Journal of Traditional and Complementary Medicine 14 (2024) 403–413

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: www.elsevier.com/locate/jtcme

The therapeutic effect and metabolic mechanism analysis of Guilingji on idiopathic oligo-asthenoteratozoospermia

Yuming Feng [a,b,1], Shanmeizi Zhao [b,c,1], Rong Ju [d,1], Jianbo Li [e,1], Chengshuang Pan [f,1], Qinglai Tang [g], Zhichuan Zou [b], Jun Jing [b], Yao Xu [h], Hualong Ding [b], Jinzhao Ma [b], Zhou Li [i], Ting Tang [b], Yong Shao [b], Li Chen [b,**], Xuefeng Huang [f,***], Xiaohong Wang [e,****], Bing Yao [a,b,c,*]

[a] The First School of Clinical Medicine, Southern Medical University, Guangzhou, 510515, Guangdong, China
[b] Department of Reproductive Medicine, Jinling Hospital, Affiliated Hospital of Medical School, Nanjing University, Nanjing, 210002, Jiangsu, China
[c] Jiangsu Key Laboratory for Molecular and Medical Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing, 210023, Jiangsu, China
[d] Department of Obstetrics and Gynecology, The Affiliated Jiangning Hospital with Nanjing Medical University, Nanjing, 211100, Jiangsu, China
[e] Center for Reproductive Medicine of Obstetrics and Gynecology, Tangdu Hospital, Air Force Medical University, Xi'an, 710032, Shaanxi, China
[f] Reproductive Medicine Center, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, 325000, Zhejiang, China
[g] Department of Urology, The Affiliated Jiangning Hospital of Nanjing Medical University, Nanjing, 211100, Jiangsu, China
[h] State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, 211116, Jiangsu, China
[i] Department of Reproductive Medicine, Nanjing Jinling Hospital, School of Medicine, Jiangsu University, Zhenjiang, 212000, Jiangsu, China

ARTICLE INFO

Keywords:
Guilingji
Oligo-asthenoteratozoospermia
Metabolomics
Glucose-6-phosphate
Traditional Chinese medicine

ABSTRACT

Introduction: Guilingji, a famous traditional Chinese medicine (TCM) formula, has been used to combat aging and male sexual dysfunction in China for centuries. To date, there has been little evidence-based clinical research on the use of Guilingji to treat idiopathic oligo-asthenoteratozoospermia (OAT), and the therapeutic mechanism from a metabolic perspective needs to be investigated further.

Methods: This was a multicenter, double-blind, randomized controlled clinical study of 240 patients with idiopathic OAT recruited from four hospitals between January 2020 and January 2022. Patients were randomly assigned in a 1:1 ratio to receive oral Guilingji capsules or placebo for 12 weeks. The total progressive motile sperm count (TPMSC) was considered the primary outcome, and the other sperm parameters, seminal plasma parameters and serum hormones were considered the secondary outcome. A nontargeted metabolomics analysis of serum from OAT patients before and after Guilingji administration was performed by HPLC–MS to identify key metabolites. Furthermore, we used a rat model to show spermatogenesis phenotypes to validate the effect of the key metabolites screened from the patients.

Results: At weeks 4, 8 and 12, TPMSC and other sperm parameters were significantly improved in the Guilingji group compared with the placebo group ($P < 0.05$ for all comparisons). At week 4, superoxide dismutase (SOD) and acrosomal enzyme activity of seminal plasma were significantly elevated in the Guilingji group compared with the placebo group, while reactive oxygen species (ROS) levels were significantly reduced ($P < 0.05$). Lactate dehydrogenase-X (LDHX) levels appeared to be significantly increased after 12 weeks continuous medication compared with Placebo group ($P = 0.032$). The metabolomics analysis of serum from OAT patients before and after Guilingji administration showed that the glucose-6-phosphate (G6P) concentration in patients' serum was significantly elevated after Guilingji treatment. Compared to the control, when Kidney-Yang deficiency model rats were treated with Guilingji or its key intermediate metabolite G6P, their sperm concentration and spermatozoic activity were improved similarly, and their structural damage of rat's testicular and epididymal tissues were recovered.

Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.
* Corresponding author. Department of Reproductive Medicine, Jinling Hospital, 305 Zhongshan East Road, Nanjing, 210002, China.
** Corresponding author.
*** Corresponding author.
**** Corresponding author.
E-mail address: yaobing@nju.edu.cn (B. Yao).
[1] The first five authors contributed equally to this manuscript.

https://doi.org/10.1016/j.jtcme.2024.01.001
Received 9 May 2023; Received in revised form 29 November 2023; Accepted 5 January 2024
Available online 5 January 2024

Conclusion: This study provided valuable clinical evidence for the utility of Guilingji as a treatment for OAT. These findings thus demonstrate that G6P is involved in the therapeutic mechanism of Guilingji in OAT treatment based on clinical and rat intervention studies.

Abbreviations

OAT	oligo-asthenoteratozoospermia
TCM	traditional Chinese medicine
HPG	hypothalamic–pituitary–gonadal
G6P	glucose-6-phosphate
TPMSC	the total progressive motile sperm count
DFI	sperm DNA fragmentation index
PR	progressive motility
BMI	body mass index
GnRH	gonadotropin-releasing hormone
INHB	inhibin B
T	testosterone
LH	luteinizing hormone
FSH	follicle-stimulating hormone
ROS	reactive oxygen species
LDHX	lactate dehydrogenase-X
γ-L-GT	γ-L-glutamyl transpeptidase
SOD	superoxide dismutase
CoA	acetyl-CoA
PDH	pyruvate dehydrogenase
LDH	lactate dehydrogenase
NADH	nicotinamide adenine dinucleotide plus hydrogen
CTL	control
MOD	model
GLJ	Guilingji

1. Introduction

Male infertility has rapidly emerged as a global public health issue in recent decades. Idiopathic oligo-asthenoteratozoospermia (OAT), characterized by abnormal sperm counts (such as oligozoospermia or cryptozoospermia), abnormal motility (such as asthenozoospermia or necrozoospermia) or teratozoospermia,[1,2] is one of the major causes of infertility in men.[3] Multiple etiological factors, including hormone dissonance, imbalanced metabolic exchange, and spermatogenic microenvironment dysfunctions, contribute to the pathogenesis of OAT. This condition is usually treated through empirical approaches, such as antioxidant treatment and hormonal treatment.[2–4] Notably, clinical evidence for the effectiveness of empirically selected drugs in OAT patients is limited.[5] In China, according to the dialectical theory of traditional Chinese medicine (TCM), Kidney-Yang deficiency is an important cause of male infertility, and it is common to treat OAT with TCM formulas.[6]

Guilingji is a well-known TCM formula that originated during the Ming dynasty and is composed of *Ginseng Radix et Rhizoma Rubra, Cornu Cervi Pantotrichum, Fructus Lycii, Caryophylli Flos, Radix Achyranthis Bidentatae, Herba Cynomorii, Herba Cistanches, Glycyrrhizae Radix, Fructus Amomi, Hippocampus kelloggi, Rhizoma Rehmanniae,* and *Manis pentadactyla* Linnaeus. This formula has been applied to treat Kidney-Yang deficiency for hundred years. However, it is unknown whether Guilingji is efficacious and reliable as a treatment for idiopathic OAT. Therefore, to provide strong evidence of this effect, we proposed a multicenter, randomized, controlled clinical trial.

As a TCM formula, Guilingji is characterized by multiple components, multiple targets, and a dynamic and integrated nature, and its pharmacological mechanism is difficult to explain.[7] Intriguingly, evidence that male diseases are due to metabolic factors promotes a consideration of the metabolic factor that underlies the therapeutic effect of Guilingji treatment on idiopathic OAT. Metabolomics is an important branch of "-omics" technologies and represents an approach to obtaining information from whole organism functional integrity.[8,9] Metabolomic testing of blood samples from clinical trials be needly used to identify the key metabolites involved after Guilingji is absorbed by the human body, which may explain the potential mechanism under Guilingji for treating OAT.

Although emerging mechanistic rat studies have shown that Guilingji can modulate the hypothalamic–pituitary–gonadal (HPG) axis and oxidant–antioxidant balance,[10–12] improve the cholinergic system,[13] regulate amino acid metabolism and adjust pyruvate metabolism,[14] the specific mechanisms have not been reported. At present, most studies on the therapeutic mechanism of Guilingji are based on correlation researches and network pharmacology studies, and some holistic mechanisms and deeper exploration are needed.

In this study, we conducted a randomized, double-blind, placebo-controlled, multicenter clinical trial to assess the clinical effect of oral Guilingji over the course of 12 weeks in patients with idiopathic OAT. After Guilingji administration, we screened its key serum metabolites by metabolomic testing and verified in Kidney-Yang deficiency rat models to provide some metabolic mechanistic explanation of its therapeutic effect.

