Original Article

Metabolomic profiling and alleviation of oxidative stress of Huangjing Zanyu capsule treating oligoasthenospermia

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Background: The traditional Chinese medicine (TCM) patent medicine Huangjing Zanyu capsule (HJZY capsule) has achieved satisfactory clinical effects in the treatment of oligoasthenospermia (OAS). This study aimed to elucidate the impact of HJZY capsule on the reproductive system, focusing on oxidative stress and metabolism profiling during the intervention, to clarify the therapeutic mechanism of HJZY capsule in treating OAS.

Methods: Cyclophosphamide was used to establish OAS model rats. Time-sequence specimen collection was applied to monitor the dynamic development of the pharmacological effect of HJZY capsule. Superoxide dismutase (SOD), glutathione peroxidase (GPX), and malonaldehyde (MDA) were evaluated by biochemistry kits to examine the impact of HJZY capsule on oxidative stress. Non-targeted metabolomics was conducted for urine and testis samples, respectively, to investigate metabolic pathways through which the HJZY capsule takes effect.

Results: The HJZY capsule elevated sperm density from 62.1±8.28, passing 68.4±7.52, to 75.9±8.48× 10^6/mL, and sperm motility from 62.0%±3.94%, passing 70.8%±9.72%, to 68.8%±4.37%. Meanwhile, SOD ($P<0.05$ in week 2) and GPX activity levels of HJZY groups were elevated to a certain degree, respectively, and lipid oxidation was attenuated, as shown by decreased MDA content ($P<0.05$ in week 2). Metabolomics results showed that the HJZY capsule could modulate pathways including taurine metabolism, purine and pyrimidine metabolism, glycerolipid and glycerophospholipid metabolism, and multiple amino acid metabolisms, among others. The cluster analysis results showed that urinary and testicular metabolomics differed in the strength of discrimination between rats in the OAS model and the HJZY groups.

Conclusions: The HJZY capsule exerts a comprehensive effect on OAS through influencing various metabolic pathways. Non-targeted metabolomics provides an effective way for profiling complex TCM prescriptions.

Keywords: Oligoasthenospermia (OAS); Huangjing Zanyu capsule (HJZY capsule); oxidative stress; urine metabolomics; testis metabolomics

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Introduction

Male infertility has challenged the population worldwide. An epidemiological study conducted by Vander Borght *et al.* reported that 8–12% of married couples experience reproductive disorders, among which males contribute up to 50% of cases (1). As suggested by the World Health Organization (WHO), sperm density lower than 15×10^6/mL can be diagnosed as oligospermia, and progressive motility rate lower than 32% is considered as asthenospermia (2). Oligospermia and asthenospermia often go hand in hand. Although first study concerning oligospermia can be dated back to the 1950s, decades later, oligoasthenospermia (OAS) remains an unsolved problem for doctors and researchers. Huangjing Zanyu capsule (HJZY capsule) has shown a remarkable curative effect in the treatment of OAS (3). However, how to evaluate the efficacy of HJZY capsule in treating OAS at the mechanism level is a problem that is waiting to be sloved. Firstly, the mechanism is not fully understood; secondly, the efficacy evaluation criteria at the mechanism level are non-uniform. To address this problem, we used metabonomics to explore the mechanism of HJZY capsule in treating OAS. Concurrently, we detected the effect of drugs on the mechanism of antioxidant stress to provide supplementary evidence.

As a high-throughput technique, metabolomics, especially urine metabolomics, is used in the study of the pathogenesis and treatment mechanism of reproductive diseases for screening of toxic substances and identifying biomarkers with diagnostic significance for clinical practice (4-6). Accumulating evidence suggests that changes of metabolites could indicate the formation and course of OAS (7-9). For complex traditional Chinese medicine (TCM) prescriptions consisting of multiple herbal medicines, metabolomics methods can comprehensively characterize pharmacological effects in the treatment of OAS (10). In recent years, metabolomics has been widely used in the research of TCM (11-13). However, it is insufficient to illustrate drug mechanism solely by urine and plasma metabolomics, as it lacks the specificity for investigating specific reproductive organs, namely the testis. Researchers are beginning to apply metabolomics to tissues or body fluids with reproductive functions. Boguenet *et al.* (14) used metabolomics to analyze seminal plasma metabolites in oligoasthenospermia patients and explored the correlation between sperm quality and metabolic changes. Jarak *et al.* (15) used NMR-based metabolomic methods to elucidate the molecular basis of metabolic changes associated with testicular aging and fertility. However, some researchers found that the differential metabolites in testicular tissue are different from those in urine or serum (16). Therefore, there is still the necessity to examine the homogeneity of urine and testis samples. In our study, both urine and testis tissue were collected for metabolomics analysis to explore the mechanism of HJZY capsule in the treatment of OAS. Intervention time was also considered as we performed three consecutive specimen collections at 0, 2, and 4 weeks after modeling and intervention to obtain dynamic information of the medicine's treatment effect. Meanwhile, we comprehensively explored the mechanism of HJZY capsule in the treatment of OAS by combining sperm quality, testicular pathological information, and the oxidative stress indicators of sodium dismutase (SOD), glutathione peroxidase (GPX) and malonaldehyde (MDA).

The present study was designed to perform detailed metabolomics analysis of HJZY capsule in the treatment of OAS to reveal the medicinal efficacy at the mechanism level. We assessed the histological, molecular, and metabolic changes in OAS rats treated with HJZY capsule for 0, 2, and 4 weeks. The aim of this study was to provide a more objective evaluation method for the efficacy of TCM prescriptions. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-22-293/rc).

Methods

Drugs and reagents

Cyclophosphamide was obtained from Hengrui Corp. (Jiangsu, China). The HJZY capsules were obtained from Shanghai Hanjiang New Asia Pharmaceutical Corp. (Yangzhou, China). Sodium corboxymethyl cellulose (CMC-Na) was obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Levocarnitine was obtained from Merro Pharmaceutical Co., Ltd. (Dalian, China). Medium (M)199 culture medium was from HyClone (Beijing, China). Nembutal was from Sigma-Aldrich (St. Louis, MO, USA).

The HJZY capsules comprise 19 herbal and animal medicine products: *Polygonum multiflorum Thunb.* (Heshouwu), *Polygonatum sibiricum Redouté* (Huangjing), *Lycium barbarum L.* (Gouqizi), *Cuscuta australis R.Br.* (Tusizi), *Schisandra chinensis (Turcz.) Baill.* (Wuweizi), *Rehmannia glutinosa* (*Gaertn.*) *Libosch. ex Fisch. & C.A. Mey.* (Dihuang), *Cistanche deserticola Y.C.Ma* (Roucongrong), *Epimedium*

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brevicornu Maxim. (Yinyanghuo), *Dipsacus asper Wall. ex C.B. Clarke* (Xuduan), *Codonopsis pilosula* (*Franch*.) *Nannf*. (Dangshen), *Angelica sinensis* (*Oliv*.) *Diels* (Danggui), *Salvia miltiorrhiza Bunge* (Danshen), *Taraxacum mongolicum Hand.-Mazz*. (Pugongying), *Patrinia villosa Juss*. (Baijiangcao), *Cnidium monnieri* (*L*.) *Cusson* (Shechuangzi), *Plantago asiatica L*. (Cheqianzi), *Vespae Nidus* (Fengfang), *Hirudo nipponica Whitman* (Shuizhi), and *Ostrea gigas Thunberg* (Muli). These plant names were checked with http://www.theplantlist.org on 24 January, 2022. It is necessary to emphasize that *Vespae Nidus*, *Hirudo nipponica Whitman*, and *Ostrea gigas Thunberg* belong to the category of animal medicine products (wasp nest, blood-sucking leech, and oyster shell, respectively).

