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Seminal plasma proteomics of asymptomatic COVID-19 patients reveals disruption of male reproductive function

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Abstract

Background A considerable proportion of males suffer from asymptomatic SARS-CoV-2 infection, while the effect on reproductive function and underlying pathomechanisms remain unclear.

Results The total sperm count decreased evidently after asymptomatic infection, yet all semen samples were tested to be SARS-CoV-2 RNA negative. Through label-free quantitative proteomic profiling, a total of 733 proteins were further identified in seminal plasma from 11 COVID-19 patients and seven uninfected controls. Of the 37 differentially expressed proteins, 23 were upregulated and 14 were downregulated in the COVID-19 group compared with control. Functional annotations in Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome showed that these proteins were highly enriched in infection, inflammation, and immunity-related pathways as well as spermatogenesis-associated biological process. Four proteins were significantly correlated with one or more semen parameters in Spearman's coefficient analysis, and seven were filtered as potential hub proteins from the interaction network by MCODE and Cytohubba algorithms. Furthermore, we verified the proteomic results by Western blot analysis of three representative proteins (ITLN1, GSTM2, and PSAP) in the validation cohort.

Conclusions In summary, our study showed that acute asymptomatic COVID-19 could alter the seminal plasma protein profile without direct testicular infection and consequently lead to impaired semen quality. These novel findings should enlighten the physicians about the adverse effects of SARS-CoV-2 infection on male fertility, and provide valuable resources for reproductive biologists to further decipher the molecular functions.

Keywords COVID-19, SARS-CoV-2, Seminal plasma, Proteomics

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Background

On May 5, 2023, the World Health Organization (WHO) declared end of the coronavirus disease 2019 (COVID-19) global health emergency, but the risks remained high as an ongoing public health issue [1]. As of February 11, 2024, the pandemic has resulted in nearly 775 million confirmed cases and over 7.0 million deaths worldwide [2]. Notably, asymptomatic infections represent as high as 43.7% of cases, and continue to increase with the genomic mutation and variant evolution of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) [3].

SARS-CoV-2 shares a 79% sequence identity with the earlier SARS-CoV virus but differs in pathogenic mechanisms [4]. The SARS-CoV-2 mainly interacts with the angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) receptors, triggering systemic hyperinflammation and a cascade of cytokines that affect multiple organs [5–8]. The male reproductive system, particularly the testis, is a potential target for the virus [9, 10]. Single-cell RNA sequencing analysis has shown high expression levels of ACE2 in spermatogonia, Leydig cells, Sertoli cells, and sperm [11]. In addition, TMPRSS2 protein is also expressed in the prostate, epididymis, and seminal vesicles [12], suggesting their susceptibility to SARS-CoV-2 infection.

Among severely affected COVID-19 individuals, postmortem examinations of testes within 1 h of death have revealed significant damage to seminiferous tubules, marked decrease in Leydig cells, and inflammatory infiltration in the interstitium [13]. Further proteomic studies also reported dramatic reductions in Leydig cell biomarker insulin-like 3 along with five cholesterol biosynthesis-related proteins and two key proteins associated with spermatogenesis and sperm motility [7]. The observed injuries may be attributed to the breakdown of the blood-testis barrier, thus allowing the virus to penetrate the testicular tissue [14]. For mild/asymptomatic COVID-19 patients, recent systematic review and meta-analyses also showed that sperm concentration, count and motility all decreased significantly after infection [15, 16]. Nonetheless, SARS-CoV-2 RNA was undetectable in these semen samples [16], and the underlying pathogenic mechanisms remain to be clarified.

Seminal plasma is primarily secreted by the testis, cauda epididymis, and accessory sexual glands, and contains a large diversity of proteins that are essential for sperm function and fertilization potential [17]. As the primary nutritive and protective medium for sperm, it also contains about 30% of sperm proteins, which are indicative of its functional state and thus used for the discovery of potential biomarkers [18]. Both label-free and multiplexed labelled techniques (e.g., tandem mass tag) have been applied for seminal plasma proteomics [19, 20]. In the present study, the label-free quantification approach was selected due to its simplicity, cost-effectiveness, flexibility, and compatibility with modern mass spectrometry [21]. We aim to evaluate the proteomic profiling of seminal plasma in asymptomatic COVID-19 patients and further identify the correlation between differentially expressed proteins (DEPs) with semen quality.

