Study Protocol and Statistical Analysis Plan

Hesperidin Improves Cognitive Function in Middle-Aged and Older Adults with Overweight or obesity: A Randomized Controlled Trial

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1 Background

Overweight and obesity are prevalent among middle-aged and older adults, and are associated with impaired cognitive abilities including memory and attention. Hesperidin is a natural constituent abundant in citrus fruits. It possesses a favorable safety profile, and animal experiments have confirmed its anti-inflammatory and neuroprotective properties. Clinical trials in humans have further demonstrated that hesperidin can regulate blood glucose and blood pressure in older adults. However, it remains unclear whether hesperidin could improve cognition in overweight or obese middle-aged and older adults, and what role gut microbiota may play in this process.

This study will enroll 105 overweight or obese participants aged 55 to 75 years. Subjects will be randomly allocated into three groups: a placebo group receiving corn starch, a medium-dose hesperidin group (300 mg per day), and a high-dose hesperidin group (600 mg per day). The intervention period will last 10 weeks. Cognitive domains including executive function, memory and attention will be assessed at baseline and upon study completion. Blood, urine and stool samples will be collected for analyses of gut microbiota, metabolites and inflammatory biomarkers. The findings will clarify the effects of hesperidin on brain health in overweight and obese populations, and reveal the potential mediating role of gut microbiota.

2 Study Objectives

(1) To determine the effect of hesperidin on cognitive function in overweight and obese individuals, as well as its dose-response relationship.

(2) To investigate the impact of hesperidin on gut microbiota composition and its metabolites.

(3) To elucidate the potential pathways through which hesperidin improves cognitive function in overweight and obese populations.

3 Subject Enrollment, Withdrawal, and Data Management

3.1 Inclusion Criteria

Age 55-75 years.

BMI ≥ 24 kg/m², or waist circumference ≥ 90 cm (male) / ≥ 85 cm (female).

Willing to participate in the study and to sign the informed consent form.

3.2 Exclusion Criteria

Baseline MMSE score < 20 (i.e., excluding patients with moderate or severe cognitive impairment). Presence of neurological diseases (e.g., Parkinson's disease, Alzheimer's disease). History of stroke or cerebrovascular disease. Malignant tumors. Severe liver, kidney, or gastrointestinal diseases (e.g., severe constipation, ulcerative colitis). Major surgery or severe trauma within the last 6 months. Current or recent use of weight-loss medications (including over-the-counter products or dietary supplements). Current use of antidepressants (statins are not excluded). Strict vegetarians (as their baseline gut microbiota differs significantly from the target population, which may introduce confounding). Binge eating disorder as defined by DSM-5 (e.g., recurrent episodes of eating significantly more food than most people would in a similar period, with a sense of loss of control, occurring at least weekly over the past 3 months). Alcohol consumption >14 g per day in females or >28 g per day in males, 14 g of alcohol is approximately equivalent to 350 mL of beer (5% alcohol by volume), 150 mL of wine (12% vol), or 45 mL of spirits (40% vol); the thresholds for males are twice these quantities. Following a weight-loss program within the last 3 months, that could interfere with the intervention within the next 6 months. Participation in other lifestyle or drug intervention studies.

3.3 Withdrawal Criteria

During the intervention period, if any of the following conditions occur, the investigator will advise the subject to withdraw from the study and record the reason and time of withdrawal. Persistent or severe intolerance reactions, e.g., severe diarrhea, vomiting, abdominal pain, rash, or other symptoms that, make intervention inappropriate.

Poor adherence: A new capsule box will be distributed weekly, and the previous week's empty capsule box will be collected. Adherence is assessed by checking the remaining capsules in each compartment (Monday to Sunday, 7 compartments, each containing 2 capsules for that day, total 14 capsules per week). Subjects who take ≤8

capsules per week will be considered as poor adherence. If poor adherence persists for two consecutive weeks, or if the subject actively states unwillingness to continue the intervention, the subject will withdraw, with the reason recorded as ‘poor adherence’.