Modeling and grouping of animals

Animal experiments were performed under a project license (No. BUCM-4-2019112502-4067) granted by the Ethics Committee of Beijing University of Chinese Medicine, in compliance with Chinese national guidelines for the care and use of animals. A total of 90 male Sprague Dawley (SD) rats [specific-pathogen-free (SPF), 7-week-old, 200–220 g] were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China; Certification No. SCXK-Jing-2016-0006) and housed in Experimental Animal Center of Beijing University of Chinese Medicine (temperature, 18–22 ℃; humidity, 38–40%; 12/12 illumination cycle). The OAS rat model was induced by 35 mg/kg/d cyclophosphamide (i.p.) for 5 consecutive days. Then, animals were distributed randomly into 10 groups according to treatment methods and time durations, as shown in *Table 1*. For HJZY groups, 0.31 g/kg/d HJZY capsule suspension in CMC-Na was given by gavage, with 0.226 mg/kg/d L-carnitine as the positive control group. The intervention lasted 4 weeks in total. Animals were anesthetized by nembutal (3%; 0.2 mL/100 g) and sacrificed via blood extraction at 0 (model verification), 2, and 4 weeks (*Figure 1*).

It should be noted that our research group conducted preliminary exploratory research on the optimal dose of HJZY capsule in the early experiments and found that 0.31 g/kg/d was the optimum. The details are in Supplementary Files (Appendix 1).

Table 1 Grouping of animals (n=100)

Treatments	Time duration		
	0 week	2 weeks	4 weeks
Blank	10 (K0)	10 (K2)	10 (K4)
Model	10 (M0)	10 (M2)	10 (M4)
HJZY	–	10 (H2)	10 (H4)
L-carnitine	–	10 (T2)	10 (M4)

HJZY, Huangjing Zanyu.

Evaluation of sperm quality

Rats in each group were sacrificed via blood extraction from

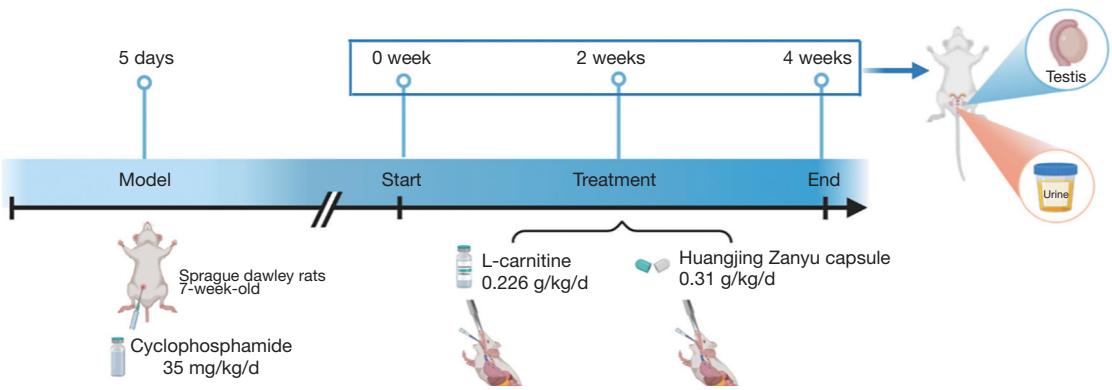

Figure 1 Schematic diagram of animal study design. Adult SD rats were intraperitoneally injected with 35 mg/kg/d cyclophosphamide for 5 consecutive days. After successfully establishing the OAS rat model, the HJZY group was given 0.31 g/kg/d HJZY capsule suspension in CMC-Na by gavage, and the positive control group was given 0.226 mg/kg/d L-carnitine in the same way. The samples of testis tissue and urine were collected after sacrifice at 0, 2, and 4 weeks, respectively. SD, Sprague Dawley; HJZY, Huangjing Zanyu; CMC-Na, sodium carboxymethyl cellulose; OAS, oligoasthenospermia.

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the abdominal aorta, and separation of testis and epididymis was performed. Both ends of the epididymis were cut open, allowing sperm diffusion in 37 ℃ 1 mL M199 culture medium. Then, 10 μL of the mixture was smeared on a counting board. The sperm quality examination system (test temperature 37 ℃, WL-9000, Beijing Weili Co., Ltd., Beijing, China) was used for sperm quality analysis. A total of 5 views were randomly selected within 2 minutes. Sperm density (10^6/mL), progressive (PR) and non-progressive (NP) sperm rates (%), and sperm motility (%) were recorded.

Hematoxlin and eosin staining for histopathological examination

Testicular tissue sections were cut into 3 μm thick sections, dewaxed, and hydrated. Then, the sections were washed with running water for 5 minutes and soaked in deionized water for 2 minutes. After hematoxylin and eosin (HE) staining, testicular tissue was rapidly immersed in 95% ethanol and anhydrous ethanol for dehydration, then hyalinized with xylene. Finally, the resin was used to seal the film. The histopathological observation was conducted with an optical microscope (Primo Star, Carl Zeiss).

SOD, MDA, GPX examination

Testicular tissues from 5 animals in each group were extracted according to the methods of the SOD, MDA, and GPX kits (Solarbio Corp., Beijing, China) to prepare test samples and the absorbance data of the samples at the corresponding wavelengths were collected (SOD: 560 nm; GPX: 412 nm; MDA: 450 nm, 532 nm, 660 nm). The bioactivities of SOD, GPX, and MDA content were examined according to the formulae given in the instructions.

Urine and testicular tissue metabolomics analysis

Urine and testicular tissue metabolome profiling was performed using an ultra-high performance liquid chromatography (UHPLC) system (Vanquish; Thermo Fisher Scientific, Waltham, MA, USA) along with Q Exactive HFX mass spectrometer (Orbitrap MS; Thermo, USA). First, 50 μL urine sample was transferred into 200 μL of extract solution (acetonitrile: methanol =1:1) containing isotopically labelled internal standard mixture. Following 30 s vortexing, samples were sonicated for 10 min in ice-water baths. After incubation for 1 hour at −40 ℃, the samples were centrifuged at 12,000 rpm for 15 minutes at 4 ℃. Similar to urine samples, testicular samples are handled the same way. We weighted 50 mg of testis sample into 1,000 μL extract solution (acetonitrile: methanol: water =2:2:1, with isotopically labeled internal standard mixture). After a 30 s vortex, the samples were homogenised (35 Hz, 4 min) and sonicated (5 min) in ice-water bath 3 times. The incubation and centrifugation conditions were consistent with urine samples. Then, the resulting supernatant was transferred for analysis. We mixed an equal aliquot of the supernatants from all the samples for urine and testis to make the quality control (QC) sample.