Materials and methods

Study subjects

The study was approved by the Reproductive Medicine Ethics Committee of Jiangxi Maternal and Child Health Hospital, and conducted in accordance with the Declaration of Helsinki. All subjects signed informed consents after enrollment from October 2022 to June 2023, and were intended to undergo assisted reproductive treatment due to female infertility factors (e.g., tubal obstruction). Eligible males were non-smokers aged between 20 and 40 years, had normal body mass index, and were normozoospermic at their first test. The asymptomatic COVID-19 group included men who were tested to be SARS-CoV-2 positive by nasopharyngeal and/or oral swab RT-qPCR (Daan Gene, China) on the day of their second semen test but had no obvious clinical symptoms (e.g., fever, headache, or sore throat) during infection, while those with no COVID-19 history were categorized into the control group. The time interval between patients' first and second semen analyses was limited within one month. The exclusion criteria were as follows: (1) exposure to harmful radiation, environmental chemicals, or external trauma; (2) history of chronic diseases such as type 2 diabetes; (3) use of supportive medication such as antiviral drugs and steroids; and (4) any genetic defect, sexually transmitted disease, varicocele or reproductive tract inflammation.

Semen collection and handling

Semen samples were collected by masturbation after sexual abstinence for two to five days, and partially sent for SARS-CoV-2 RNA detection by RT-qPCR immediately. After liquefaction for 30 min at 37 °C, computer-assisted semen analysis (CASA; Suijia Software, China) was performed by experienced technicians as per the WHO laboratory procedures [22]. For samples with high (>100 million/mL) sperm concentration, manual sperm count was additionally conducted for validation. In this study, the manual and CASA analyses were highly comparable and the difference of all sperm parameters was limited within 2.0%. Therefore, we adopted the CASA data for final comparison. The left-over samples were centrifuged at 1000×g for 7 min to remove sperm, and further centrifuged at 10,000×g for 30 min at 4 °C to produce cell debris-free seminal plasma. The clear supernatant was aspirated and stored at -80 °C for use. For label

free proteomic profiling, we enrolled 11 asymptomatic COVID-19 patients and seven uninfected controls. For protein expression validation, another seven patients were included in each group.

Protein extraction and quality control

Weighted seminal plasma samples were transferred into 2 mL centrifuge tubes and added with lysis solution containing 8 M Urea/50mM Tris-HCl and cOmplete™ Protease Inhibitor Cocktail (Roche, Switzerland). After homogenization using a tissue lyser and centrifugation at 20,000×g for 15 min, the supernatant was collected and added with 10 mM dithiothreitol and 20 mM iodoacetamide sequentially. The Bradford method was then used for protein quantitation using BSA as a standard. In addition, SDS-PAGE was simultaneously applied for quality control. Briefly, 10 μg protein from each sample was mixed with loading buffer, heated at 95 °C for five minutes, and centrifuged at 20,000×g for five minutes. The supernatant was loaded onto a 4–12% SDS-PAGE gel and electrophoresed at 80 V for 20 min, followed by 120 V for another 60 min before staining, destaining, and photographing.