Withdrawal: The subject requests to withdraw from the study at any time without providing a reason.

Loss to follow-up: The subject misses at least two consecutive follow-up visits and cannot be contacted despite repeated attempts by the investigator.

Other circumstances: Any other situation in which the investigator considers it necessary to terminate participation for the subject's safety and benefit, e.g., development of another serious illness requiring urgent treatment.

3.4 Post-withdrawal handling of data and biological samples

For research data that have already been collected (including baseline and completed follow-up assessments), identifiable information will be removed and the data will be retained for final analysis, where it may be used as adjusting factors. For biological samples (blood, stool, urine) that have been collected but not yet processed: if a participant withdraws consent and specifically asks for their samples to be destroyed, the research team will follow that request, document the destruction in the Sample Disposal Log, and then dispose of the samples. If the participant does not request destruction, these samples may be used for further testing as part of this study, depending on the circumstances.

After withdrawal, if any adverse reactions that might be linked to the intervention occur, participants are free to contact the research team at any time. We will continue to provide necessary medical advice and discuss possible next steps.

4 Randomization and Blinding

4.1 Sample size

Sample size was estimated using G*Power 3.1.9.7. A repeated-measures ANOVA model was assumed, with an effect size of 0.20, a significance level (α) of 0.05, and a power of 90%. The design included three groups, two repeated measurements, and a correlation coefficient of 0.5 between repeated measures. The estimated sample size

was 84 participants. Accounting for an expected 20% dropout or loss to follow-up, we plan to enroll a total of 105 participants, who will be allocated in a 1:1:1 ratio to the three groups (35 per group).

4.2 Randomization

Random sequence generation: An independent statistician not involved in the clinical part of the study will generate the random sequence using the RAND function in Excel and a random number table. The allocation ratio is 1:1:1, meaning 105 participants will be assigned to each of the three groups: placebo, medium-dose (300 mg/day), and high-dose (600 mg/day).

Randomization method: To ensure baseline comparability across the three groups in key characteristics such as age, sex, and cognitive level, a stratified block randomization method will be used. Cognitive level is stratified into three categories: high (28-30), medium (26-27), and relatively low (20-25). Age is stratified into three groups: 55-64 years, 65-69 years, and 70-75 years. Sex is stratified as male and female. This gives 3 (cognitive level) × 3 (age) × 2 (sex) = 18 strata.

Within each stratum, an independent statistician not involved in the clinical part of the study will generate the allocation sequence using the RAND function in Excel and a random number table. Block randomization will be applied with a block size of 6 (each block contains 2 participants for the placebo group, 2 for the medium-dose group, and 2 for the high-dose group). This ensures that the three groups remain balanced in sample size and key baseline characteristics throughout the enrollment period. The randomization results will be kept in sealed, opaque envelopes or kept by a third-party researcher who has no contact with the participants, so that investigators cannot predict the next assignment during recruitment and screening, thus preventing selection bias.

After all participants have been enrolled, comparability of baseline characteristics across the three groups will be checked. One-way ANOVA will be used for continuous variables (age, MMSE score), and the chi-square test for categorical variables (sex). If any statistically significant difference is found between groups ($p < 0.05$), subsequent primary outcome analyses will be adjusted using analysis of

covariance (ANCOVA), with age, sex, and baseline MMSE score as covariates.

4.3 Blinding

This study will use a double-blind design. Hesperidin capsules and placebo (corn starch) capsules are identical in appearance, color, smell, taste, and packaging, and will be packaged uniformly by the same manufacturer (Hebei Kangtai Pharmaceutical Co., Ltd.). Participants, intervention providers, outcome assessors, and data analysts will all be blinded to group assignment. Only the personnel who fill the capsules will know the capsule content and will fill them according to each participant's group based on their sequential number. The staff distributing the capsules will not know the content; they will only distribute the capsules according to the numbered containers.