2. Material and methods

Ethical approval

The Clinical Ethics Review Committee of Nanjing Jinling Hospital approved the study procedures (2019NZKY-005-03). The procedures were implemented in compliance with the International Ethical Guidelines for Research Involving Human Subjects as stated in the Declaration of Helsinki. Written informed consent was obtained from all participants. The occurrence of any adverse events during the treatment course was recorded and monitored by the Clinical Ethics Review Committee of Nanjing Jinling Hospital.

The trial was registered in the China Clinical Trial Registry (ID: ChiCTR2100054837; http://www.chictr.org.cn/showproj.aspx?proj=145405).

2.1. Participants

This multicenter, randomized, double-blind, placebo-controlled study was conducted between January 2020 and January 2022 at four sites (Jinling Hospital, Nanjing Jiangning Hospital, Xi'an Tangdu Hospital and The First Affiliated Hospital of Wenzhou Medical University) in China. All eligible patients had an established diagnosis of idiopathic OAT.[2] For inclusion, patients were required to have a diagnosis of idiopathic OAT according to the WHO Laboratory Manual for Human Semen Examination and Treatment (5th edition).[15] Furthermore, initial examination and re-examination of abnormal semen quality were required to fulfil at least one of the following conditions: sperm concentration <15 million/mL or total sperm count <39 million; proportion

of forward motile sperm <32 % or total sperm viability <40 %; sperm morphology (normal forms) < 4 %. Exclusion criteria included leucospermia (with orchitis, epididymitis, prostatitis, severe genital trauma, testicular torsion, urethritis, cryptorchidism, varicocele, inguinal or genital surgery or serious internal medicine diseases such as diabetes, tumor, hepatobiliary disease, severe renal insufficiency); chromosomal karyotype abnormalities; endocrine diseases; other clinical diseases; or a history of taking medication known to reduce fertility. Additionally, patients were excluded if they had a testicular volume <12 mL, a body mass index (BMI) < 18.5 or >32 kg/m^2, or exposure to an occupation or environment with reproductive toxicity or if they had received pharmacotherapy to improve semen quality in the past 2 weeks. According to TCM dialectics, it is difficult to select patients with complete Kidney-Yang deficiency, but we excluded patients with Kidney-Yin deficiency syndrome, which have dry mouth or throat, easy to oral ulcer or tongue slightly dry.

2.2. Trial design

2.2.1. Outcomes

The Total progressive motile sperm count (TPMSC) was considered the primary outcome in our study, the other sperm parameters, seminal plasma parameters and serum hormones were considered the secondary outcome.

2.2.2. Sample size

The primary objective of this trial was to demonstrate the optimal effectiveness of Guilingji compared to placebo treatment for idiopathic OAT based on the TPMSC. Based on our previous small sample size data of TPMSC before and after Guilingji treatment for four weeks approved by The Clinical Ethics Review Committee of Nanjing Jinling Hospital. With continuity correction, the study required a total of 240 participants (120 per group) to preserve 85 % power for a 5 % two-sided test while accounting for a 20 % dropout rate.

2.2.3. Randomization and blinding

The software package SAS 9.2 (SAS Institute Inc., Cary, NC, USA) was used to generate random numbers. All participants were randomly and evenly divided between the Placebo group (n = 120) and the Guilingji group (n = 120) using blocked randomization (block = 60, rand = 4). The random numbers were assigned to the participants by an independent statistician at the GCP Center of Jinling Hospital. Drugs were numerically labeled and sequenced according to the random numbers, and the patients were assigned to groups in accordance with the sequence. The clinicians and participants were blinded to the assignment until completion of the study. Allocation concealment was performed using sealed opaque envelopes. The database was locked after all data were entered, and group information was revealed when statistical analyses were conducted. Unblinding was allowed only in the case of a patient emergency.

2.2.4. Interventions

There were 240 eligible patients; they were randomly assigned in a 1:1 ratio to receive oral Guilingji capsules (0.6 g, twice daily) or placebo (0.6 g, twice daily) for 12 weeks. Guilingji capsules and placebo were provided by Shanxi Guangyuyuan Pharmaceutical Co. Ltd. (Batch No.: 3911908001; China), The placebo matched the experimental drug in appearance, texture, smell, and packaging. At baseline and throughout the study (week 4, week 8, and week 12), blood samples were taken to measure reproductive hormones; sperm samples were also collected at these timepoints.

2.3. Clinical and biochemical measurements

Semen samples were obtained in the hospital by masturbation after 3-7 days of sexual abstinence and placed at room temperature until liquefied. After liquefaction, the samples were analyzed to determine the sperm parameters according to the WHO guidelines using a computer-aided sperm analysis (CASA) system (WLJY-9000, Beijing Weili Co., Ltd., Beijing, China), and sperm morphology was evaluated using the Shorr staining method. Biochemical analysis of seminal plasma was measured according to routine clinical methods (kit from XINDI Biological Pharmaceutical Engineering Co., Ltd., Nanjing, China). The sperm DNA fragmentation index (DFI) was assessed by the sperm chromatin structure assay (SCSA, 20160051, CellPro Biotech Co., Ltd., Zhejiang, China) and performed in strict accordance with the manufacturer's instructions.

The serum levels were measured at the laboratory of Jinling Hospital. Testosterone (T, 33560, Beckman Coulter, USA), luteinizing hormone (LH, 33510, Beckman Coulter, USA), follicle-stimulating hormone (FSH, 33520, Beckman Coulter, USA) and inhibin B (INHB, C86024, YHLO, China) were determined by chemiluminescent immunoassay.

2.4. Nontargeted metabolomics

To extract metabolites from serum samples, 400 μL of cold extraction solvent methanol/acetonitrile/water (2:2:1, v/v/v) was added to 120 mg of sample and adequately vortexed. After being vortexed, the samples were incubated on ice for 20 min and then centrifuged at 14,000×*g* for 20 min at 4 °C. The supernatant was collected and dried in a vacuum centrifuge at 4 °C. For HPLC–MS analysis, the samples were redissolved in 100 μL acetonitrile/water (1:1, v/v) solvent and transferred to HPLC vials. Nontargeted metabolomics was performed with an ultrahigh-performance liquid chromatography system (UHPLC Agilent 1290; Agilent, USA) equipped with a quadrupole time-of-flight mass spectrometer (ESI/Triple TOF 5600; AB Sciex, Concord, Canada) at the facilities of Shanghai Applied Protein Technology Co., Ltd. (Shanghai, China).

2.5. Animals and experimental design

Adult (42-day-old) male rat of the SD strain (Jiangsu ALF Biotechnology Company) were randomly divided into different groups, housed 3-4 per cage, and maintained under controlled temperature (20-26 °C), humidity (40-70 %) and lighting (lights on at 08:00 a.m. and off 08:00 p.m.) conditions in a standard laboratory environment; they had free access to rodent feed and water. All animal protocols were approved by the Institutional Animal Care and Use Committee of Jinling Hospital. All experiments were carried out in accordance with the guidelines approved by Jinling Hospital. The hydrocortisone-induced Kidney-Yang deficiency rat model was established based on a previously described procedure.[11] Rats were intraperitoneal injection treated with hydrocortisone for the experimental groups (10 mg/kg body weight every day for 20 days; hydrocortisone, hydrocortisone + Guilingji and hydrocortisone + G6P groups) or with PBS for the control groups. After hydrocortisone treatment, rats were dosed intragastrically (i.g.) with Guilingji (150 mg/kg body weight), L-G6P (20 mg/kg body weight), M-G6P (40 mg/kg body weight), H-G6P (80 mg/kg body weight) or vehicle (PBS) every day for 28 days. The Guilingji treatment protocol was slightly modified from that of a previous animal study by Guilingji.[11,12]

2.6. Reagents

Hydrocortisone (20 ml:100 mg) was provided by Sinopharm Rongsheng Pharmaceutical Co., LTD. The supply of Guilingji used for this study (Shanxi Guangyuyuan Pharmaceutical Co. LTD. Z14020687) met the standard requirements as described in the Chinese Pharmacopoeia (Version 2015). Before the rat experiment, Guilingji was suspended in PBS to prepare 50 mg/mL (for doses of 150 mg/kg body weight) suspensions and homogenized with a magnetic stirrer at maximum speed for 30 s at room temperature. D-Glucose 6-phosphate disodium salt hydrate (G6P, V900924, Sigma, USA) was combined with PBS to a

concentration of 5 mg/mL (for doses of 20 mg/kg, 40 mg/kg and 80 mg/kg body weight) and dissolved with a magnetic stirrer at maximum speed for 10 s at room temperature.