The mobile phase consisted of acetonitrile (A) and water containing ammonium acetate and ammonia hydroxide (pH =9.75) (B). The conditions of electrospray ionization (ESI) were set as follows: 25 Arb of sheath gas flow rate, 20 ARB of Aux gas flow rate, 350 ℃ of capillary temperature, 60,000 of full MS resolution, 7,500 of MS/MS resolution, 10/30/60 of collision energy in normalized collisional energy mode, and spray voltage as 3.6 kV (ESI+) but −3.2 kV (ESI−).

Statistical analysis

Normality and homogeneity of all the data were tested. Shaprio-Wilk test was used to check the normality; Hartley's test was used for homogeneity test of the data. Data of sperm quality, oxidative stress indexes, and differential metabolites between groups was analyzed and visualized with GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) was performed if the data were normally distributed and the Wilcoxon test was performed if data were abnormally distributed. A P value <0.05 was considered statistically significant.

To maximize identification of differences in metabolic profiles between groups, principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were performed to determine the natural inter-relationship among the groups and further develop their potential differences. Student's *t*-test was used to compare the different levels of metabolites between groups. The variable importance in the projection (VIP) of the first principal component of the OPLS-DA model was used to screen the different metabolites. Variables with P<0.05 and VIP >1 were included in further analysis. Differential metabolites were screened between the following pairs: K0-M0, K2-M2, K4-M4, H2-M2, H4-

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M4, T2-M2, and T4-M4. Then, pathway enrichment and topological analysis of differential metabolites were performed based on Kyoto Encyclopedia of Genes and Genomes (KEGG) and PubChem databases with species restricted to *Rattus norvegicus* (RAT). Statistical and network topology analysis was performed on the obtained pathway and its metabolites. The P value and impact value of the importance to the metabolic network of each metabolite were calculated. For result visualization, a volcano plot, tree plot, and heatmap of metabolite clusters were drawn.

The unit of analysis for each dataset was a single animal. There were 10 rats in each group. There were no adverse events caused during the study. The baseline data are presented in *Table 2*.

Table 2 Baseline data of animals (n=100)

Group	Number of animals alive		
	0 week	2 weeks	4 weeks
K0	(10/10)	–	–
M0	(10/10)	–	–
K2	(10/10)	(10/10)	–
M2	(10/10)	(10/10)	–
T2	(10/10)	(10/10)	–
H2	(10/10)	(10/10)	–
K4	(10/10)	(10/10)	(10/10)
M4	(10/10)	(10/10)	(10/10)
T4	(10/10)	(10/10)	(10/10)
H4	(10/10)	(10/10)	(10/10)

Results

Efficacy of HJZY capsule on sperm quality and testicular tissue

The sperm quality and testicular tissue of 7 rats were tested from each group (7/10). In the 0 week groups, fragments of epididymal tissue were found in 5 samples during semen detection testing (3 samples from the K0 group and 2 samples from the M0 group), which were detected as sperm by the machine. This situation could have caused errors in the test results, so we decided to eliminate these abnormal data and normalize the test sample size of each group.

After the intervention, the HJZY capsule could improve sperm quality in multiple ways. In terms of sperm density, the HJZY capsule showed no significant difference from the model group in the second week, yet improvement was observed. In the fourth week, sperm density was elevated (P<0.0001). The improvement in total sperm motility was similar to that in sperm density. At the end of 4 weeks, the sperm samples of rats in the HJZY group were significantly improved compared with the model group (P<0.0001) and were close to the level of the blank group in the same week. As for the progressive motility (PR) and non-progressive motility (NP) sperm rate, it was found that the PR sperm rate in the H2 and H4 groups could be maintained at a relatively stable level, and there was no statistical difference between the H4 and the blank group at 4 weeks (*Table 3*,

Table 3 Grades a, b sperm and sperm density (mean ± SD, n=7)

Groups	Sperm density (×10^6/mL)	PR sperm rate (%)	NP sperm rate (%)	Sperm motility rate (%)
K0	105.0±17.10	69.0±6.73	12.7±8.41	81.8±6.43
K2	104.0±7.55	70.0±5.87	11.7±5.66	81.7±8.23
K4	102.0±7.08	68.3±5.22	10.2±3.66	78.5±3.18
M0	62.1±8.28$^{\#}$	52.8±3.43$^{\#}$	9.11±2.28	62.0±3.94$^{\#}$
M2	59.4±11.20$^{\#}$	54.6±4.43$^{\#}$	8.98±4.67	63.5±8.08$^{\#}$
M4	52.1±5.69$^{\#}$	48.5±2.20$^{\#}$	2.87±1.93$^{\#}$	51.4±3.32$^{\#}$
T2	72.2±9.76$^{\#,*}$	63.2±4.45$^{\#,*}$	11.6±7.18	74.8±10.1*
T4	76.7±8.00$^{\#,*}$	58.3±4.47$^{\#,*}$	11.8±5.56*	70.1±7.34*
H2	68.4±7.52$^{\#}$	61.1±5.53$^{\#,*}$	9.76±5.25	70.8±9.72$^{\#}$
H4	75.9±8.48$^{\#,*}$	62.2±5.09*	6.65±2.23	68.8±4.37$^{\#,*}$

*, means P<0.05 *vs.* M group; $^{\#}$, means P<0.05 *vs.* K group from the same week. PR, progressive; NP, non-progressive.

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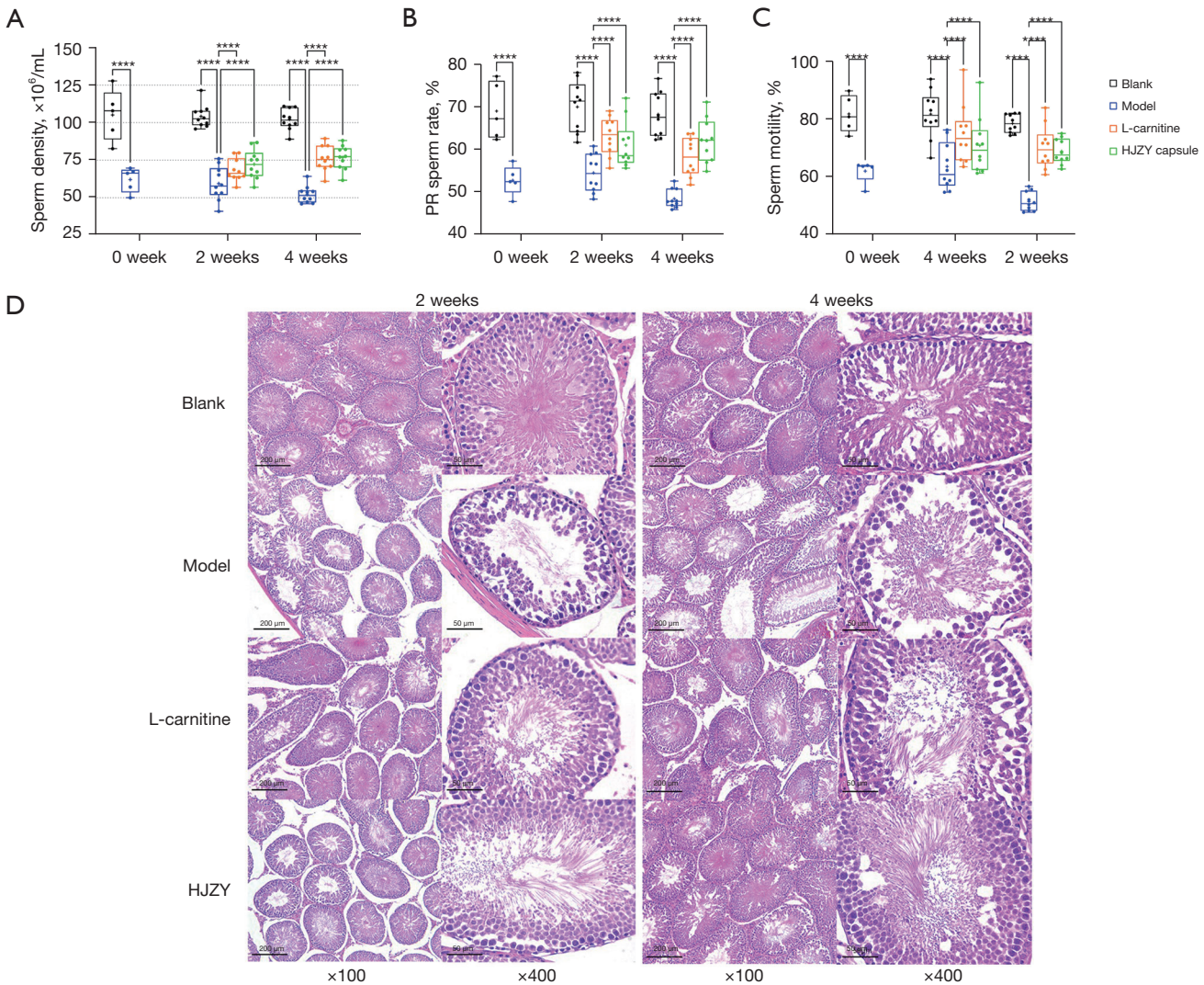

