Study on the Molecular Mechanism of Berberine to Improve Type 2 Diabetes Mellitus Complicated With Depression (SBDD)

Study Protocol with Statistical Analysis Plan

NCT06979440

18/5/2025

Study Protocol with Statistical Analysis Plan

1. Introduction

1.1 Lack of Specific Therapeutic Interventions for Type 2 Diabetes Complicated with Depression

According to the International Diabetes Federation, approximately 536.6 million individuals aged 20–79 worldwide were afflicted with diabetes in 2021, of whom 90% had type 2 diabetes (T2D)[1]. Depression has emerged as a significant comorbidity of T2D, with epidemiological studies revealing that approximately 28% of T2D patients suffer from comorbid depression—a prevalence twice that of the general population[2]. The coexistence of T2D with depression exacerbates disease progression, impairs quality of life, diminishes treatment adherence, elevates mortality risk, and imposes substantial burdens on families and society.

The underlying mechanisms linking T2D and depression remain incompletely elucidated. Notably, while depression incidence is notably higher in diabetic patients, no significant difference exists between individuals with impaired glucose metabolism and healthy controls, suggesting that hyperglycemia and related metabolic disturbances may not be the primary triggers of depression[3]. Moreover, antidepressant therapy alone fails to sustainably ameliorate hyperglycemia in T2D patients. Furthermore, several classes of antidepressants (e.g., selective serotonin reuptake inhibitors, SSRIs) are associated with weight gain, which may exacerbate T2D risk[4]. Given the absence of targeted pharmacotherapies for this comorbidity, deciphering its pathogenic mechanisms and identifying novel therapeutic targets hold urgent clinical significance.

1.2 Gut Microbiota-Derived Metabolite 5-AVAB Exacerbates Hippocampal Neuronal Damage and Depression-like Behaviors in T2D with Depression

The hippocampus serves as a critical neuropathological substrate in T2D with comorbid depression; hippocampal atrophy is a distinctive neuroimaging hallmark in affected patients[2]. Preclinical evidence demonstrates that diabetes diminishes hippocampal neurogenesis and neuroplasticity, thereby fostering the development of depressive symptoms[5]. The gut microbiota, a shared etiological factor in both T2D and depression[6], modulates hippocampal

function through metabolic and multifactorial pathways[7]. Dysbiosis and increased gut microbial metabolites contribute to hippocampal dysfunction and behavioral impairments[8]. Nevertheless, the roles and mechanisms of gut microbiota and their metabolites in hippocampal neuronal injury in T2D with depression remain obscure. Thus, profiling characteristic gut microbiota and microbial metabolites in this comorbidity and exploring microbiota-targeted therapeutic strategies for hippocampal injury bear profound scientific and clinical implications.

Among microbial metabolites, **5-aminovaleric acid betaine (5-AVAB)** has garnered substantial attention as a novel bioactive molecule. 5-AVAB is a gut microbiota-derived metabolite increasingly implicated in metabolic diseases. A recent randomized controlled trial demonstrated that elevated plasma 5-AVAB levels correlate strongly with diabetes[9]. Notably, 5-AVAB crosses the blood-brain barrier and induces neuronal dysfunction[10]. Furthermore, it promotes lipidotoxicity by attenuating mitochondrial β-oxidation, which may drive obesity-related metabolic disorders[11]. These findings suggest that 5-AVAB may serve as a crucial mediator in diabetes-associated neuronal pathology.