2.7. Analysis of rat sperm motility and concentration

Sperm motility and counts were measured as reported previously.[16] Briefly, the left caudal epididymis of each rat was shredded and suspended in sperm incubation solution (0.5 mL HTF medium containing 0.5 % bovine serum albumin) at 37 °C for 5 min. Then, the sperm suspension was placed on a hemocytometer under a light microscope. Sperm motility was determined immediately using the standard method. The number of motile spermatozoa and the total number of spermatozoa were counted in a unit area and used to calculate the percentage of sperm motility. Sperm count was also determined using a hemocytometer, and the results were expressed in millions per milliliter of suspension.

2.8. Enzyme-linked immunosorbent assays

For the rat experiment, blood samples were collected in 1.5 mL Eppendorf tubes and left to clot for 1 h at 4 °C; the tubes were centrifuged at 500×*g*/4 °C for 10 min to harvest serum. Serum samples and conditioned cell culture media were stored at −80 °C for subsequent serum determinations. The levels of GnRH (LOT20230105A; mlbio; China), INHB (LOT20230117A; mlbio; China), total T (TT, LOT20230105A; mlbio; China), LH (LOT20230105A; mlbio; China), and FSH (LOT20230117A; mlbio; China) were determined using enzyme-linked immunosorbent assay kits for rat specimens.

2.9. G6P quantification assay

G6P was quantified using a glucose-6-phosphate kit (ab155892, Abcam, USA) according to the manufacturer's instructions. In brief, the sample preparation protocol for rat testis tissues was as follows. First, testis tissues were weighed and homogenized with 400 μL cold PBS by using a low-temperature tissue grinder (KZ-III-F, Servicebio, China). After the homogenate was centrifuged at 18,000×*g*/4 °C for 10 min, the supernatant was filtered through 10 kDa molecular weight cut off spin columns (FUF051, Beyotime, China) to remove interfering materials. The filtered samples were collected to measure the concentration of G6P. The serum and filtered samples were used for subsequent colorimetric detection by reading the optical density at a wavelength of 450 nm using a microplate reader (Power Wave 340, BioTek, USA).

2.10. Histopathological analysis

Testicular and epididymal tissues were quickly collected, fixed in 4 % paraformaldehyde (PFA), placed in 75 % ethanol, dehydrated and embedded in paraffin. Paraffin blocks were cut longitudinally into serial sections with a thickness of 3 μm (HM 325; Thermo, USA) and subjected to hematoxylin and eosin (H&E) staining.

The H&E-stained slices were scanned with an upright optical microscope (ECLIPSE 80i, Nikon, Japan). For immunofluorescence staining, the paraffin sections were dewaxed, dehydrated, and incubated overnight at 4 °C with primary antibodies. Then, the tissues were incubated with secondary antibodies for 1 h in the dark and mounted in antifade mounting medium with DAPI (H1200, Vector, Laboratories, USA) as a counterstain for nuclei. Immunofluorescence signals were visualized and recorded using a laser scanning confocal microscope (TCS SP8, Leica, Germany).

2.11. Statistical analysis

The full analysis set, consisting of all randomized and consenting patients, was included according to intention-to-treat principles for the efficacy analyses. We also performed prespecified per-protocol analyses for the primary outcome, sequentially excluding subgroups of patients who did not meet our eligibility criteria or did not adhere completely to their assigned medication regimens. Missing primary outcomes were imputed as the worst possible score. The Shapiro–Wilk test was used to assess the normality of continuous variables. Continuous variables are presented as the mean and standard deviation (if normally distributed) or the median and interquartile range (if nonnormally distributed). If a continuous variable did not conform to a normal distribution and had heterogeneity of variance, logarithmic transformation was performed. All *P* values are for two-sided tests, and results with *P* values of less than 0.05 were considered statistically significant. Continuous variables were compared using one-way analysis of variance when the assumptions of normality and homogeneity of variance were satisfied. Otherwise, the Kruskal–Wallis H test was applied. Analysis of covariance was used to compare the clinical data of the groups before and after week 4 of treatment. Two-way repeated-measures analysis of variance and subsequent multiple comparisons were performed among the parameters after 12 weeks of continuous treatment. If the Time × Group interaction had a *P* value $\geq$ 0.05, we observed only the main effects; otherwise, we analyzed the simple effects. SPSS software (version 25.0; IBM, Armonk, USA) was used for analysis. Statistical analyses and visualization of the experimental data were performed using GraphPad Prism 9.0. Detailed descriptions of the statistical tests are specified in the Results section and in the Figure Legends.

3. Results

3.1. Participant flow

Between January 2020 and January 2022, 240 patients with idiopathic OAT underwent randomization: 120 were assigned to receive placebo, and 120 were assigned to receive Guilingji. In total, 204 (85.0 %) finished the treatment, and 204 (85.0 %) completed the follow-up. The dropout rates at 4 weeks after the baseline visit were 15.83 % (19 of 120) in the placebo group and 14.17 % (17 of 120) in the Guilingji group. The reasons for dropout are listed in Fig. 1. The baseline characteristics of the patients were similar ($P > 0.05$, Table S1), although the subjects who received Guilingji (31.20 ± 4.48 years) were, on average, older than those who received placebo (30.84 ± 4.65 years). No Guilingji-associated adverse events among the participants were reported in this study.

3.2. Guilingji treatment improves TPMSC and other sperm parameters in patients with idiopathic OAT

The total progressive motile sperm count (TPMSC), as our primary outcome, is considered an indicator of high-quality sperm because sperm concentration and PR readily fluctuate over a wide range. At week 4, idiopathic OAT patients who received Guilingji had a more favorable set of semen parameter outcomes except semen volume than those who received placebo (Table 1). Sperm parameters, including TPMSC, concentration, PR and rate of normal morphology, were significantly elevated in the Guilingji group at weeks 8 and 12 compared with the placebo group ($P < 0.05$ for all comparisons) (Table 4). DFI is an index of DNA damage in sperm, with a higher DFI indicating poorer sperm quality. At week 12, the Guilingji group had a significant reduction in DFI compared to the placebo groups, which means that it achieved better sperm quality outcomes (Table 4).

There are multiple causes of idiopathic OAT, including endocrine, lifestyle, and immune factors.[3] In particular, the HPG axis is integral to the function of the reproductive system. To assess the therapeutic effect from the perspective of the HPG axis, reproductive hormone levels were measured every four weeks after intake of Guilingji. At week 4, the serum INHB levels of the Guilingji group were significantly elevated compared to those of the placebo group (106.89 ± 51.45 ng/L versus