Figure 2 Sperm quality and oxidative stress markers measurements. (A-C) demonstrate sperm density, PR sperm rate, and total motility rate, respectively. (D): representative pathological sections of rat testes in different groups were taken under ×100 and ×400. Staining methods: hematoxylin-eosin staining (H&E). ****, represent P<0.0001. HJZY, Huangjing Zanyu; PR, progressive motility.

Figure 2A-2C).

Meanwhile, pathological analysis of testicular tissues showed that HJZY capsule significantly improved the testicular lesions of model animals (*Figure 2D*). After a 2-week intervention, the HJZY capsule demonstrated curing efficacy by restoring Sertoli cell arrangement and spermatogenesis to a certain degree compared to the model group. Consequently, in the fourth week, rat testes in the HJZY group recovered significantly from the symptoms compared to the model group in cell alignment and sperm density in the middle of seminiferous tubules. Combined with the improvement of sperm quality mentioned above, the HJZY capsule can be considered to positively affect the recovery of injured testicular tissue, alleviating the symptoms of OAS and ensuring the production of more high-quality sperm.

Oxidative stress alleviation

There were 5 rats in each group (5/10). The SOD, GPX activity, and MDA content in the testes of each group are shown in *Table 4*. Compared with the model group in the same week, the L-carnitine group at 2 weeks and 4 weeks and the HJZY capsule at 2 weeks was shown to increase the

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Table 4 SOD, GPX activity, and MDA content of each group (mean ± SD, n=5)

Time	Groups	SOD (U/g)	GPX (U/mg)	MDA (nmol/g)
0 week	Blank	262.775±37.414	3.746±0.893	79.490±17.560
	Model	255.156±17.755	3.322±1.005	118.345±29.365
2 weeks	Blank	274.692±41.080	4.515±1.364	104.542±15.010
	Model	216.125±25.311	2.904±1.184	138.185±17.821#
	L-carnitine	309.945±94.868*	4.343±2.449	116.332±18.196
	HJZY	297.257±34.374*	3.857±0.848	109.805±24.688*
4 weeks	Blank	278.731±41.698	3.660±1.070	61.739±11.239
	Model	227.598±43.037	2.737±1.813	107.973±13.718#
	L-carnitine	306.983±52.867*	3.906±1.183	93.835±28.002#
	HJZY	276.467±51.833	4.423±1.713	84.830±25.252

*, means P<0.05 *vs.* M group; #, means P<0.05 *vs.* K group from the same week. SOD, sodium dismutase; GPX, glutathione peroxidase; MDA, malonaldehyde; SD, standard deviation; HJZY, Huangjing Zanyu.

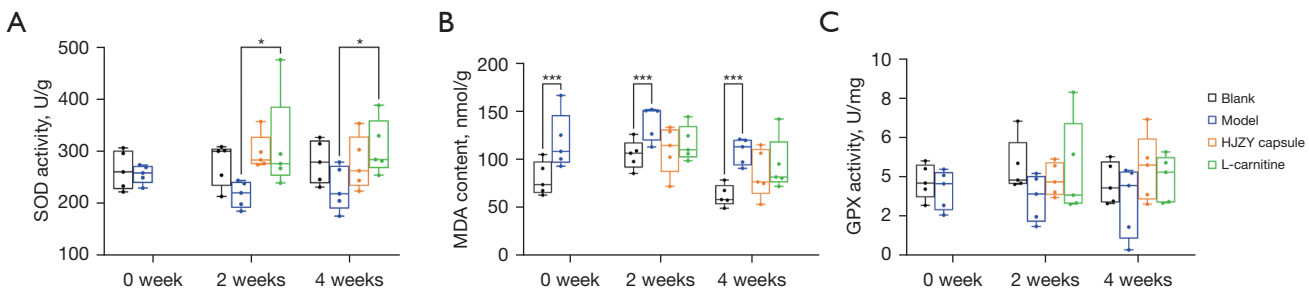

Figure 3 (A) and (C) show SOD and GPX activity while (B) shows MDA content in the testis. * and ***, represent P<0.05, 0.001, respectively. SOD, sodium dismutase; MDA, malonaldehyde; GPX, glutathione peroxidase; HJZY, Huangjing Zanyu.

SOD activity in OAS model rats' testes (P<0.05). There was no statistical difference in GPX activity in each week, but the rats in the HJZY capsule group had the highest testicular GPX activity at 4 weeks. In terms of MDA content, the HJZY capsule was shown to significantly reduce the MDA level in the testis of model rats at 2 weeks (P<0.05), and it also displayed a more obvious reduction effect at 4 weeks (*Figure 3A-3C*).

Basic information of differentially expressed metabolites

There were 8 rats in the 0-week group (8/10), 10 rats in the 2-week group and 4-week groups (10/10). 400/161 metabolites in testis samples and 835/325 metabolites in urine samples were detected in total under ESI+/ESI– mode, respectively. Focusing on H2-M2 and H4-M4 comparisons, 58/15 (ESI+/ESI–), 81/7, 137/53, and 287/121 differential metabolites were obtained (*Figure 4*), and 71, 89, 184, and 392 metabolites remained valid. OPLS-DA analysis showed that all comparing pairs were separated significantly.