Endogenous 5-AVAB synthesis involves enzymes such as **5-aminovaleramidase (DavA)** and **L-lysine monooxygenase (DavB)** and is positively associated with *Pseudomonas*, *Bilophila*, *Coriobacteriaceae*, and *Enterococcus faecalis*[10,12]. Reported microbial pathways for 5-AVAB production include the **trimethyllysine (substrate)-DavB-DavA** cascade in *Pseudomonas* (Fig. 1-1)[13]. Our preliminary metabolomic analyses revealed **significantly elevated fecal 5-AVAB levels** in T2D with depression mice compared to controls. Moreover, **oral gavage of 5-AVAB in C57BL/6J mice led to rapid cerebral accumulation**, peaking within **5 minutes**. In a **high-fat/high-sugar diet + streptozotocin + chronic unpredictable mild stress (CUMS)-induced T2D with depression model**, **4-week 5-AVAB administration (100 mg/kg) markedly exacerbated depression-like behaviors** (see Preliminary Data 1). Thus, **5-AVAB represents a potential novel biomarker promoting T2D with depression progression**. Given the absence of prior investigations in this domain, further mechanistic exploration is imperative.

Building upon our previous findings, we propose to collect serum and fecal samples from healthy controls and T2D with depression patients to establish **a clinical microbiota model**—validating elevated 5-AVAB levels and elucidating its molecular mechanisms.

References

[1] Ong K L, Stafford L K, McLaughlin S A, et al. Global, regional, and national burden of diabetes from 1990 to 2021, with projections of prevalence to 2050: a systematic analysis for the Global Burden of Disease Study 2021[J]. *The Lancet*, 2023, 402(10397): 203-234.

[2] Tomic D, Shaw J E, Magliano D J. The burden and risks of emerging complications of diabetes mellitus[J]. *Nature Reviews Endocrinology*, 2022, 18(9): 525-539.

[3] Nouwen A, Nefs G, Caramlau I, et al. Prevalence of Depression in Individuals With Impaired Glucose Metabolism or Undiagnosed Diabetes[J]. *Diabetes Care*, 2011, 34(3): 752-762.

[4] Gold S M, Köhler-Forsberg O, Moss-Morris R, et al. Comorbid depression in medical diseases[J]. *Nature Reviews Disease Primers*, 2020, 6(1): 69.

[5] Ho N, Sommers M S, Lucki I. Effects of diabetes on hippocampal neurogenesis: Links to cognition and depression[J]. *Neuroscience & Biobehavioral Reviews*, 2013, 37(8): 1346-1362.

[6] Warma S, Lee Y, Brietzke E, et al. Microbiome abnormalities as a possible link between diabetes mellitus and mood disorders: Pathophysiology and implications for treatment[J]. *Neuroscience & Biobehavioral Reviews*, 2022, 137: 104640.

[7] Kuijer E J, Steenbergen L. The microbiota-gut-brain axis in hippocampus-dependent learning and memory: current state and future challenges[J]. *Neuroscience & Biobehavioral Reviews*, 2023, 152: 105296.

[8] Liu G, Yu Q, Tan B, et al. Gut dysbiosis impairs hippocampal plasticity and behaviors by remodeling serum metabolome[J]. *Gut Microbes*, 2022, 14(1): 2104089.

[9] Haberbosch L, Kierszniowska S, Willmitzer L, et al. 5-Aminovaleric acid betaine predicts impaired glucose metabolism and diabetes[J]. *Nutrition & Diabetes*, 2023, 13(1): 17.

[10] Mossad O, Nent E, Woltemate S, et al. Microbiota-dependent increase in δ-valerobetaine alters neuronal function and is responsible for age-related cognitive decline[J]. *Nature Aging*, 2021, 1(12): 1127-1136.

[11] Liu K H, Owens J A, Saeedi B, et al. Microbial metabolite delta-valerobetaine is a diet-dependent obesogen[J]. *Nature Metabolism*, 2021, 3(12): 1694-1705.

[12] Haikonen R, Kärkkäinen O, Koistinen V, et al. Diet- and microbiota-related metabolite, 5-aminovaleric acid betaine (5-AVAB), in health and disease[J]. *Trends in Endocrinology & Metabolism*, 2022, 33(7): 463-480.

[13] Zhao M, Zhao L, Xiong X, et al. TMAVA, a Metabolite of Intestinal Microbes, Is Increased in Plasma From Patients With Liver Steatosis, Inhibits γ-Butyrobetaine Hydroxylase, and Exacerbates Fatty Liver in Mice[J]. *Gastroenterology*, 2020, 158(8): 2266-2281.e27.