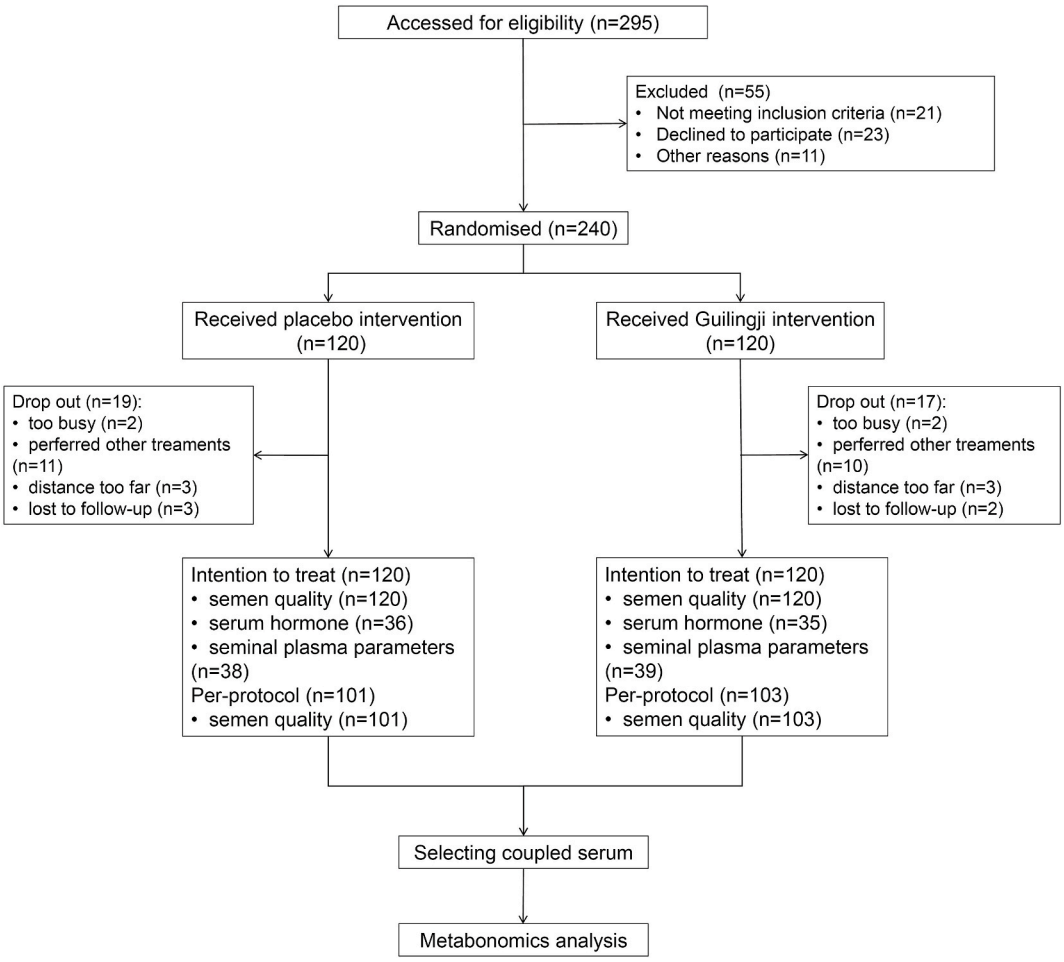

Fig. 1. Clinical trial flowchart of placebo and Guilingji treatment. Between January 2020 and January 2022, 240 patients with idiopathic OAT underwent randomization; 120 were assigned to receive placebo, and 120 were assigned to receive Guilingji. In total, 204 (85.0 %) finished the course of treatment, and 204 (85.0 %) completed the follow-up. The dropout rates as of 4 weeks after the baseline visit were 15.83 % (19 of 120) in the placebo group and 14.17 % (17 of 120) in the Guilingji group.

Table 1
Semen parameters of each group before and after 4 weeks treatment. All data are presented as the mean ± SD or the median and interquartile range, and the differences were tested by analysis of covariance (ANCOVA).

	Placebo treatment (n = 120)		Guilingji treatment (n = 120)		F	*P* value
	Before	After	Before	After		
Semen volume (mL)	4.25 ± 1.58	4.30 ± 1.49	4.20 ± 1.88	4.14 ± 1.61	0.686	0.408
Sperm concentration (× 10^6/ml)	42.14 ± 38.68	34.05 ± 30.78	41.99 ± 41.65	48.36 ± 36.12	26.407	< 0.001
Progressive motility (PR, %)	22.90 ± 15.55	23.83 ± 13.17	20.77 ± 13.70	26.37 ± 15.78	6.556	0.011
Total progressive motile sperm count (TPMSC, × 10^6)	19.58 (12.17–43.01)	20.71 (9.45–41.71)	17.25 (7.50–34.17)	30.52 (14.07–62.27)	19.905	< 0.001
Sperm morphology (normal forms, %)	3.82 ± 1.64	3.93 ± 1.64	4.16 ± 1.97	4.59 ± 1.97	6.339	0.012
Sperm DNA fragmentation index (DFI%)	28.89 ± 12.54	30.60 ± 13.70	32.30 ± 14.22	27.39 ± 11.95	14.824	< 0.001

Table 2
Serum hormone levels of each group before and after 4 weeks treatment. All data are presented as the mean ± SD, and the differences were tested by analysis of covariance (ANCOVA). Abbreviations: INHB, inhibin B; TT, total testosterone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

	Placebo treatment (n = 36)		Guilingji treatment (n = 35)		F	*P* value
	Before	After	Before	After		
INHB (ng/L)	108.73 ± 52.10	96.64 ± 45.93	91.21 ± 44.12	106.89 ± 51.45	10.112	0.002
TT (nmol/L)	13.89 ± 4.43	13.42 ± 4.30	14.21 ± 4.73	14.84 ± 4.19	2.173	0.145
LH (IU/L)	4.46 ± 2.17	3.93 ± 1.32	4.52 ± 1.84	3.94 ± 1.29	0.002	0.964
FSH (IU/L)	5.47 ± 2.66	5.43 ± 2.34	5.62 ± 2.94	5.30 ± 2.43	0.840	0.363

96.64 ± 45.93 ng/L, $P < 0.05$), but there was no significant difference at week 8 (98.68 ± 39.05 ng/L versus 96.39 ± 46.48 ng/L, $P > 0.05$) or week 12 (97.62 ± 45.88 ng/L versus 99.34 ± 50.02 ng/L, $P > 0.05$) (Table 2). These results might indicate that Guilingji slightly stimulated INHB production in the short term, after which the levels normalized. In addition, serum FSH levels showed a steady and continuous downward trend after Guilingji administration, while the average serum TT and LH levels showed no difference between the groups (Table 2 and Supplementary Table S2).

3.3. Guilingji treatment normalizes seminal plasma oxidation, acrosomal enzyme activity and lactate dehydrogenase-X levels in idiopathic OAT patients

Given that seminal plasma biochemistry indices are strongly correlated with sperm quality and are considered classic biomarkers for assessing male factor infertility, we sought to identify the changes in seminal plasma biochemistry biomarkers before and after Guilingji treatment. We found that oxidative stress and acrosomal enzyme activity were significantly improved after treatment with Guilingji. At week 4, superoxide dismutase (SOD) and acrosomal enzyme activity were significantly elevated in the Guilingji group compared with the placebo group, while reactive oxygen species (ROS) levels were significantly reduced (Table 3). In addition, as shown in Table 4, patients who received Guilingji for 12 weeks had higher levels of lactate dehydrogenase-X (LDHX) than those who received placebo.

3.4. Changes in serum metabolites after Guilingji treatment

To further explore the changes that occur in serum metabolites after Guilingji treatment, the serum samples collected from patients before and after Guilingji administration were analyzed by HPLC–MS, and the data were processed using SIMCA software.

With this approach, we identified 26 metabolites whose levels changed after Guilingji administration, of which five metabolites presented increased levels and 21 metabolites presented decreased levels (Fig. 2A). To gain insight into the therapeutic effects of Guilingji, all upregulated differential metabolites were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (Fig. 2B). The changes in differential metabolites in the two groups can be clearly observed in the form of a heatmap (Fig. 2C). These results indicated that Guilingji significantly restored five metabolites in serum. Six metabolic pathways were identified: inositol phosphate metabolism; protein digestion and absorption; glycine, serine, and threonine metabolism; aminoacyl-tRNA biosynthesis; ATP-binding cassette (ABC) transporters; and biosynthesis of amino acids. Analysis of serum metabolite content revealed an increasing trend in G6P after Guilingji treatment (Fig. 2C and D) by HPLS-MS and colorimetric detection. G6P is known to be a key metabolite involved in glucose metabolism, the pentose phosphate pathway, and the regulation of the mitochondrial respiratory chain[17–19]; these pathways are closely associated with spermatogenesis energy metabolism.[20–22]

3.5. Guilingji and its key metabolite G6P both bring the positive effect for OAT symptoms in the Kidney-Yang deficiency rat model

In this study, we designed experimental strategies to evaluate the therapeutic effect of Guilingji and its key intermediate metabolite G6P (Fig. 3A). The G6P-treated groups of rats were divided into three subgroups with different dose regimens. The Body weight of each group was measured (Fig. 3B and C). After hydrocortisone administration, the rats in the MOD showed emaciated, depilate, the signs of exhaustion such as languorous, sluggish, and crouched occurred in the MOD group as well. There was no significant difference in the weight of rats in each group in the beginning. During the disposal of hydrocortisone, rats in the model group showed a slower growth trend compared with that of the control group (Fig. 3B). On the day 27 and 55, the weight of rats in the model, GLJ, G6P groups was significantly lower than that in the CTL group ($P < 0.01$) (Fig. 3C). Guilingji and G6P (20, 40, 80 mg/kg/d) administration significantly ameliorated hydrocortisone-induced Kidney-Yang deficiency subfertility, as assessed by sperm concentration and sperm activity (Fig. 3D). To observe the effects of Guilingji and G6P on pathological changes in testicular and epididymal tissues, we performed histopathological analysis of H&E-stained tissue. In the MOD group, the basal layer of the testicular seminiferous tubules became thinner with the destruction of tissue, and the number of spermatozoa in the epididymis was reduced. In the MOD + Guilingji and MOD + G6P groups, the testicular seminiferous tubules were complete and well formed; there were well-developed spermatogenic cells and Sertoli cells close to the basement membrane in the seminiferous tubules, and the nuclei of the cells were clearly visible (Fig. 3E). Collectively, these results suggest that G6P treatment relieves signs of OAT in the Kidney-Yang deficiency rat model, as does Guilingji treatment. We examined the concentrations of testis G6P and found that they were significantly increased in the MOD + GLJ and MOD + G6P groups compared with the MOD group (Fig. 4A).