5. Study procedures

5.1 Enrollment

Eligible middle-aged and older adults will be recruited from community health service centers in Shanghai. All potential participants will undergo initial screening and a detailed assessment. Before enrollment, each participant will sign an informed consent form, and the research staff will clearly explain the study aims, procedures, and significance to ensure good cooperation. Those who meet the inclusion and exclusion criteria will be formally enrolled after signing the consent form.

5.2 Baseline data collection

Before randomization, baseline data will be collected from all enrolled participants to assess demographic characteristics, physical status, and baseline cognitive function. Both baseline epidemiological surveys and follow-up assessments will be carried out by trained research staff. All data will be entered into an encrypted database accessible only to authorized personnel. To protect participant privacy, any directly identifiable personal information (such as name, ID number, and contact details) will be de-identified during data entry and replaced with a unique study ID. The analytical database will contain no personal identifiers, ensuring anonymity throughout data analysis. All materials involving participant privacy will be securely stored and will not be disclosed to any third party without the participant's permission

or approval from the ethics committee. The specific items to be collected are as follows:

① Questionnaire

Demographic characteristics and socioeconomic status will be collected, including sex, date of birth, education level, occupational history, family structure, and annual income. Dietary assessment will include food frequency questionnaire and 24-hour dietary recall. Physical activity will be assessed using the International Physical Activity Questionnaire (IPAQ).

② Anthropometric and biochemical measurements

Height (cm) and weight (kg) will be measured using standard methods. Waist and hip circumferences will be measured with a tape measure to calculate the waist-to-hip ratio. Blood pressure (average of two measurements) and heart rate will be measured using a sphygmomanometer. Fasting glucose, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) will be analyzed using an automated biochemical analyzer.

③ Cognitive function tests

Cognitive function, including executive function, will be assessed using the following tools: the Alzheimer's Disease Assessment Scale - Cognitive subscale - Memory (ADAS-Cog-M), the Wechsler Memory Scale, the Mini-Mental State Examination (MMSE), the Montreal Cognitive Assessment (MoCA), the Wechsler Digit Symbol Substitution Test, Digit Span Test, Verbal Fluency Test, and the Trail Making Test (TMT-A and TMT-B).

④ Baseline biological sample collection and storage

Morning urine (10 mL) will be collected, aliquoted into 1.5 mL centrifuge tubes, and stored at -80°C. Fasting blood will be collected, and plasma and red blood cells will be separated by centrifugation, aliquoted into 1.5 mL cryotubes, and stored at -80°C. Participants will collect fresh stool samples using stool collection tubes. Stool samples will be processed within 6 hours of collection. Each sample will be aliquoted into sterile screw-cap centrifuge tubes. Bacterial DNA will be extracted from the stool

samples following the kit instructions and stored at -80°C for gut microbiota analysis. For metabolomics, 1 g of stool will be mixed with PBS buffer, the supernatant collected, and stored at -80°C.

5.3 Study design and intervention protocol

The intervention period lasts 10 weeks. The placebo group will receive two 300 mg capsules of corn starch powder (total 600 mg per day), which are identical in appearance, smell, and packaging to the hesperidin capsules. The medium-dose group will take two capsules: one 300 mg capsule of hesperidin powder and one 300 mg capsule of corn starch powder. The high-dose group will receive two 300 mg capsules of hesperidin powder (total 600 mg per day). The doses were chosen based on previous human intervention studies. Eghtesadi et al. showed that 500 mg/day of hesperidin for 8 weeks improved metabolic parameters. Taking into account the safety profile of hesperidin and existing literature, we set 300 mg/day as the medium dose and 600 mg/day as the high dose, allowing us to assess a potential dose-response relationship.

During the intervention, research staff will pre-fill a weekly supply of capsules into portable pill boxes (seven days' worth per box). Each participant will take 2 capsules per day, which have already been placed into the boxes. The boxes will be distributed to participants on the morning of the first day of each week, and participants will pick them up in person and sign a log.