2. Study Design

2.1 Research purpose and significance

(1) Establish clinical microbiota and animal models to assess the efficacy of berberine-mediated inhibition of gut microbial 5-AVAB in ameliorating T2D with depression.

(2) Validate 5-AVAB elevation in patients and delineate its mechanistic contributions.

2.2 Study Framework

(1) Investigate berberine's inhibitory effect on 5-AVAB production in in vitro gut microbiota from T2DM with depression patients

① Enroll T2DM with depression (T2DD) and T2DM-only (Control) subjects. Collect fecal and serum samples to compare 5-AVAB levels.

② Evaluate the ability of different concentrations of berberine (10/50/100 μg/mL) to inhibit 5-AVAB production in fecal microbiota from T2DD patients.

(2) Mechanistic Study of Berberine's Suppression of Key 5-AVAB-Producing Bacteria

① Compare gut microbiota structure in fecal samples from clinical T2DD patients versus Control subjects. Perform correlation analysis to identify 5-AVAB-associated characteristic microbial taxa.

② Identify and isolate 5-AVAB-producing bacterial strains. Screen for the key 5-AVAB-producing bacteria.

2.3 Study Subjects

Control group: Patients with type 2 diabetes mellitus (T2DM) only

T2DD group: Patients with type 2 diabetes mellitus complicated with depression (T2DD)

2.4 Research Procedures

(1) Study on berberine's inhibitory effect on 5-AVAB production by gut microbiota in T2DD patients

This study will enroll two groups of participants, divided into control group and T2DD patient group, with 20 individuals in each group. Whole blood (5 mL) and fresh feces (5 g) will be collected from each participant, with whole blood used for subsequent serum extraction. LC-MS/MS will be employed to measure and compare 5-AVAB content in samples from each group. The fecal samples will be homogenized with anaerobic culture medium, then incubated with berberine (10 μg/mL, 50 μg/mL, 100 μg/mL) for 2, 6, 12, and 24 hours respectively, followed by 5-AVAB measurement using LC-MS/MS.

(2) Mechanism study of berberine's inhibitory effect on key 5-AVAB-producing bacteria

Following metagenomic sequencing, bioinformatics analysis will compare gut microbiota in clinical T2DD patients and control subjects, analyzing differences in microbial species composition between groups. Highly abundant gut microbial taxa will be screened and correlated with fecal 5-AVAB content to identify the most significantly associated genera. Using culturomics, 5-AVAB-producing bacterial strains will be identified and obtained. Single colonies will be picked into LB liquid medium for growth at 37°C, supplemented with TML-d9 substrate (50 mg/mL) for continued culture. Concentration-time curves of 5-AVAB-d9 will be plotted for different bacterial strains to screen for key producers showing highest AUC values. Using sterile pure medium as control, the selected bacterial strains will be co-cultured with berberine (0, 50 mg/mL) for 12 hours in vitro. UV-Vis spectrophotometer UV-2600 (Shimadzu, China) will compare OD620nm values between berberine-treated and control groups, while LC-MS/MS will measure 5-AVAB-d9 production.

2.5 Evaluation Criteria

No additional interventions are implemented in this study. Fecal samples are collected post-defecation, and blood collection volume (5 mL) complies with ISO international standards.

2.6 Data Management, Statistical Analysis Plan & Confidentiality Measures

All study data will be stored on password-protected encrypted hard drives within dedicated research computers and retained for three years after study completion, accessible exclusively to authorized research personnel. Upon request, government regulatory bodies, institutional review boards, or ethics committees may access the data in accordance with regulations. De-identified participant information and questionnaire responses may be utilized for future scientific research.III. Participant Recruitment (n=40–60).