3.6. Guilingji and G6P treatments ameliorate disturbance of sexual hormone

Disturbance of sexual hormone is an important manifestation of Kidney-yang deficiency syndrome, which is the important cause of male infertility. We found significant serum hormone abnormalities in MOD group compared with CTL group ($P < 0.05$) (Fig. 4B–F), including T, INHB, FSH, LH and gonadotropin-releasing hormone (GnRH). After Guilingji and G6P treatments, serum T, INHB, FSH, LH and GnRH were improved, especially in MOD + GLJ group and MOD + H-G6P group ($P < 0.05$) (Fig. 4B–F). This finding indicates that Guilingji and G6P treatments could regulatory HPG axis and Sertoli cell function.

Table 3
Seminal plasma parameters of each group before and after 4 weeks treatment. All data are presented as the mean ± SD or the median and interquartile range, and the differences were tested by analysis of covariance (ANCOVA). Abbreviations: ROS, reactive oxygen species; LDHX, lactate dehydrogenase-X; γ-L-GT, γ-L-glutamyl transpeptidase; SOD, superoxide dismutase.

	Placebo treatment (n = 38)		Guilingji treatment (n = 39)		F	*P* value
	Before	After	Before	After		
ROS (IU/mL)	501.51 ± 183.00	490.03 ± 153.49	554.70 ± 175.40	423.16 ± 128.46	9.658	0.003
LDHX (mU/10^6 sperm)	3.55 (1.20–6.13)	3.37 (1.00–8.04)	3.62 (2.41–6.23)	4.53 (2.99–8.94)	2.522	0.117
Citric acid (mmol/L)	19.75 ± 16.20	21.13 ± 14.72	23.48 ± 17.05	20.35 ± 13.49	0.136	0.714
α-Glycosidase (U/L)	325.98 ± 136.36	322.36 ± 145.22	341.53 ± 135.90	318.23 ± 127.76	0.153	0.697
Zn (mmol/L)	2.95 ± 1.04	2.83 ± 1.12	2.58 ± 1.00	2.91 ± 1.26	1.770	0.187
Fructose (mmol/L)	18.14 ± 8.15	17.37 ± 7.21	16.29 ± 8.96	15.69 ± 8.51	0.146	0.704
γ-L-GT (U/L)	1661.49 ± 604.69	1699.58 ± 462.43	1853.40 ± 525.09	1725.77 ± 628.20	0.421	0.518
Acrosomal enzyme activity (IU/10^6 sperm)	41.95 ± 23.55	46.17 ± 24.02	38.09 ± 22.94	50.66 ± 22.56	0.841	0.035
SOD(U/L)	130.87 (56.75–181.35)	134.63 (42.81–177.20)	79.98 (41.00–170.00)	138.00 (54.00–250.00)	5.182	0.026

Table 4

Multiple comparisons of semen, serum and seminal plasma parameters from each group at weeks 4, 8, and 12. All data are presented as the mean ± SD or the median and interquartile range, and the differences were tested by two-way repeated-measures analysis of variance. If the Time × Group interaction had a *P* value ≥ 0.05, we observed only the main effect; otherwise, we analyzed the simple effects denoted by a, b, and c. a, $P < 0.05$ compared with the placebo group at the same treatment time; b, $P < 0.05$ compared with the 4-week treatment; c, $P < 0.05$ compared with the 8-week treatment. Abbreviations: INHB, inhibin B; FSH, follicle-stimulating hormone; ROS, reactive oxygen species; SOD, superoxide dismutase; LDHX, lactate dehydrogenase-X.

	Group	Untreated	Time			F	*P* value
			week 4	week 8	week 12		
Semen parameters: (Placebo: n = 120; Guilingji: n = 120)							
Sperm concentration (× 10^6/mL)	Placebo	42.14 ± 38.68	34.05 ± 30.78	36.70 ± 32.50	35.09 ± 30.98		
	Guilingji	41.99 ± 41.65	48.36 ± 36.12[a]	52.73 ± 37.50[a]	53.17 ± 37.60[a]		
	Main effect of Group					8.112	0.005
	Main effect of Time					2.393	0.099
	Time × Group interaction					14.916	< 0.001
Progressive motility (PR, %)	Placebo	22.90 ± 15.55	23.83 ± 13.17	24.13 ± 13.32	24.70 ± 12.99		
	Guilingji	20.77 ± 13.70	26.37 ± 15.78[a]	27.99 ± 15.73[a]	27.97 ± 15.15[a]		
	Main effect of Group					1.288	0.258
	Main effect of Time					17.591	< 0.001
	Time × Group interaction					7.665	0.001
Total progressive motile sperm count (TPMSC, × 10^6)	Placebo	19.58 (12.17–43.01)	20.71 (9.45–41.71)	20.44 (9.74–47.63)	20.14 (9.74–48.78)		
	Guilingji	17.25 (7.50–34.17)	30.52[a] (14.07–62.27)	32.52[a] (32.52–71.61)	35.30[a] (18.14–73.82)		
	Main effect of Group					7.078	0.008
	Main effect of Time					26.266	< 0.001
	Time × Group interaction					11.808	< 0.001
Sperm morphology (normal forms, %)	Placebo	3.82 ± 1.64	3.93 ± 1.64	3.93 ± 1.63	3.97 ± 1.51		
	Guilingji	4.16 ± 1.97	4.59 ± 1.97[a]	4.85 ± 2.06[a]	4.75 ± 1.97[a]		
	Main effect of Group					9.982	0.002
	Main effect of Time					11.442	< 0.001
	Time × Group interaction					5.312	0.006
Sperm DNA fragmentation index (DFI, %)	Placebo	28.89 ± 12.54	30.60 ± 13.70	29.61 ± 13.59	29.37 ± 13.54		
	Guilingji	32.30 ± 14.22	27.39 ± 11.95[a]	25.32 ± 10.45[a]	24.21 ± 11.03[ab]		
	Main effect of Group					1.859	0.174
	Main effect of Time					11.826	< 0.001
	Time × Group interaction					15.623	< 0.001
Serum hormone: (Placebo: n = 36; Guilingji: n = 35)							
INHB (ng/L)	Placebo	108.73 ± 52.10	96.64 ± 45.93	96.39 ± 46.48	99.34 ± 50.02		
	Guilingji	91.21 ± 44.12	106.89 ± 51.45[a]	98.68 ± 39.05	97.62 ± 45.88		
	Main effect of Group					0.047	0.828
	Main effect of Time					0.267	0.802
	Time × Group interaction					3.929	0.016
FSH (IU/L)	Placebo	5.47 ± 2.66	5.43 ± 2.34	5.50 ± 2.65	5.51 ± 2.33		
	Guilingji	5.62 ± 2.94	5.30 ± 2.43	4.87 ± 1.78[a]	4.62 ± 1.55[ab]		
	Main effect of Group					0.508	0.478
	Main effect of Time					3.297	0.037
	Time × Group interaction					4.161	0.016
Seminal plasma parameters: (Placebo: n = 38; Guilingji: n = 39)							
ROS (IU/mL)	Placebo	501.51 ± 183.00	490.03 ± 153.49	490.97 ± 148.22	493.16 ± 164.59		
	Guilingji	554.70 ± 175.40	423.16 ± 128.46[a]	420.07 ± 137.05[a]	414.17 ± 144.33[a]		
	Main effect of Group					2.027	0.159
	Main effect of Time					9.338	<0.001
	Time × Group interaction					6.959	0.002
Acrosomal enzyme activity (IU/10^6 sperm)	Placebo	41.95 ± 23.55	46.17 ± 24.02	38.40 ± 22.20	43.77 ± 21.24		
	Guilingji	38.09 ± 22.94	49.21 ± 21.12[a]	48.42 ± 22.75[a]	50.66 ± 22.56[a]		
	Main effect of Group					0.881	0.351
	Main effect of Time					4.810	0.006
	Time × Group interaction					3.330	0.031
SOD (U/L)	Placebo	130.87 (56.75–181.35)	134.63 (42.81–177.20)	113.38 (39.57–178.74)	116.68 (50.60–181.75)		
	Guilingji	79.98 (41.00–170.00)	138.00[a] (54.00–250.00)	143.22[a] (64.56–248.00)	153.46[a] (67.00–290.00)		
	Main effect of Group					1.180	0.281
	Main effect of Time					3.896	0.017
	Time × Group interaction					5.864	0.002
LDHX (mU/10^6 sperm)	Placebo	3.55 (1.20–6.13)	3.62 (2.41–6.23)	3.92 (1.00–5.92)	3.84 (1.99–6.13)		
	Guilingji	3.37 (1.00–8.04)	4.53 (2.99–8.94)	5.00 (3.52–8.14)	5.00 (3.42–7.72)		
	Main effect of Group					4.793	0.032
	Main effect of Time					2.402	0.087
	Time × Group interaction					1.145	0.325