Protein digestion and peptide fractionation

For protein digestion, 150 μg of protein was taken from each sample and added with 3 μg trypsin for incubation at 37 °C. After 14–16 h, the enzymatically digested peptides were desalted using Waters solid-phase extraction cartridges, vacuum-dried, and redissolved in pure water for storage at -20 °C. Equal amounts of peptide from all samples were diluted with solvent A (5% acetonitrile, pH 9.8) and injected into the Agilent ZORBAX 300Extend-C18 column. The peptide was fractionated on UltiMate™ 3000 binary rapid separation system (Thermo Scientific, USA) using a gradient elution in solvent B (95% acetonitrile, pH 9.8) at a flow rate of 0.3 mL/min. The elution peaks were monitored at a wavelength of 214 nm, and one fraction was collected every minute. Based on the chromatographic elution profiles, the samples were combined to obtain 10 fractions, which were then freeze-dried.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The dried peptide samples were redissolved in 0.1% formic acid (FA) and centrifuged at 20,000×g for 10 min. The collected supernatant was then injected into a C18 column and separated using the EASY-nLC™ 1200 system (Thermo Fisher Scientific, USA) with a linear gradient of solvent B (98% acetonitrile, 0.1% FA) at a flow rate of 0.3 mL/min. The separated peptides were ionized by nano-ElectroSpray Ionization and then transferred to Orbitrap Explori™ 480 mass spectrometer (Thermo Fisher Scientific, USA) for data-dependent acquisition (DDA) detection mode. The ion source voltage was set at 2.2 kV, and the resolution for primary and secondary MS was 60,000 and 15,000, respectively.

Protein identification and quantification

The DDA label-free MS/MS raw data were analyzed using the MaxQuant software (version 2.1.4.0). The main parameters in MaxQuant were set as follows: MS mode as standard, enzyme as Trypsin/P, fixed modification as Carbamidomethyl (C), and variable modifications as Oxidation (M) and Acetyl (protein N-term). In addition, both "Match between runs" and "Second peptide search" options were enabled, with all other parameters set to default. The Uniprot database was used for protein identification in corresponding species. For filtering, the threshold of false discovery rate was set as 1% at both peptide spectrum match and protein levels. Proteins from contaminant and decoy databases were additionally removed.

Bioinformatic analysis

Raw intensity values of proteins were normalized by the "medium" method. Subcellular localization analysis of proteins was performed using the WoLF PSORT software. Significant DEPs between COVID-19 and control groups were identified using the *metaX* package in R software (version 4.0), and further visualized by volcano plot and heatmap using *ggplot2* and *pheatmap* packages as previously described [23]. The criteria were defined as a P-value < 0.05 from the t-test and a fold change > 1.5. The screened DEPs were then subjected to hypergeometric-based analyses with Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome [24–26]. Functional terms with a P-value < 0.05 were considered as significantly enriched. Additionally, the protein-protein interaction (PPI) network of DEPs was constructed via the Search Tool for the Retrieval of Interacting Genes (STRING) database and visualized with Cytoscape (version 3.9.1) [27, 28]. We also evaluated the relationship between DEPs as well as their relationship with semen parameters using Spearman's correlation.

Western blot

Three proteins (intelectin-1 [ITLN1], glutathione S-transferase mu 2 [GSTM2], and prosaposin [PSAP]) were selected for validation by western blot. In line with previous selection criteria [29, 30], these proteins have a well-described function in inflammation, oxidative stress or sperm-oocyte interaction, and are closely associated with male fertility [31–33]. Briefly, 50 μg of seminal plasma proteins were separated on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% (w/v) skimmed

milk at 37 °C for 1 h and subsequently incubated with the primary antibodies of 1:1000 diluted anti-ITLN1 (Rabbit Polyclonal, ab118232, Abcam, Cambridge, USA), 1:500 diluted anti-GSTM2 (Rabbit Polyclonal, ab175282, Abcam, Cambridge, USA), 1:500 diluted anti-PSAP (Rabbit Polyclonal, ab180751, Abcam, Cambridge, USA), or 1:5000 diluted anti-β-actin (Rabbit Polyclonal, 20536-1-AP, Proteintech, Wuhan, China) at 4 °C overnight. After TBST washing for three times, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (SA00001-2, Proteintech, Wuhan, China, diluted 1:5000) at room temperature for 1 h. The immunoreactive proteins were visualized with enhanced chemiluminescence detection reagents (Pierce, Rockford, USA), and filmed with a Z320 scanner (Founder, Beijing, China). All images were quantified via ImageJ software (National Institutes of Health). The expression levels of the three proteins were normalized to β-actin.

