe the behavioral effects of JTW in CUMS rats, we measured changes in rat body weight, SPT, and OFT (Fig. 1). Before the experiment, body weight did not differ significantly between groups (Fig. 1a). On day 21, the M group weighed significantly less than the C group [t(df) = 8, F = 3.699, p = 0.036; Fig. 1a). After 14 d of continuous administration, rat weights in the FLX, JTW.H, JTW.L, CR.H, CR.L, CC.H, and CC.L groups significantly increased (Fig. 1a).

Sucrose preference rate did not differ between groups before the experiment (Fig. 1b). On days 7, 14, and 21, sucrose preference rates gradually decreased among all treatment groups, compared with the C group (Fig. 1b). After 14 d of continuous drug administration, sucrose preference rate of rats in the FLX, JTW.H, JTW.L, CR.H, CR.L, CC.H, and CC.L groups significantly increased, differing significantly from the M group (Fig. 1b). Although there was no statistical difference between the M group and the treatment group, we could see from the change trends that drug treatments improved CUMS rats (Fig. 1b).

Rat autonomous behavior in the OFT did not differ before the experiment (Fig. 1c,d). After 35 d, however, differences emerged [t(df) = 8, p < 0.001]. Rats in the M group had significantly lower upright times than the C group [t(df) = 8, p < 0.001; Fig. 1c]. Additionally, compared with the M group, the FLX, JTW.H, JTW.L CR.H CR.L CC.H and CC.L groups had significantly higher upright times [t(df) = 8, F = 5.421, p < 0.001; t(df) = 8, F = 5.421, p < 0.001; t(df) = 8, F = 5.421, p = 0.003; t(df) = 8, F = 5.421, p = 0.008; t(df) = 8, F = 5.421, p = 0.03; t(df) = 8, F = 5.421, p = 0.009; t(df) = 8, F = 5.421, p = 0.042; Fig. 1c]. Furthermore, the M group exhibited significantly reduced horizontal movements compared with the C group [t(df) = 8, p < 0.001; Fig. 1d]. The FLX, JTW.H, and JTW.L groups had significantly higher horizontal movements than the M group [t(df) = 8, p = 0.001; t(df) = 8, p = 0.017; t(df) = 8, p = 0.603; Fig. 1d].

Similarity of gut microflora among CUMS rats

A petal diagram was generated from common and unique microflora between groups to observe their distribution across treatments. The petal diagram directly reflects similarity of OTU components between samples. Each petal represents a group of samples, and different colors represent different groups. The core number in the middle represents the number of OTUs common to all samples, while petal count represents the number of OTUs unique to a group. We found 427 common OTUs across groups (Fig. 2), and OTU components were similar between the nine groups.

JTW alters alpha diversity of gut microflora in CUMS rats

We used alpha diversity to measure variation in species richness and diversity of microbial communities. To test the sufficiency of our gut microflora data, we constructed rarefaction curves, rank abundance curves, and species accumulation boxplots according to the OTU number at different sequencing depths (Fig. 3a, b, c). Our results indicated that the sequencing data were sufficient to reflect most microbial species in the samples (Fig. 3a); richness and evenness were relatively high for every group (Fig. 3b), samples were sufficient, and species richness was high (Fig. 3c).

We used the Simpson index to assess species diversity, along with the Chao1 index and observed species to assess species richness. A higher Simpson index indicates lower diversity. Higher observed species and Chao1 index indicates greater abundance. We found that the M group had higher gut microflora diversity than the JTW.H, JTW.L, CR.H, and CR.L groups, a difference that was significant ($p < 0.001$; Fig. 4a). Gut microflora diversity did not differ across the five groups (Fig. 4a). Additionally, the M group had significantly greater species richness than the JTW.H, JTW.L, CR.H, and CR.L groups ($p < 0.001$; Fig. 4b and 4c). These results showed that CUMU significantly altered alpha diversity of gut microflora, as did JTW and CR administration.