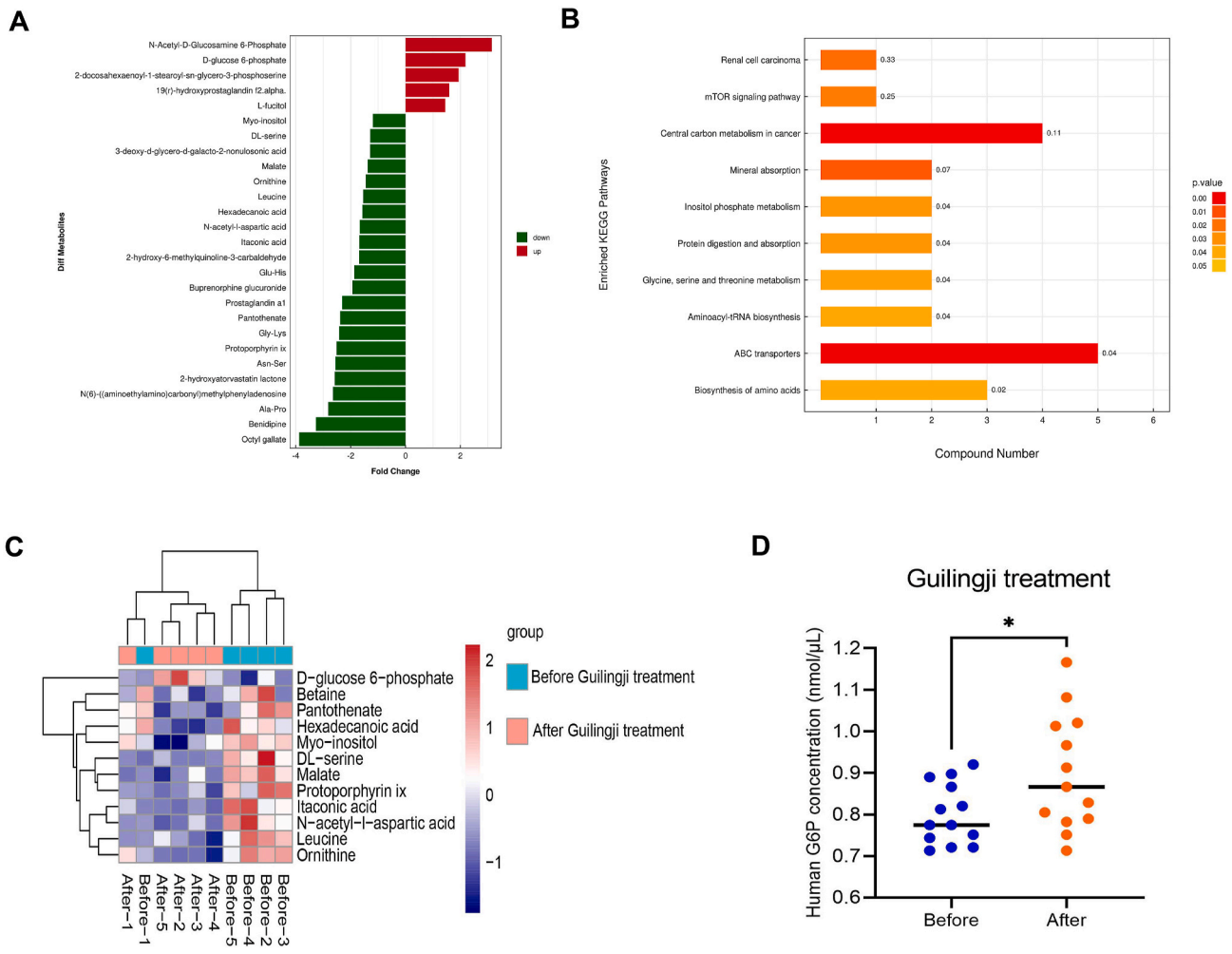

Fig. 2. Serum nontargeted metabolomics analysis of OAT patients before and after Guilingji treatment. (**A**) Differential metabolites from the serum of OAT patients before and after Guilingji treatment. Green represents downregulation; red represents upregulation. (**B**) Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of upregulated metabolites. Red represents positive correlation coefficients between metabolites, while blue represents negative correlation coefficients. The color scale represents the strength of the correlation as measured by Pearson's correlation coefficient. (**C**) Heatmap of significant changes in metabolite content in serum samples. The scale from −1 to 2 represents the relative content of the differential metabolites from low to high. (**D**) Serum G6P concentrations before and after Guilingji administration in patients with OAT (n = 13 per group). Data were analyzed by one-way analysis of variance (ANOVA). *, $P < 0.05$. Abbreviations: G6P, glucose-6-phosphate.

4. Discussion

Our preclinical findings were validated by a multicenter, randomized, double-blind trial, which involved a long-term observation period of 12 weeks. This study provided empirical clinical evidence of the effectiveness of Guilingji for OAT treatment. Then, we focused on the key serum metabolite G6P and demonstrated its protective effect on spermatogenesis. It is worth mentioning that G6P achieved the same salvage effect as Guilingji in the Kidney-Yang deficiency rat model. The mechanisms involved in this effect of G6P may be an important research direction to clarify the therapeutic effect of Guilingji on OAT.

In this trial, the primary outcome, TPMSC, was significantly improved after the first four weeks of Guilingji treatment and remained stable for the next eight weeks. The Guilingji and placebo groups also had significant differences with respect to most secondary endpoints, such as sperm DFI. The process of spermatogenesis is closely related to the HPG axis. An unexpected finding was that there was a transient increase in INHB after the start of Guilingji intake. Other hormones did not show significant changes except for FSH. INHB, secreted by Sertoli cells, is an important biomarker to assess spermatogenic function. FSH is a hormone that is secreted by the pituitary gland and stimulates Sertoli cells in the testes, thus promoting spermatogenesis. There is a significant negative correlation between serum INHB levels and FSH in adult males, and INHB has a negative feedback effect on FSH.[23,24] After taking Guilingji, OAT patients showed a minor decrease in serum FSH, indicating that testicular damage was alleviated and hormone homeostasis was achieved through the HPG axis. These results are consistent with rat models. Oxidative stress is one of the important pathogenetic factors in oligo-asthenozoospermia.[25] The SOD and ROS levels in the OAT patients' seminal plasma declined significantly after they began Guilingji treatment. This finding suggests that Guilingji may regulate the local oxidant–antioxidant balance of the testes, which is consistent with a mechanism study on the effect of ginseng from Guilingji and a multiple-chemical components of Guilingji study.[12,26]

Some studies have suggested that Guilingji is helpful in normalizing disordered amino acid levels in the testes of model rats, which might be due to enhancement of the energy supply.[10] LDHX is an enzyme that is necessary for sperm glucose metabolism and provides sufficient energy for sperm movement in the reproductive tract. This enzyme also plays an important role in meiosis and differentiation of pachytene spermatocytes into mature spermatozoa.[27] The LDHX content of seminal plasma increased after Guilingji administration, suggesting that Guilingji may act on spermatocytes by enhancing the availability of energy.