Statistical analysis

Continuous data were presented as means with standard deviations. After examination of normality by Shapiro-Wilk test, unpaired t-test or Mann-Whitney U test was used for comparison between COVID-19 and control groups. To evaluate the semen parameter change from the first to second test in the same participants, data were analyzed by paired t-test or Wilcoxon signed-rank test as appropriate [34]. All analyses were performed with SAS version 9.4 (SAS Institute, USA) and a two-tailed $P<0.05$ was considered as statistically significant.

Results

Semen quality assessment

The baseline characteristics of asymptomatic COVID-19 and control groups are presented in Table S1. No significant differences were observed in age, body mass index, frequency of sexual activity, sex hormone profile, as well as semen parameters at the first test. However, the total sperm count decreased significantly after infection (276.7 ± 183.3 vs. 111.5 ± 65.3 million in proteomics cohort, $P=0.026$; 254.4 ± 198.8 vs. 81.1 ± 46.5 million in validation cohort, $P=0.040$) (Table 1). In addition, there was an evident post-infection reduction of semen volume in proteomics cohort (4.1 ± 1.9 vs. 2.8 ± 1.2 mL, $P=0.026$) and sperm concentration in validation cohort (66.0 ± 48.8 vs. 28.9 ± 15.4 million/mL, $P=0.043$). Contrarily, the total and progressive sperm motility did not show significant changes. For the control group, all semen parameters were comparable between the two tests. The collected samples were consistently tested to be SARS-CoV-2 RNA negative.

Identification of deps in seminal plasma

A total of 733 proteins were identified from COVID-19 ($n=11$) and control ($n=7$) samples through LC-MS/MS (Figure S1**A**). These proteins were primarily derived from chromosome 1 and 11, mainly distributed in 20–60 kDa of molecular weight, and mostly located in extracellular region and cytoplasm (Figure S1**B-D**). Pearson correlation analysis showed that all samples were well correlated in protein intensity ($r=0.844$ to 0.931) (Figure S1**E**, **F**). After comparative analysis, 37 proteins were identified to be differentially expressed, in which 23 were upregulated and 14 were downregulated in the COVID-19 group. The volcano plot and heatmap of DEPs are shown in Fig. 1, with specific names listed in Table 2.

Functional enrichment analysis of deps

Through GO, KEGG, and Reactome functional analyses, the identified 733 proteins were found to be mainly enriched in metabolic process, infectious disease, and immune system (Figure S2). For the 37 DEPs, a consistent enrichment was also observed in infection, inflammation, and immunity-related pathways (Fig. 2). Specifically, the GO analysis demonstrated significant enrichment in the positive regulation of NF-kappaB transcription

Table 1 Semen parameter change of COVID-19 and control patients

	COVID-19			Control		
	First sampling	Second sampling	*P*-value	First sampling	Second sampling	*P*-value
For proteomics	*n* = 11	*n* = 11		*n* = 7	*n* = 7	
Semen volume (mL)	4.1 ± 1.9	2.8 ± 1.2	0.041	3.4 ± 0.8	3.1 ± 1.0	0.448
Sperm concentration (million/mL)	82.1 ± 68.8	39.5 ± 13.5	0.056	48.0 ± 15.9	41.4 ± 13.5	0.523
Total sperm count (million)	276.7 ± 183.3	111.5 ± 65.3	0.026	158.0 ± 54.2	137.1 ± 86.2	0.572
Total sperm motility (%)	50.5 ± 14.2	48.6 ± 16.1	0.746	49.4 ± 9.5	52.1 ± 13.2	0.454
Progressive sperm motility (%)	38.5 ± 11.9	39.1 ± 15.3	0.903	41.2 ± 8.8	43.6 ± 14.4	0.446
For validation	*n* = 7	*n* = 7		*n* = 7	*n* = 7	
Semen volume (mL)	3.8 ± 1.3	2.9 ± 1.0	0.231	3.6 ± 2.0	3.3 ± 2.6	0.836
Sperm concentration (million/mL)	66.0 ± 48.8	28.9 ± 15.4	0.043	37.1 ± 11.0	17.5 ± 16.4	0.533
Total sperm count (million)	254.4 ± 198.8	81.1 ± 46.5	0.040	137.0 ± 88.1	49.4 ± 43.7	0.930
Total sperm motility (%)	50.2 ± 19.7	50.0 ± 11.2	0.831	39.6 ± 19.3	36.6 ± 22.6	0.121
Progressive sperm motility (%)	37.5 ± 14.6	40.7 ± 13.0	0.634	31.0 ± 16.4	30.8 ± 18.6	0.143