JTW alters beta diversity of gut microflora in CUMS rats

To determine variation in the overall structure of gut microflora, we used the weighted UniFrac distance to analyze sample PCoA. We found that the first two dimensions of the PCoA map described the weighted UniFrac distance across groups. Microbial communities of JTW.H, JTW.L, CR.H, and CR.L groups differed significantly from the M group along the positive direction of PCoA1, explaining 49.16% of total variability (Fig. 5). Therefore, as a whole, the M group could be separated from the other four groups along PCoA1, while the FLX group was closer to the C group. In sum, the FLX, JTW.H, JTW.L, CR.H, and CR.L groups had some influence on CUMS-induced changes to gut microflora structure.

JTW changed relative abundance of gut microflora in CUMS rats

We used LEfSe to determine dominant bacterial taxa in our rat groups. This analysis is used to reflect the composition of different species in two or more biological communities. We identified the largest differences (LDA score > 4.0) in gut microbiota among the nine groups. Differential flora in the C group were Firmicutes, Peptostreptococcaceae, and *Romboutsia* (Fig. 6). Differential flora in the M group were *Bacillus* and Lactobacillales. The only differential flora of the FLX group was *Dubosiella*. The most differentially abundant bacterial taxa in the JTW.H group were Proteobacteria, Erysipelotrichia, Klebsiella, and *Citrobacter*, while in the JTW.L group, they were were Clostridia, Verrucomicrobiae, Lachnospiraceae, and Akkermansiaceae. Differential flora in the CR.H group were *Bacteroides* and Muribaculaceae; in the CR.L group, they were *Blautia*, *Lachnoclostridium,* and *Coprobacillus*. The CCH and CC.L groups had Ruminococcaceae and *Allobaculum*, respectively, as the only differentially abundant bacterial taxon. These results showed that different doses of JTW, CR, and CC in CUMS rats changed the abundance of their gut microbiota.

To further identify alterations in the fecal microbial community, we created a heatmap of the microbial community structure per rat group. Each group differed considerably in the relative abundance of gut microbiota (Fig. 7). For example, the JTW.L group was more abundant in Lachnospiraceae, *Bacteroides*, and *Akkermansia*, whereas Ruminococcaceae was reduced. We also depicted between-group variation in gut microflora using bar graphs (Fig. 8).

Discussion

Here, we found that JTW improved the body weight, SPT, and OFT of CUMS rats, suggesting that the herbal remedy attenuated CUMS-induced depression-like behaviors in rats. Our petal map and alpha diversity analysis showed that gut microflora across the nine groups had highly similar and very rich species composition. Additionally, PCoA analysis revealed considerable differences in the species composition structure across groups. Finally, LEfSe and heatmaps indicated that JTW increased Lachnospiraceae, *Bacteroides*, and *Akkermansia* abundance, but decreased Ruminococcaceae abundance.

Previous studies have largely focused on the relationship between gut microflora and depression. Recent research has demonstrated that gut microflora can affect brain neurobiochemistry and behavioral phenotype through the gut-brain axis, thus altering mood and behavior (Raimondi et al. 2019). Haghighat et al. (2019) found that probiotics improves patient depressive symptoms through increasing serum BNDF levels. Moreover, Sun et al. (2018) found that probiotics function similarly to antidepressants in CUMS rats through increasing 5-HT and BNDF levels via the brain-gut axis (Sun et al. 2018). With the help of correlation statistics, we screened microflora with changed relative abundance at each taxonomic level to explore the influence of JTW in CUMS rats. Earlier results suggested that JTW, CR, and CC may have antidepressant effects (Xiang et al. 2020; Liu et al. 2017; Wang et al. 2020). In this study, CUMS rats had lower body weight, SPT, and OFT. We set up the JTW, CR, and CC groups to compare the effects of several drugs in CUMS rats. We calculated the “low-dose group” based on clinical dosage. Each group was divided into high-and low-dose for comparison, and the drugs had greater effects in CUMS rats.

Bacteroides are important anaerobes at the beginning of life. Some strains have long been used as probiotics (Silvia et al. 2015). Cheng et al. (2020) found that *Bacteroides* was associated with depression using a microbiota-related gene set enrichment analysis. In this study, JTW increased *Bacteroides* abundance in CUMS rats, consistent with a previous study (Xiao et al. 2020). *Bacteroides* can also produce large amounts of GABA (Philip et al. 2019). Wu et al. (2020) revealed that HPP GABA-enriched soybeans exert modulatory effects on the behaviors of depressed mice. In addition, *Bacteroides* might be an indicator of depression-related intestinal inflammation (Rong et al. 2019). However, the relationship between *Bacteroides* and severity of intestinal inflammation remains unclear. Our findings here suggest that JTW may play an antidepressant role by regulating *Bacteroides* abundance, GABA, and inflammatory factors.