Despite valuable clinical evidence of the utility of Guilingji as a

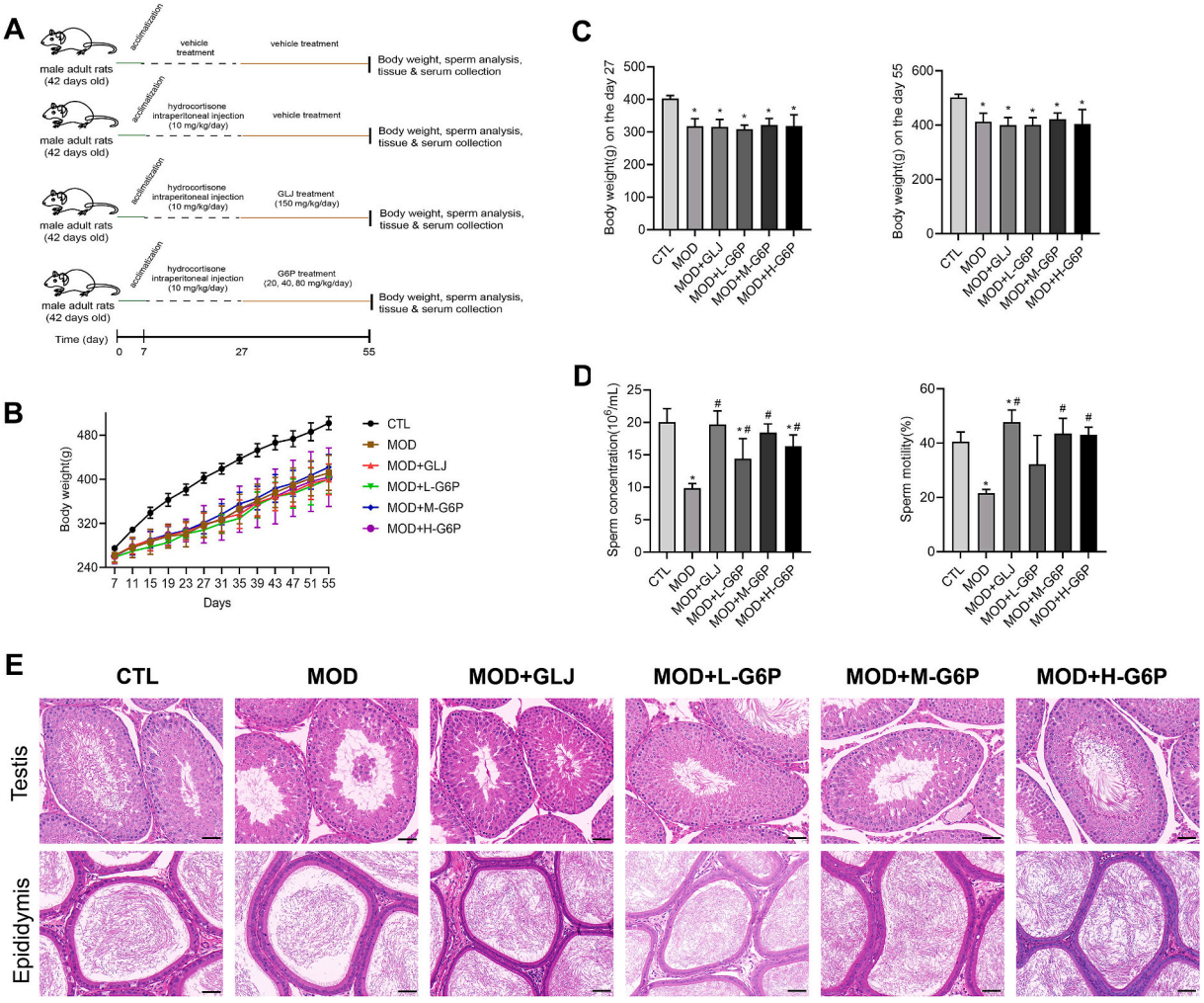

Fig. 3. Guilingji and G6P rescue sperm concentration and motility in the Kidney-Yang deficiency rat model. (A) Timeline for the rats subjected to Guilingji or G6P administration after intraperitoneal injection with hydrocortisone. (**B**) Weight change of each group (n = 5 per group). (**C**) Body weight of each group on the day 27 and 55. (**D**) Sperm concentration and motility in the CTL, MOD, GLJ and G6P with different concentration gradient groups (n = 5 per group). (**E**) Representative hematoxylin and eosin (H&E) staining of transverse sections from testicles and epididymal tissues of each group. Scale bar = 50 μm. Data were presented as the mean ± SD and analyzed by one-way analysis of variance (ANOVA). *, $P < 0.05$, compared with CTL group; #, $P < 0.01$, compared with MOD group. Abbreviations: CTL, control; MOD, model; GLJ, Guilingji; G6P, glucose-6-phosphate.

treatment for male infertility in this clinical study, the mechanism of the therapeutic effect of Guilingji is not well understood. Considering that Guilingji is characterized by multiple components, unraveling the detailed mechanism remains a unique challenge. In this study, we performed metabolomics analysis of serum samples before and after Guilingji treatment from clinical trials and tried to identify some common and key factors to explain the therapeutic effect of Guilingji treatment. Herein, we identified G6P as one of the most differentially abundant metabolites in serum after Guilingji intake, as confirmed in the rat model. The possible reason why serum and testis local G6P concentrations increased after Guilingji intake is that intestinal microbiota effect by regulating the activities of enzymes involved in G6P metabolism.[28,29]

We demonstrated that the structures of the testis and epididymis recovered from hydrocortisone-induced Kidney-Yang deficiency impairment after the animals received G6P to validate the effect of G6P. The increase of G6P in testis actively supports the glycolytic pathway of Sertoli cells, regulates lactate metabolism, and further supply the energy for spermatocyte meiosis. Spermatocyte meiosis, including the generation of programmed double strand breaks (DSBs) and homologous recombination, is an important process in spermatogenesis.[30] If this process is impeded or delayed, oligo-asthenozoospermia and an increased rate of DFI occur as a result.[31] Combined with the DFI indices from patients after Guilingji treatment, we can speculate that supplementation with Guilingji and G6P may improve spermatocyte function and influence the spermatocyte meiosis process. These potential mechanisms may be: 1) The increase of G6P can promote glycolysis and eventually produce pyruvate, which can be converted into acetyl-CoA (CoA) by pyruvate dehydrogenase (PDH) and enter the mitochondrial tricarboxylic acid cycle[32,33]; 2) It can also be converted from lactate dehydrogenase (LDH) to lactic acid, accompanied by oxidation of nicotinamide adenine dinucleotide plus hydrogen (NADH) to NAD+.[34,35] These potential mechanisms could function individually or cooperatively. As discussed above, these potential mechanisms involve a lot of enzymes, enzyme activities, and the feedback of metabolism pathways to keep a balance of oxidative stress and a balance of energy metabolism. This is the insufficiency of this study, and it is also the focus of our future research.

This study has some limitations: 1) The dataset regarding seminal plasma and hormone levels was relatively small. More seminal plasma and hormone tests are required to confirm these findings. 2) In this study, we focused only one metabolite of interest, G6P; we did not examine any other metabolites.

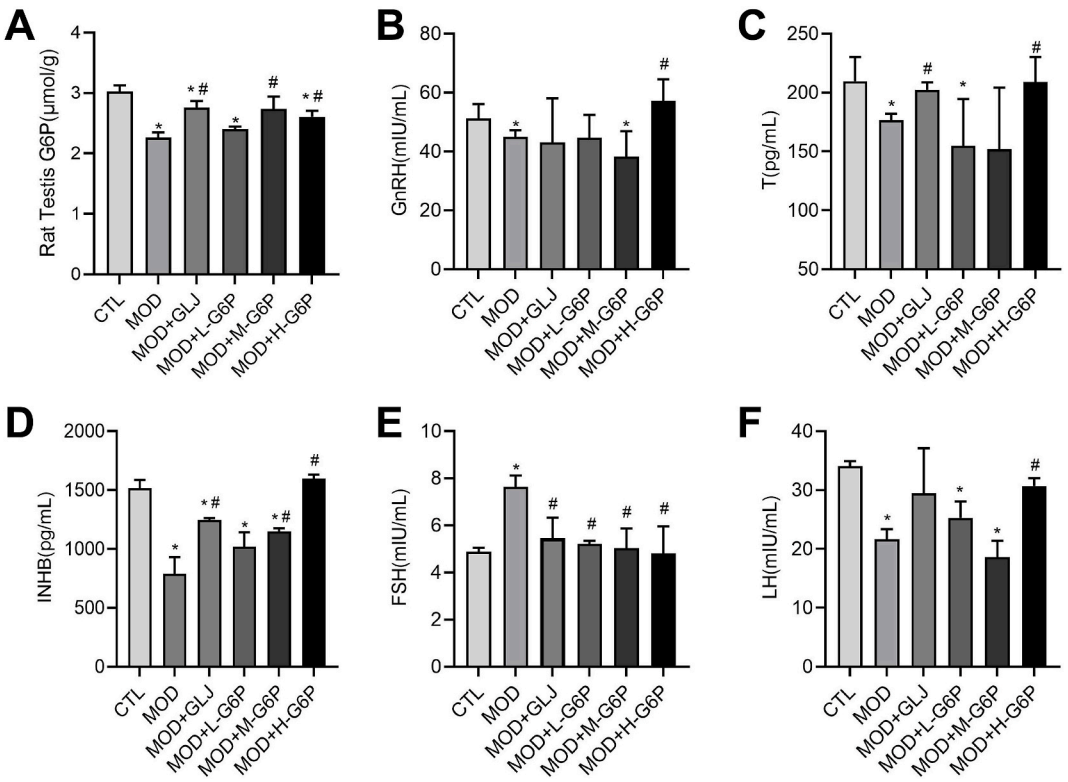

Fig. 4. Testis G6P and serum hormone levels in the Kidney-Yang deficiency rat model after administration of GLJ or G6P. (**A**) Content of G6P in the testis tissues of each group. (**B**) Serum GnRH level of each group. (**C**) Serum total T level of each group. (**D**) Serum INHB level of each group. (**E**) Serum FSH level of each group. (**F**) Serum LH level of each group. Data were presented as the mean ± SD and analyzed by one-way analysis of variance (ANOVA). *, $P < 0.05$, compared with CTL group; #, $P < 0.01$, compared with MOD group. Abbreviations: CTL, control; MOD, model; GLJ, Guilingji; G6P, glucose-6-phosphate; GnRH, gonadotropin-releasing hormone; INHB, inhibin B; T, testosterone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

5. Conclusions

In conclusion, this study is the first to investigate the role of the TCM formula Guilingji for OAT treatment using a randomized, double-blind, placebo-controlled, multicenter clinical trial from a metabolic perspective and provide valuable clinical evidence for the utility of Guilingji as a treatment for male infertility. These findings thus demonstrate that key intermediate metabolite G6P is involved in the therapeutic mechanism of Guilingji in OAT treatment based on clinical and rat intervention studies; this insight could provide new ideas for further clinical and mechanistic research on TCM.