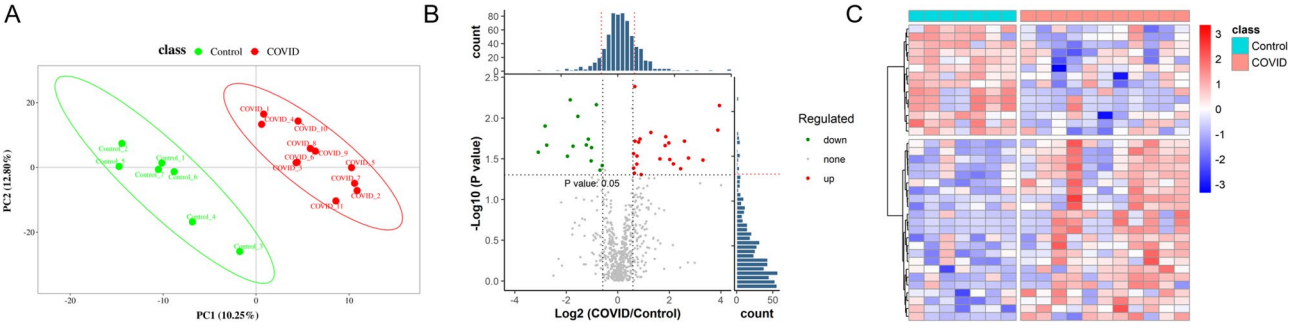

Fig. 1 Identification of differentially expressed proteins (DEPs) in seminal plasma. (**A**) Volcano plot of the 34 DEPs between COVID-19 ($n=11$) and control ($n=7$) groups. (**B**) Heatmap of the 34 DEPs between COVID-19 ($n=11$) and control ($n=7$) groups. The criteria for DEPs are a fold change > 1.5 and a *P*-value < 0.05 from the t-test

Table 2 List of differentially expressed proteins in seminal plasma (COVID-19 *versus* Control)