Pathway analysis of urine and testis metabolomics

According to the KEGG pathway database, differential metabolites in each group were enriched into metabolic pathways. Pathways with impact values greater than 0.1 in each group were obtained and arranged in descending order according to impact value (*Tables 5,6*, only pathways with impact >0.1 are displayed). In common pathways of the 2 metabolomics, shared metabolites (underlined ones) were taurine and hypo-taurine from taurine and hypo-taurine pathway; urocanic acid from histidine metabolism pathway; L-isoleucine and L-valine from valine, leucine,

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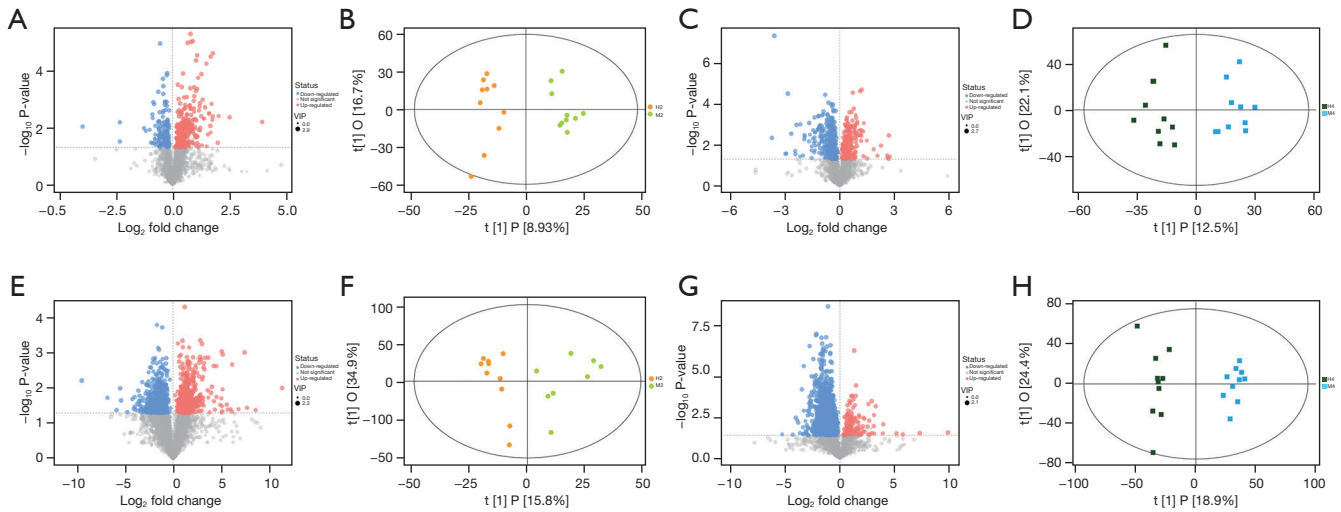

Figure 4 Volcano plots and OPLS-DA score plots of urine and testis differential metabolites (positive mode results as an example). (A) and (B): testis sample of H2 *vs.* M2; (C) and (D): testis sample of H4 *vs.* T4; (E) and (F): urine sample of H2 *vs.* M2; (G) and (H): urine sample of H4 *vs.* T4. In volcano plots, red dots represent upregulated metabolites, blue dots for downregulated ones. The X-axis indicates log2 fold change, and the Y-axis measures -log10 P value. OPLS-DA, orthogonal partial least-squares discriminant analysis.

Table 5 Pathways enriched in testis metabolomics

Pathway	Hits/total	Impact	Compounds detected
Taurine and hypo-taurine metabolism	1/8	0.29	Hypo-taurine
Histidine metabolism	1/15	0.15	Urocanic acid
Phenylalanine, tyrosine, and tryptophan biosynthesis	1/4	0.50	L-Phenylalanine
Phenylalanine metabolism	1/9	0.41	L-Phenylalanine
Valine, leucine, and isoleucine biosynthesis	1/11	0.33	L-Isoleucine

The shared metabolites from two metabolomics' common pathways in testis metabolomics was underlined.

and isoleucine biosynthesis pathway; and L-phenylalanine from phenylalanine metabolism pathway. The tree plots in *Figure 5* show pathways enriched in different comparisons.

Although the number of pathways screened in testis metabolomics was relatively small, these results are more responsible for what happened in the target organ of the HJZY capsule. Apart from amino acids metabolism and energy metabolism, lipid metabolism and purine/pyrimidine metabolism emerged as 2 essential categories which play an essential role in the reproduction system. As for pathways screened in urine samples, multiple amino acids metabolism pathways were dominant pathways, together with taurine, pyruvate metabolism pathways, and some vitamin metabolism pathways, which is in accordance with the characteristic of urine metabolite mostly being biomolecular degradation.

The results above suggest that the HJZY capsule can modulate various metabolic pathways and has an accumulative effect with the advancement of the treatment, as it demonstrated a more comprehensive influence in the 4th week than in the 2nd week.

Correlation between metabolites and sperm quality

The correlation between metabolites and sperm quality is shown in *Figure 6*. Hypo-taurine, L-isoleucine, and phenylalanine were negatively correlated with sperm density, yet the relationship between the 3 and sperm motility was not evident in this study. Valine was positively correlated with sperm density and negatively correlated

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Table 6 Pathways enriched in urine metabolomics

Pathway	Hits/total	Impact	Compounds detected
D-Glutamine and D-glutamate metabolism	2/5	1.00	L-Glutamic acid; L-Glutamine
Taurine and hypo-taurine metabolism	1/8	0.43	Taurine
Alanine, aspartate, and glutamate metabolism	2/24	0.41	L-Glutamic acid; L-Glutamine
Phenylalanine metabolism	1/9	0.24	Phenylpyruvic acid
Arginine and proline metabolism	5/44	0.20	L-Glutamine; L-Glutamic acid; L-Proline; Guanidoacetic acid; 4-Aminobutyraldehyde
Tryptophan metabolism	2/41	0.11	N-Acetylserotonin; L-3-Hydroxykynurenine
Taurine and hypo-taurine metabolism	2/8	0.71	Taurine; Hypo-taurine
Vitamin B6 metabolism	1/9	0.49	Pyridoxal
Thiamine metabolism	1/7	0.40	Thiamine
Ascorbate and aldarate metabolism	2/9	0.40	D-Glucuronic acid; Ascorbic acid
Valine, leucine, and isoleucine biosynthesis	2/11	0.33	L-Valine
Riboflavin metabolism	1/11	0.33	Flavin Mononucleotide; Riboflavin
Nicotinate and nicotinamide metabolism	2/13	0.24	Niacinamide; Nicotinic acid
Biotin metabolism	1/5	0.17	Biotin
Cysteine and methionine metabolism	3/28	0.16	S-Adenosylmethionine; L-Methionine; 2-Aminoacrylic acid
Pyruvate metabolism	1/22	0.16	S-Acetyldihydrolipoamide-E
Glyoxylate and dicarboxylate metabolism	2/16	0.15	Cis-aconitic acid; Isocitric acid
Histidine metabolism	1/15	0.15	Urocanic acid
Pyrimidine metabolism	7/41	0.14	Uridine; Dihydrouracil; Deoxycytidine; dTDP; Orotic acid; Uracil; Deoxyribose 1-phosphate
beta-Alanine metabolism	3/19	0.13	Dihydrouracil; Anserine
Phenylalanine metabolism	1/9	0.13	Phenylacetaldehyde
Histidine metabolism	1/15	0.11	Hydroxypropionic acid; Dihydrouracil; Uracil
Citrate cycle (TCA cycle)	3/20	0.11	1-Methylhistamine

The shared metabolites from two metabolomics' common pathways in urine metabolomics was underlined.

with sperm motility, while urocanic acid was positively correlated with sperm motility.