Our data are similar to recent reports of reductions in relative *Akkermansia* abundance of mice with depressive-like behavior (McGaughey et al. 2019). *Akkermansia* is widely present in the intestinal tract of healthy people, playing a role in protecting the intestinal barrier and attenuating inflammation (Belzer et al. 2012; Swidsinski et al. 2011). Through correlating differentially abundant gut microflora with depression indicators and metabolite levels, Ma et al. (2019) found that alterations in *Akkermansia* abundance were closely related to depression symptoms and inflammatory cytokines. Similarly, Wu et al. (2020) identified significant positive correlations between *Akkermansia* and 5-HT; the latter is closely related to depression and is widely considered a target of most antidepressants (Wu et al. 2020). These

results demonstrated that the treatment mechanism of JTW might be related to increasing *Akkermansia* abundance and the corresponding influence on 5-HT.

Short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate are closely related to gut microbiota. Indeed, some significant correlations between differential bacteria taxa, SCFAs and neurotransmitters in depressed mice were identified. (Wu et al. 2020). Polyphenols promote SCFA production through influencing Bacteroidetes in gut microbiota (Zhou et al. 2020). Additionally, *Akkermansia* is significantly and positively correlated with acetic acid levels (Wu et al. 2020), while Lachnospiraceae is a producer of SCFAs in the intestinal tract (Zhuang et al. 2020). Taken together, these findings suggest that JTW may affect SCFA synthesis through adjusting gut microbial abundance; this then generates some antidepressive effects. However, the role of gut microflora and SCFAs in JTW's therapeutic function requires further study.

Both Lachnospiraceae and *Blautia* are associated with depression (Cheng et al. 2020; Humbel et al. 2020). In this study, we observed an increased abundance of both taxa. A previous study found that Lachnospiraceae and *Blautia* decreased in patients with depression, but increased after treatment (Gu et al. 2020; Yang et al. 2020; Wong et al. 2016). We also observed a decrease in Ruminococcaceae abundance. Similarly, in a study using mice as the depression model, Ruminococcaceae was the most differentially abundant taxon between control and depressed mice (Wu et al. 2020). Another study showed that ketamine significantly decreased Ruminococcaceae abundance in depressed rats (Getachew et al. 2018). Together, these data suggest that the antidepressant effect of JTW may be related to changes in Lachnospiraceae, *Blautia*, and Ruminococcaceae, but more research is needed to better understand the relationship between depression and such changes to gut microflora.

Conclusion

In summary, JTW may exert antidepressant effects through regulating relative abundance of gut microflora. We found that JTW increased Lachnospiraceae, Bacteroides, and *Akkermansia* abundance, while decreasing Ruminococcaceae abundance. Changes in the gut microflora may be one of the ways that JTW can treat depression. The novel outcome of JTW improving dominant gut bacteria abundance in a rat model of depression may help clarify the mechanism of antidepressants *in vivo*. The herbal remedy has the potential to be effective in the clinical treatment of depression. However, we acknowledge that the intestinal environments of rats and humans differ considerably. Therefore, future studies should focus on investigating how JTW influences the gut microflora of patients with depression.

Declarations

Data Availability Statements

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

Chunquan Yu, Shan Gao, and Lin Li provided the study concept and design. Yueer Wang and Zhu Li conducted analyses and wrote the manuscript. Man Feng, Yue Li, Tongyao Ni, Yilan Xu, and Shuming Gao participated in research. All authors have read and approved the final manuscript.

Compliance with ethical standards

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Conflict of interest

No potential conflict of interest was reported by the authors.

Ethical approval

All the animal experiments were approved by the Ethical Committee for Animal Experimentation of Tianjin University of Traditional Chinese Medicine (May 9, 2019, TCM-LAEC2019066), and were conducted in compliance with the Guidelines for Animal Experimentation of the university.

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