Funding

This study was funded by the National Key Research and Development Program of China (grants 2018YFC1004700 and 2018YFC1003800), the National Natural Science Foundation of China (grants 81701431, 82001618, 81973965, and 81971373), the Jiangsu Provincial Medical Key Discipline Cultivation Unit (JSDW202215) and the 2022 Annual Foundation of Nanjing Jinling Hospital (22JCYYYB4).

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We sincerely thank Prof. Sun Haixiang and Prof. Wang Haibin for supporting this study. We thank Ms. Zhu Yanran, Gan Wenjun and Mr. Chen Weiqing for their assistance in collecting clinical specimens, and we also thank the other members of Dr Yao's laboratory for their discussion and insights. Finally, we sincerely thank the members of the clinical laboratory at the reproductive center of Jinling Hospital.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2024.01.001.

References

1. Klaudinyova V, Klaudiny J, Baumgartner J, et al. Isolation and partial characterization of Japanese quail oviduct mRNAs. *Folia Biol.* 1986;32:273–281.
2. Minhas S, Bettocchi C, Boeri L, et al. European association of urology guidelines on male sexual and reproductive health: 2021 update on male infertility. *Eur Urol.* 2021;80:603–620.
3. Colpi GM, Francavilla S, Haidl G, et al. European academy of andrology guideline management of oligo-astheno-teratozoospermia. *Andrology.* 2018;6:513–524.
4. Wang M, Wang Q, Du Y, et al. Vitamins combined with traditional Chinese medicine for male infertility: a systematic review and meta-analysis. *Andrology.* 2020;8:1038–1050.
5. Agarwal A, Baskaran S, Parekh N, et al. Male infertility. *Lancet.* 2021;397:319–333.
6. Zhang Z, Yang J, Kong T, et al. Traditional Chinese Medicine syndrome elements of male infertility revealed by latent tree model analysis. *J Tradit Chin Med.* 2018;38:926–935.
7. Zhang Y, Gao Z, Cai Y, et al. A novel strategy integrating gas phase fractionation with staggered mass range and LC-MS/MS molecular network for comprehensive metabolites profiling of Gui Ling Ji in rats. *J Pharm Biomed Anal.* 2023;222, 115092.
8. Liu F, Sun Z, Hu P, et al. Determining the protective effects of Yin-Chen-Hao Tang against acute liver injury induced by carbon tetrachloride using 16S rRNA gene sequencing and LC/MS-based metabolomics. *J Pharm Biomed Anal.* 2019;174:567–577.
9. Luo C, Bian X, Zhang Q, et al. Shengui sansheng san ameliorates cerebral energy deficiency via citrate cycle after ischemic stroke. *Front Pharmacol.* 2019;10:386.
10. Ding R, Ning S, Yang X, et al. Brain and testicular metabonomics revealed the protective effects of Guilingji on senile sexual dysfunction rats. *J Ethnopharmacol.* 2022;290, 115047.

11. Du K, Gao XX, Feng Y, et al. Integrated adrenal and testicular metabolomics revealed the protective effects of Guilingji on the Kidney-Yang deficiency syndrome rats. *J Ethnopharmacol*. 2020;255, 112734.
12. Wang Z, Xie Y, Chen H, et al. Guilingji protects against spermatogenesis dysfunction from oxidative stress via regulation of MAPK and apoptotic signaling pathways in Immp2l mutant mice. *Front Pharmacol*. 2021;12, 771161.
13. Zhao SJ, Liu XJ, Tian JS, et al. Effects of Guilingji on aging rats and its underlying mechanisms. *Rejuvenation Res*. 2020;23:138–149.
14. Zhao SJ, Tian JS, Tai G, et al. NMR-based metabolomics revealed the protective effects of Guilingji on the testicular dysfunction of aging rats. *J Ethnopharmacol*. 2019;238, 111839.
15. World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. fifth ed. Geneva, Swtizerland: World Health Organization; 2010.
16. Jing J, Ding N, Wang D, et al. Oxidized-LDL inhibits testosterone biosynthesis by affecting mitochondrial function and the p38 MAPK/COX-2 signaling pathway in Leydig cells. *Cell Death Dis*. 2020;11:626.
17. Villar-Palasí C, Guinovart J. The role of glucose 6-phosphate in the control of glycogen synthase. *Faseb J*. 1997;11:544–558.
18. Song J, Sun H, Zhang S, et al. The multiple roles of glucose-6-phosphate dehydrogenase in tumorigenesis and cancer chemoresistance. *Life*. 2022;12.
19. Jeon EH, Park TS, Jang Y, et al. Glucose-6-phosphate transporter mediates macrophage proliferation and functions by regulating glycolysis and mitochondrial respiration. *Biochem Biophys Res Commun*. 2020;524:89–95.
20. Song K, Yang X, An G, et al. Targeting APLN/APJ restores blood-testis barrier and improves spermatogenesis in murine and human diabetic models. *Nat Commun*. 2022;13:7335.
21. Deng C, Zhang J, Huo Y, et al. Melatonin alleviates the heat stress-induced impairment of Sertoli cells by reprogramming glucose metabolism. *J Pineal Res*. 2022;73, e12819.
22. Neto F, Bach P, Najari B, et al. Spermatogenesis in humans and its affecting factors. *Semin Cell Dev Biol*. 2016;59:10–26.
23. Andersson AM, Muller J, Skakkebaek NE. Different roles of prepubertal and postpubertal germ cells and Sertoli cells in the regulation of serum inhibin B levels. *J Clin Endocrinol Metab*. 1998;83:4451–4458.
24. Demyashkin GA. Inhibin B in seminiferous tubules of human testes in normal spermatogenesis and in idiopathic infertility. *Syst Biol Reprod Med*. 2019;65:20–28.
25. Bisht S, Faiq M, Tolahunase M, et al. Oxidative stress and male infertility. *Nat Rev Urol*. 2017;14:470–485.
26. Shi J, Gao X, Zhang A, et al. Characterization of multiple chemical components of GuiLingJi by UHPLC-MS and H NMR analysis. *J Pharm Anal*. 2022;12:460–469.
27. Casano R, Orlando C, Serio M, et al. LDH and LDH-X activity in sperm from normospermic and oligozoospermic men. *Int J Androl*. 1991;14:257–263.
28. Qin L, Yao W, Wang T, et al. Targeting gut microbiota-derived butyrate improves hepatic gluconeogenesis through the cAMP-PKA-GCN5 pathway in late pregnant sows. *Food Funct*. 2022;13:4360–4374.
29. Cabello-Olmo M, Oneca M, Pajares MJ, et al. Antidiabetic Effects of Pediococcus acidilactici pA1c on HFD-Induced Mice. *Nutrients*. 2022:14.
30. Griswold MD. Spermatogenesis: the commitment to meiosis. *Physiol Rev*. 2016;96:1–17.
31. Neale MJ, Pan J, Keeney S. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. *Nature*. 2005;436:1053–1057.
32. Luo D, Zhang M, Su X, et al. High fat diet impairs spermatogenesis by regulating glucose and lipid metabolism in Sertoli cells. *Life Sci*. 2020;257, 118028.
33. Sun S, Hu F, Wu J, et al. Cannabidiol attenuates OGD/R-induced damage by enhancing mitochondrial bioenergetics and modulating glucose metabolism via pentose-phosphate pathway in hippocampal neurons. *Redox Biol*. 2017;11:577–585.
34. Jeon E, Park T, Jang Y, et al. Glucose-6-phosphate transporter mediates macrophage proliferation and functions by regulating glycolysis and mitochondrial respiration. *Biochem Biophys Res Commun*. 2020;524:89–95.
35. Zhang X, Tao H, Li S, et al. Ldha-dependent metabolic programs in Sertoli cells regulate spermiogenesis in mouse testis. *Biology*. 2022;11.