In terms of the consistency of the urine and testicular metabolites results, as shown in the correlation heat map (*Figure 6E*), although the relative content of valine in urine was related to those of L-isoleucine and phenylalanine in the testis at the 4th week, the relative contents of the same substances in the two metabolomics were not strongly correlated. The phenomenon suggested that the urine metabolome results may not accurately reflect the metabolic status in the testis. In addition, there was a strong correlation between a few metabolites that do not belong to the same pathway, such as hypo-taurine with L-isoleucine and L-valine, urocanic acid with taurine, taurine with L-phenylalanine, and so on.

Cluster analysis was performed between model and HJZY groups to determine the interpretation strength of urine and testis metabolomics results on OAS pathology, respectively. Sperm quality alone could perfectly discriminate the 2 groups in either 2 or 4 weeks (*Figure 7A*). However, when combined with urine metabolites or testis metabolites levels, the latter demonstrated more reliable discrimination than the former using either Pearson correlation or Euclidean distance as classification method

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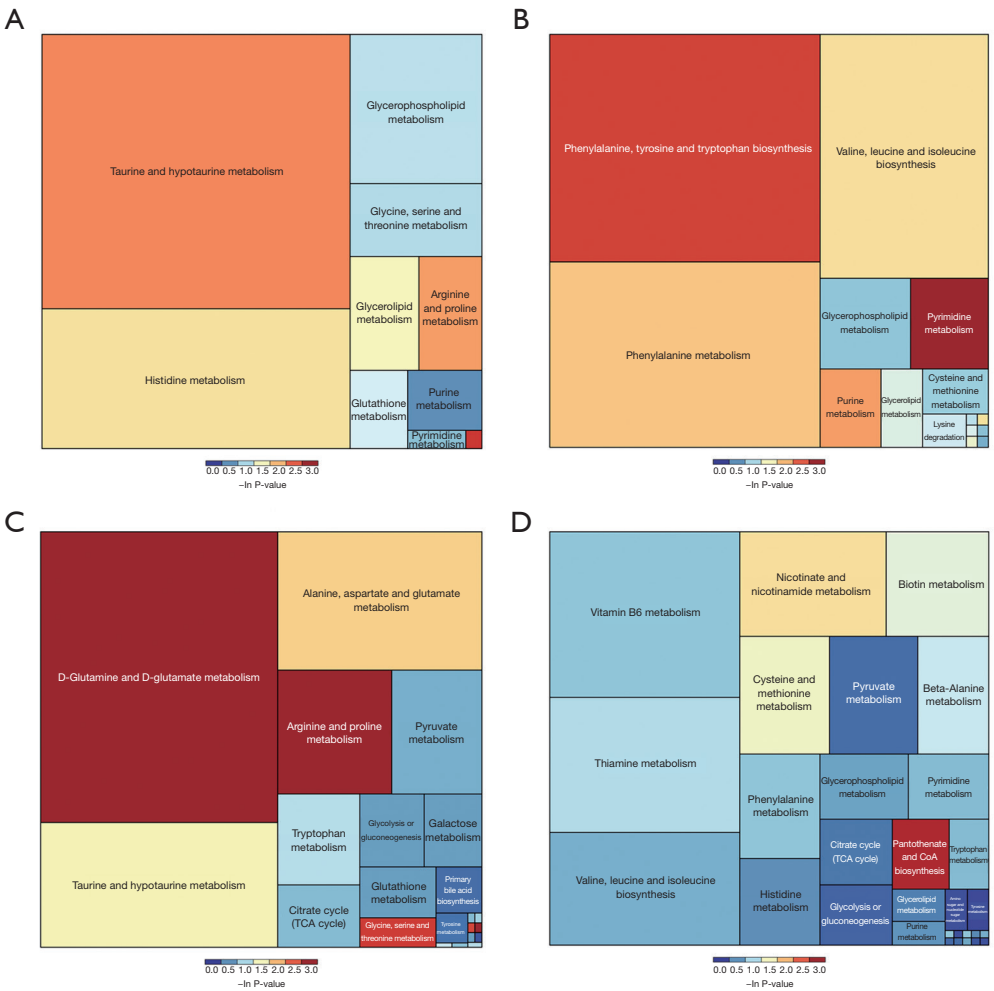

Figure 5 Overview of pathway analysis results enriched from testis (A for H2 *vs.* M2; B for H4 *vs.* M4) and urine (C for H2 *vs.* M2; D for H4 *vs.* M4) metabolites by tree plots.

(*Figure 7B-7E*), which may be due to the lack of specificity of urine samples for the reproduction system diseases compared with testis, a reproductive organ.

Discussion

Cyclophosphamide is a widely used anti-cancer agent and immunosuppressant (17,18). However, numerous studies have shown its toxic properties acting on the male reproductive system (19-22). It has been reported that cyclophosphamide can increase the expression of apoptosis-related proteins and inhibit the expression of meiosis, blood-testosterone barrier, and sperm motility related proteins, leading to the disruption of germ cell division and differentiation and the failure of blood-testosterone barrier functions, affecting sperm quality in many aspects (23). Model rats had darker hair color, dispirited reaction, and diarrhea, which reflected the effect of cyclophosphamide.

The sperm quality indexes of the model group continued to decrease during the experimental period, while those of the HJZY group continued to increase. On the one hand, the modeling method had good stability, and self-healing had little impact during the experiment. On the other hand, it shows that the HJZY capsule has a good recovery effect on OAS. As shown in the pathological sections, the structure of Sertoli cells and Leydig cells of rat testes in the HJZY group was reconstructed after 4 weeks of intervention, which guaranteed the structural integrity of spermatogenic tubules, therefore, it provided a stable internal environment for better spermatogenesis. Again on

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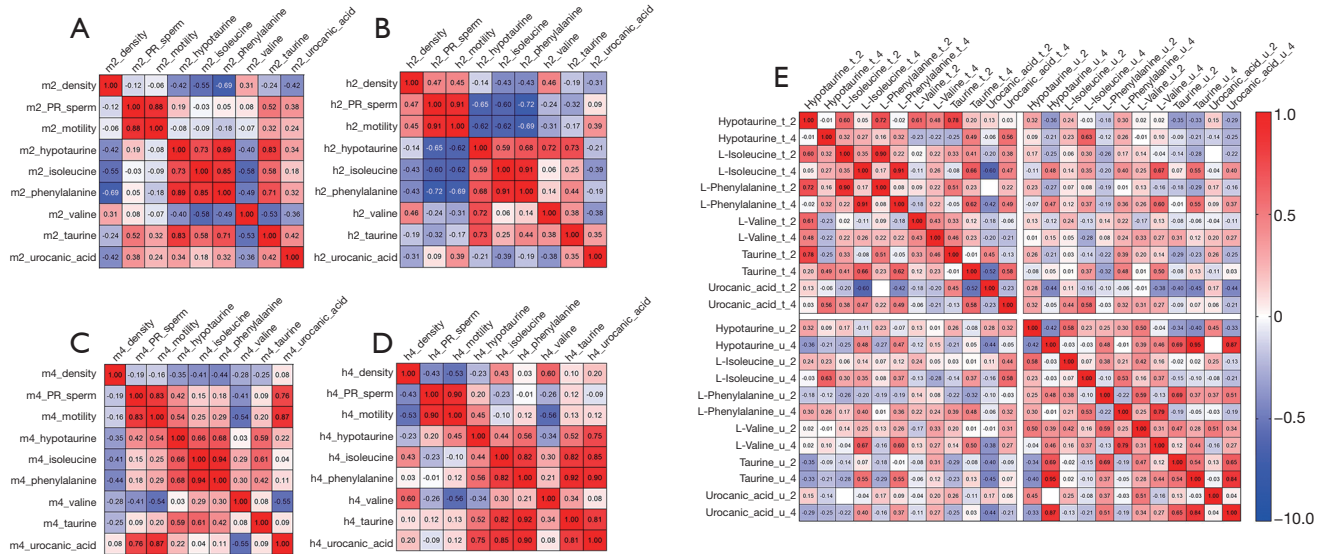