3. Participant Recruitment

3.1 Inclusion Criteria

(1) Aged 18–65 years (inclusive), regardless of gender;

(2) Control group: Diagnosed with type 2 diabetes (T2D), with no history of depression or other psychiatric disorders;

(3) T2DD patient group: Diagnosed with type 2 diabetes comorbid with depressive disorder

(T2DD), where depression meets the DSM-5 diagnostic criteria for recurrent depressive disorder without psychotic symptoms or single-episode major depressive disorder (MDD), with a total score of ≥22 on the HAMD-17 scale;

(4) Participants must read, fully understand the subject information, and sign the informed consent form.

3.2 Exclusion Criteria

(1) Those who do not meet the inclusion criteria;

(2) No use of antibiotics, prebiotics, probiotics, enteral nutrition drugs, etc. within 3 months prior to diagnosis, and no such treatments during the study period;

(3) Individuals with progressive severe diseases (e.g., cancer);

(4) Those with severe aphasia, agnosia, or apraxia;

(5) Long-term use of psychotropic medications in the previous month or participation in new drug trials in the past 3 months;

(6) Pregnant or lactating women;

(7) Individuals with alcoholism or drug abuse;

(8) Poor mental condition, unable to cooperate;

(9) Any other conditions deemed unsuitable for participation in the study.

3.3 Withdrawal and Termination Criteria

If a participant discontinues the study prior to sample collection, they will be considered withdrawn/terminated from the study.

3.4 Study Participation Duration (Per Session and Total Time)

Participation in the study is defined as the period from enrollment to the completion of sample collection.

3.5 Recruitment Process

Recruitment for this study will commence only after obtaining approval from the Ethics

Committee of Affiliated Hospital of Nantong University. Participants will be recruited from the Endocrinology and Neurology Departments of the hospital, with an anticipated enrollment of 40–60 individuals. No financial compensation will be provided.

4. Risk/Benefit Assessment

4.1 Benefits

This study aims to establish the gut microbial metabolite 5-AVAB as a novel biomarker for diagnosing type 2 diabetes with comorbid depression (T2DD). The findings may advance diagnostic techniques and the development of related testing kits, as well as facilitate the development of new therapeutics for T2DD. Additionally, this research could provide evidence for identifying therapeutic targets in the comorbidity of chronic diseases such as diabetes and depression.

4.2 Risks

Since this study does not involve additional interventions beyond standard care, participation is unlikely to cause physical, psychological, or social harm beyond routine clinical management. Furthermore, it will not negatively impact disease diagnosis or treatment.

4.3 Protection of Special Populations

No additional interventions are applied in this study. Special populations (e.g., vulnerable individuals) must review and fully comprehend the subject information with the assistance of a family member before providing written informed consent.

5. Ethical Considerations in the Study

(1) Patient Privacy Protection:

The study may involve sensitive information from participants, necessitating strict confidentiality measures.

Solution: Implement rigorous privacy protection protocols, including data anonymization and restricted data access.

(2) Informed Consent:

Participants must fully comprehend the study's purpose, methodology, potential risks, and

benefits before voluntary enrollment.

Solution: Obtain explicit informed consent prior to study commencement, accompanied by detailed participant information materials.

6. Annual Research Plan

This project is scheduled for completion over three years.

Year 1 (January 2025 – December 2025):

Recruit T2DD patients and healthy controls; prepare for clinical sample collection.

Complete annual progress report and draft relevant manuscript sections.

Year 2 (January 2026 – December 2026):

Conduct clinical sample collection and laboratory testing.

Complete annual progress report, finalize related manuscripts, and initiate journal submissions.

Year 3 (January 2027 – December 2027):

Finalize all clinical sample collection and testing.

Submit annual progress report and publish research findings.

7. Additional Notes

7.1 Collaborations

This study is funded by the National Natural Science Foundation of China (NSFC, Grant no. 82404740), Jiangsu Pharmaceutical Association ("Yao" Yan Xin Sheng Project, Grant no. 202495102).

7.2 Publication Plans & Authorship

Research findings will be disseminated through peer-reviewed scientific papers. Authorship will be determined based on intellectual contribution.