Hesperidin powder is supplied by Chengdu Oukang Company, along with a certificate of analysis ensuring quality and food safety (purity 95.8%, batch No. HAF03-2507003, CAS No. 520-26-3, heavy metals <10 ppm, total aerobic microbial count <10 CFU/g). Corn starch (test results comply with GB/T standards) is provided by Cangzhou Haihua Shunda Grain and Oil Condiment Co., Ltd. Empty capsule shells (size 0) are supplied by Zhejiang Pujiang Enkang Capsule Co., Ltd. Packaging is done by Hebei Kangtai Pharmaceutical Co., Ltd. All capsules are packaged under uniform procedures and storage conditions, ensuring that each capsule contains the specified amount of hesperidin powder (300 mg) or corn starch (300 mg). The capsules are natural with no additives and come with standard quality inspection

reports. Hesperidin is a citrus flavonoid and has generally been well tolerated in previous studies, but gastrointestinal discomfort or allergic reactions may still occur. Any adverse events will be managed promptly, and the intervention will be discontinued if necessary.

Adherence will be assessed by collecting the pill boxes each week to count remaining capsules and by measuring hesperidin metabolites in urine samples collected weekly.

Before the intervention begins, participants will be given a Huawei Band 10 activity tracker free of charge. The device records daily physical activity levels, including step count and active time. Data are synced via Bluetooth to a study-dedicated smartphone and will be used for research purposes only. The Huawei Band 10 is manufactured by Huawei Technologies Co., Ltd. It is a consumer-grade wearable device primarily designed for daily sleep trend monitoring, heart rate tracking, and activity tracking. Its sleep monitoring is based on HUAWEI TruSleep™ technology.

Participants will wear the band every day until the end of the intervention. They are asked to maintain their usual lifestyle habits, including diet, physical activity, and sleep, throughout the study. If a participant is unwilling to wear the band for reasons such as skin allergy, they should notify the research staff. After assessment, if the staff determines that the participant can still provide complete and scientifically sound data through weekly follow-ups even without the band, the band will be collected, and we will strengthen weekly follow-up observations of the participant's daily life to ensure data quality. If the assessment concludes that the quality of daily activity data cannot be guaranteed without the band, the participant may not be able to continue in the intervention trial.

During the intervention, a 20-minute nutrition sessions will be given every two weeks by a researcher with a background in nutrition. The content will be based on the Chinese Dietary Guidelines and will consist of general health education. The sessions are designed to avoid influencing participants’ eating behaviors; participants are encouraged to keep their original lifestyle habits. The purpose is to improve

adherence, not to serve as a weight loss intervention. Capsule distribution will take place at the same time as the nutrition talks. All participants will receive the same dietary guidance and schedule.

(1) Follow-up assessments during intervention

At weeks 2, 4, 6, and 8 of the intervention, each participant will have a one-on-one session with a research staff member to collect a 24-hour dietary recall, assess physical activity level using the International Physical Activity Questionnaire (IPAQ), and evaluate sleep status. These assessments aim to confirm that participants have maintained their usual lifestyle habits throughout the intervention. Any side effects or discomfort will also be recorded.

(2) Anthropometric and vital sign measurements

At weeks 2, 4, 6, and 8, research staff will measure height, weight, waist circumference, hip circumference, blood pressure, heart rate, body fat, and skeletal muscle mass.

(3) Stool collection during intervention

At weeks 2, 4, and 8, participants will collect fresh stool using a sterile container. An opened anaerobic bag will be placed inside the container, which will then be sealed, placed in a cold-chain transport bag, and delivered to the community health center.

(4) Urine collection

Urine samples (10 mL) will be collected weekly throughout the intervention.

(5) End-of-intervention assessments

After the intervention ends, cognitive function tests will be scheduled at a consistent time of day (preferably 8:00-9:00 AM) to avoid potential bias from daily variations in diet, physical activity, and other lifestyle factors. Urine (10 mL), blood (18 mL), and stool samples will also be collected.