Figure 6 Heatmaps of correlation analysis between sperm quality measurements and relative levels of common metabolites in the testis of (A) M2, (B) H2, (C) M4, and (D) H4. Consistency of urine and testis metabolomics at different time points are shown in (E). Digits in the cells are Pearson correlation coefficients between variables.

the other hand, recovery of antioxidation system in testes also played an essential role as abnormal lipid catabolism caused by oxidative stress could deal critical damage to the integrity of spermatogenic tubules and consequently sabotage physical functions of the reproduction system. A stable internal environment depends on the balance between oxidation and anti-oxidation (24). This balance could be recovered by eliminating the over-accumulated free radicals and producting inhibition (25). Administration of HJZY capsule elevated SOD and GPX activity in model rats, both being critical antioxidative enzymes. Though GPX activity elevation was insignificant in HJZY groups compared with model groups in the same week, the trend was apparent. The MDA content was decreased after HJZY capsule intervention, with a gradual decline following intervention time. To conclude, the HJZY capsule demonstrated a substantial curative effect for OAS, which could be partly explained by its recovery of testis antioxidative activity.

A study has shown differences in metabolites detected in the urine of healthy men and women, which are highly correlated with gender factors, such as α-ketoglutaric acid, 4-hydroxybutyric acid, and so on (26). In addition, the activity levels of biological processes such as energy metabolism and saturated fatty acid metabolism are also different (26). Metabolomic analysis of urine retrieved citrate, hippurate, formate, and various amino acids as potential biomarkers. Through pathway enrichment analysis, it was concluded that cyclophosphamide could affect biological processes such as amino acid biosynthesis, succinic acid metabolism, and tricarboxylic acid cycle, and so on (27). Amino acid metabolism is a major kind of metabolic process detected by both metabolomics analyses. The metabolic pathways of amino acids related to the reproductive system include alanine, aspartate, glutamate metabolism, arginine and proline metabolism, phenylalanine metabolism, beta-alanine metabolism, and lysine degradation. However widely amino acid metabolism pathways had been affected, most of the metabolites detected were of little impact to corresponding pathways, especially those from urine samples. Several amino acids were common DEMs of urine and testicular metabolites, including L-isoleucine, L-valine, L-phenylalanine, and so on. As one of the aromatic amino acids, phenylalanine can be used as a discriminant metabolite for male infertility as shown by a study on human seminal plasma (28). Another study showed that absolute phenylalanine content in male seminal plasma was downregulated (29), which is consistent with our study. Moreover, phenylalanine metabolism is closely related to energy metabolism, with involvement in synthesizing acetyl-CoA and fumaric acid. The metabolic level of fumaric acid was downregulated in the model group, which was improved in the HJZY group after 4 weeks of

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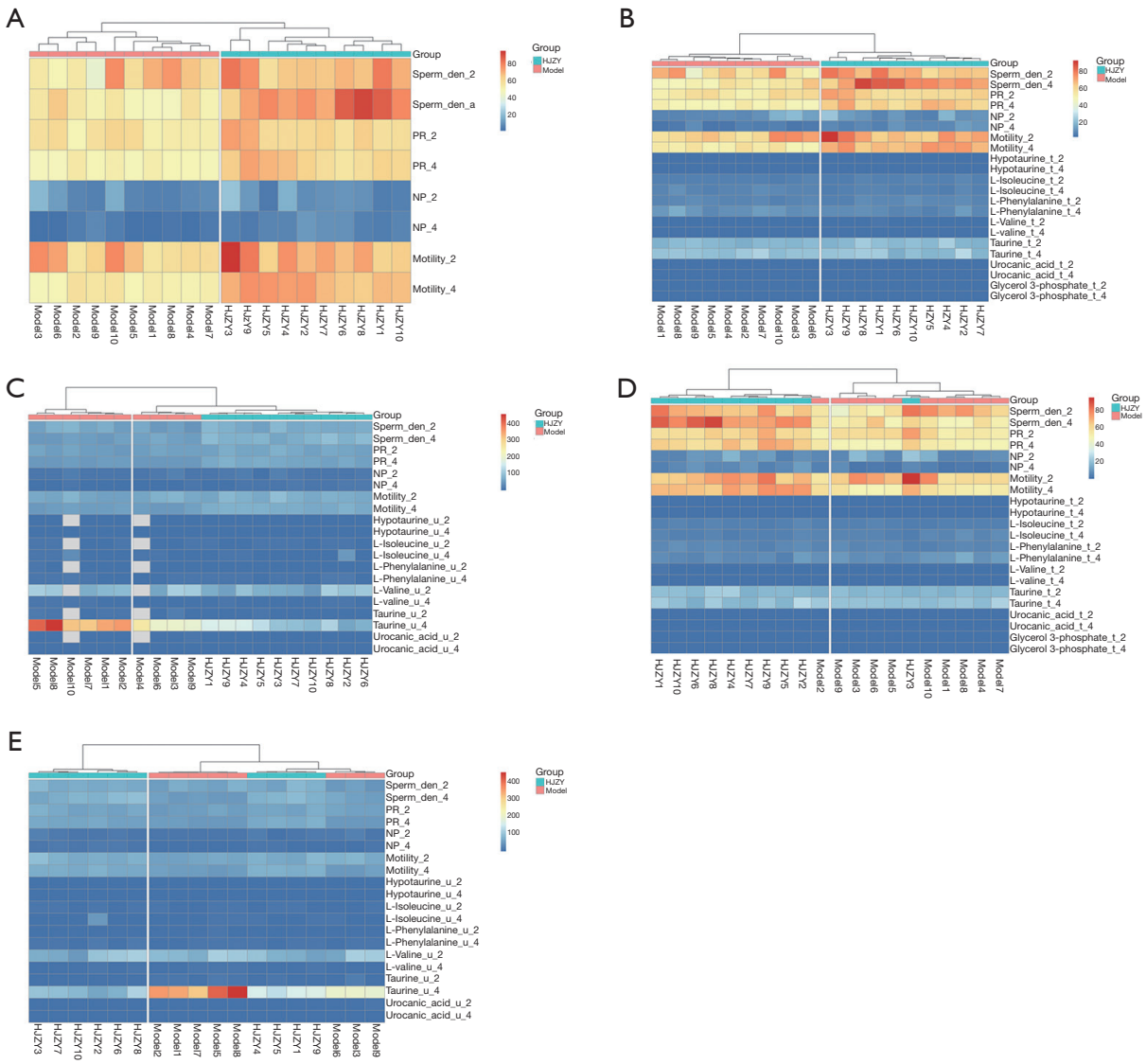

Figure 7 Cluster analyses with different variables to verify the classification strength of urine or testis metabolites. (A) Sperm quality measurement alone could separate the two groups precisely. (B,C) Euclidean distance was used as the discrimination standard. Testis DEM levels with sperm quality provided better discrimination results than urine DEMs. (D,E) Pearson correlation was used as the discrimination standard. Again, testis DEM levels with sperm quality indexes provided better discrimination results than urine DEMs. HJZY, Huangjing Zanyu; DEM, differentially expressed metabolite.

administration. In addition, beta-alanine metabolism also connects to the metabolism of uracil. Compared with the blank group, the metabolic level of uracil in the model group was increased while it decreased in the H4 group. In terms of taurine and hypo-taurine metabolism, the taurine level in the model group was higher than that in the blank group at 4 weeks, which HJZY capsule could down-regulate at 2 and 4 weeks. In combination with the fact that the level of taurine generated in the urea cycle in the arginine biosynthesis process was higher in the H4 group than in the model group, it could be implied that the utilization rate of taurine in the H4 group was improved, which is beneficial for sperm capacitation.

Apart from those shared by urine and testicular metabolomics, testicular metabolites alone are worthy of attention in that they provide a more comprehensive

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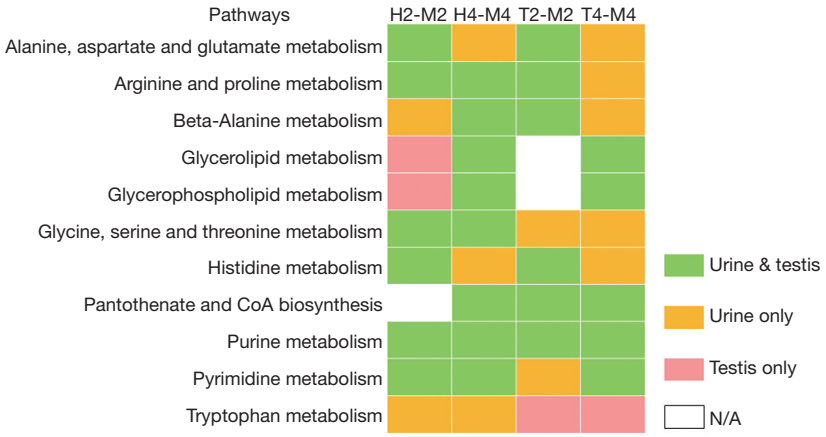

Figure 8 Pathway results consistency between urine and testis metabolomics. Group H4 demonstrates the most comprehensive effect upon OAS model rats, indicating treatment with HJZY capsule for 4 weeks achieved the most satisfying efficacy. OAS, oligoasthenospermia; HJZY, Huangjing Zanyu.

picture of what HJZY did to OAS testis. Lipid metabolism is strongly affected by oxidative stress, which causes excessive degradation of the membrane, therefore destroying healthy cells' physiological structure (30). The HJZY capsule also relieved lipid metabolic abnormality caused by cyclophosphamide modeling, mainly exerted through glycerophospholipid metabolism and glycerolipid metabolism pathways. Glycerolipid metabolism is a lipid metabolism process closely related to glycerophospholipid metabolism. Its core reaction is the acetylation of glycerol and the degradation of triacylglycerol. Acetyl-CoA is essential in the acetylation reaction. The intermediate product diacetyl-glycerol is the critical substrate in the glycerophospholipid reaction. The fatty acids produced by the degradation of triacylglycerols are converted into smaller fat molecules through their degradation and metabolism (31). Although the impact value of the glyceride metabolism pathway did not reach 0.1 in each comparison, it still plays an essential role in the lipid metabolism process as a complementary reaction of glycerophospholipid metabolism and should be given sufficient attention. Purine metabolism and pyrimidine metabolism are the core metabolic processes in the production and degradation of DNA and RNAs, which directly reflects whether the spermatogenesis process in the testicular tissue is proceeding normally, therefore being key symptom indicators of OAS. In this study, compared with the blank group, the levels of guanosine and adenine decreased in the model group at 4 weeks. Correspondingly, the levels of deoxyguanosine monophosphate and adenosine diphosphate also decreased, while xanthine and its nucleosides increased. The HJZY groups were able to correct the abnormal trend of adenine and guanosine in the model group. In pyrimidine metabolism, the cytosine level increased while the 5-methylcytosine level decreased at 4 weeks in the model group. The UMP level decreased, but the pseudouridine level increased. The HJZY capsule could counteract the changes in the level of corresponding metabolites. More quantification investigation is needed to clarify the relationship between OAS and these metabolites.

Results from urine and testis metabolomics were divergent due to the natural difference of sample sources, which explains the difference between the metabolites detected and enriched pathways. The consistency of enriched pathways is shown in *Figure 8*. The results of the H4-M4 comparison have the best consistency, followed by H2-M2, while those of L-carnitine groups are relatively low. Due to a different number of metabolites detected, urine metabolomics retrieved more pathways than testis, yet the pathways specifically obtained in testis tissue are more representative of the reproduction system. Pathways with higher consistency include amino acid biosynthesis, including the metabolism or degradation of specific amino acids, glycerophospholipid metabolism, purine metabolism, pyrimidine metabolism, and so on. It can be considered that these pathways are also closely related to the mechanism of HJZY capsule treating OAS. Amino acid metabolism should be emphasized in clinical treatment. As a marker of male infertility, L-phenylalanine could be used. Evidence points to its elevated level indicating OAS (32). Fumaric

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acid upregulation and uracil, taurine, and hypotaurine downregulation could serve as curative efficacy markers. It is important to note the need to use these markers comprehensively rather than simply observing one. Further studies of key protein expressions in these pathways are suggested. Also, given the fact that results of urine and testis metabolomics vary drastically at different time points and treatments, caution should be exercised when explaining the results of the diagnostic biomarker or risk factor studies focusing on urine metabolites collected from infertility patients, since urine samples are not necessarily responsible for reproductive diseases.

Although metabolomics has given us a lot of information, it still has technical limitations and emerging challenges. Firstly, a certain amount of information could be lost because of an overwhelming number of unidentified peaks. Secondly, incomplete metabolomes could interfere with test results, and they also leads to poor repeatability. Thirdly, some metabolites partake in multiple metabolic pathways, and we couldn't easily identify the pathway with the greatest weight through metabolomics analyses. In addition, for the reproductive system, there is no complete metabolite database in the field of male infertility, and the differential metabolites obtained by existing studies are dishevelled. Therefore, our next step is to verify the results of differential metabolites through the experiments *in vivo* and *in vitro*. When conditions permit, we consider using multi-omics analysis.

Conclusions

This study found that HJZY capsule can improve the oxidative stress damage of OAS rats' testes and can affect the levels of taurine, hypo-taurine, urocanic, valine isoleucine, and phenylalanine in corresponding pathways, respectively. In addition, we found differences in the consistency between urine and testis metabolomic results based on the detection of correlative metabolic pathways and metabolites. Thus, when using urine metabolomics to screen biomarkers, attention should be paid to its practical significance for testicular metabolism.

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Footnote

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Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-22-293/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-22-293/coif). All authors report that the study has received sample testing service and technical support from Shanghai Biotree biomedical technology Co., Ltd. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. BUCM-4-2019112502-4067) granted by the Ethics Committee of Beijing University of Chinese Medicine, in compliance with Chinese national guidelines for the care and use of animals.

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