Week	0	1	2	3	4	5	6	7	8	9	10
Physical Test	●		●		●		●		●		●
Blood Test	●										●
Urine Test	●	●	●	●	●	●	●	●	●	●	●
Stool Test	●		●		●				●		●
Cognitive and Food Frequency Table	●										●
Follow-up Questionnaire and Health Lecture			●		●		●		●		●
Capsule Distribution	●	●	●	●	●	●	●	●	●	●	●
Gift Distribution		●	●	●	●	●	●	●	●	●	●

5.4 Sample collection and laboratory analysis

(1) Blood samples

Peripheral blood (18 mL) will be collected via venipuncture into EDTA-anticoagulated tubes. An aliquot of whole blood will be set aside. The remaining blood will be centrifuged at 1000 rpm for 15 minutes. The plasma will be collected, aliquoted into cryotubes, and stored for subsequent analyses. These analyses include:

Multi-omics profiling

Cytokines: pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 (to assess inflammatory response); chemokines such as monocyte chemoattractant protein-1 (MCP-1) and IL-8

C-reactive protein (CRP) as a sensitive marker of systemic inflammation

Blood biomarkers: Aβ42/Aβ40 ratio

Biochemical parameters: triglycerides, glycosylated hemoglobin (HbA1c), blood lipids, HDL-C, LDL-C

For immune cell analysis, blood cells will be treated with ACK red blood cell lysis buffer (1×, 10 mL). The tube will be inverted gently to mix and left at room temperature for 3 minutes. Then centrifuge at 300g for 5 minutes at 4°C (acceleration 9, deceleration 7) and discard the supernatant. Add 5 mL PBS to resuspend the cells, centrifuge again at 300g for 5 minutes, and discard the supernatant. Add 1.8 mL of 0.5% BSA in PBS (0.5 g BSA dissolved, then add 100 μL DMSO per tube). The EDTA tube is centrifuged at 400g for 5 minutes at room temperature to separate

plasma and cellular components. Using fluorescent antibodies, various immune cell populations will be quantified by flow cytometry, including the proportions and absolute counts of CD4+ T cells, CD8+ T cells, Tregs, B cells, and monocyte subsets.

(2) Stool samples

After samples are brought back to the laboratory, they will be initially aliquoted, then snap-frozen in liquid nitrogen for 10 minutes and transferred to -80°C storage for subsequent metagenomic analysis. Functional gene profiling will be used to assess the collective functional potential of the microbial community, including genes involved in short-chain fatty acid (SCFA) synthesis, bile acid metabolism enzymes, and lipopolysaccharide synthesis. Microbiota-derived metabolites such as short-chain fatty acids (acetate, propionate, butyrate), secondary bile acids, and tryptophan metabolites will also be measured. We will examine microbial species, cognitive function-related genes, metabolic function genes, and microbial interactions to capture the functional activity of the gut microbiota and perform cross-omics correlations with host plasma metabolome and inflammatory markers.

(3) Urine samples

Morning urine will be collected. Within 30 minutes after collection, samples will be centrifuged at 12,000-15,000×g for 10-15 minutes at 4°C to remove cells and debris. The supernatant will then be filtered through a 0.22 μm syringe filter to further remove particulates and microorganisms. The filtered urine will be aliquoted into enzyme-free, chemically clean centrifuge tubes (e.g., polypropylene tubes) and immediately stored in a -80°C ultra-low temperature freezer. Repeated freeze-thaw cycles should be avoided. These samples will be used for metabolomics analysis.

For analysis, an aliquot of 100 μL of thawed, vortex-mixed urine will be transferred to a new centrifuge tube. Metabolites will be extracted and then gently dried using a vacuum concentrator or nitrogen blow-down evaporator. The dried residue will be reconstituted in 100 μL of initial mobile phase (e.g., water:acetonitrile = 95:5) and vortexed for 30 seconds. After another centrifugation at 14,000×g for 15 minutes at 4°C, the supernatant will be collected for metabolomics analysis.