Uniprot	Gene	Protein name	Ratio	*P*-value
Up-regulated				
P0DMV9	HSPA1B	Heat shock 70 kDa protein 1B	1.580	0.0041
Q9NVJ2	ARL8B	ADP-ribosylation factor-like protein 8B	15.414	0.0070
Q15257	PTPA	Serine/threonine-protein phosphatase 2 A activator	14.759	0.0141
O00410	IPO5	Importin-5	2.442	0.0151
P50990	CCT8	T-complex protein 1 subunit theta	3.632	0.0170
P28907	CD38	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1	1.815	0.0181
P46782	RPS5	40 S ribosomal protein S5	6.014	0.0192
P60900	PSMA6	Proteasome subunit alpha type-6	1.585	0.0193
P14625	HSP90B1	Endoplasmin	1.748	0.0199
Q9Y265	RUVBL1	RuvB-like 1	3.952	0.0203
P54652	HSPA2	Heat shock-related 70 kDa protein 2	3.578	0.0218
P08238	HSP90AB1	Heat shock protein HSP 90-beta	1.528	0.0275
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	1.676	0.0295
P62826	RAN	GTP-binding nuclear protein Ran	6.685	0.0311
P02649	APOE	Apolipoprotein E	3.102	0.0319
Q16851	UGP2	UTP-glucose-1-phosphate uridylyltransferase	4.058	0.0324
O14556	GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	9.859	0.0329
P40121	CAPG	Macrophage-capping protein	4.454	0.0366
P02774	GC	Vitamin D-binding protein	1.666	0.0370
O94985	CLSTN1	Calsyntenin-1	1.534	0.0414
Q8WWA0	ITLN1	Intelectin-1	5.410	0.0420
P05067	APP	Amyloid-beta precursor protein	1.561	0.0476
Q9Y4L1	HYOU1	Hypoxia up-regulated protein 1	1.881	0.0496
Down-regulated				
P28161	GSTM2	Glutathione S-transferase Mu 2	0.279	0.0060
Q9H3G5	CPVL	Probable serine carboxypeptidase CPVL	0.563	0.0069
P20155	SPINK2	Serine protease inhibitor Kazal-type 2	0.343	0.0096
P05109	S100A8	Protein S100-A8	0.142	0.0126
Q9NR99	MXRA5	Matrix-remodeling-associated protein 5	0.452	0.0183
O95994	AGR2	Anterior gradient protein 2 homolog	0.430	0.0215
Q7L4I2	RSRC2	Arginine/serine-rich coiled-coil protein 2	0.148	0.0216
Q07654	TFF3	Trefoil factor 3	0.301	0.0222
Q9GZX9	TWSG1	Twisted gastrulation protein homolog 1	0.495	0.0232
P59666	DEFA3	Neutrophil defensin 3	0.118	0.0264
P06702	S100A9	Protein S100-A9	0.259	0.0296
P00491	PNP	Purine nucleoside phosphorylase	0.489	0.0338
Q16610	ECM1	Extracellular matrix protein 1	0.658	0.0383
Q96BH3	ELSPBP1	Epididymal sperm-binding protein 1	0.622	0.0438

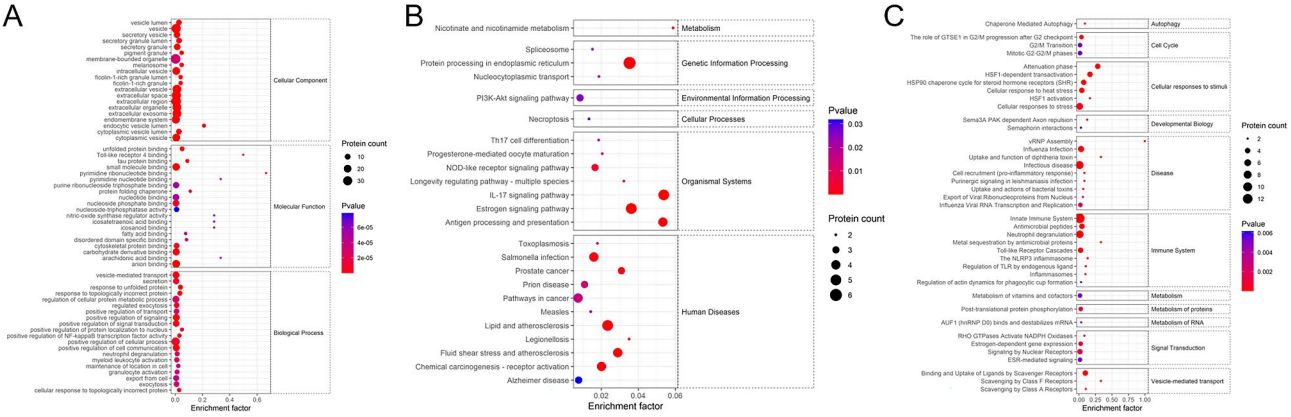

Fig. 2 Functional enrichment analyses of differentially expressed proteins in seminal plasma. (**A**) Gene Ontology enrichment analysis. (**B**) Kyoto Encyclopedia of Genes and Genomes enrichment analysis. (**C**) Reactome enrichment analysis

factor activity, myeloid leukocyte activation, neutrophil degranulation, Toll-like receptor 4 binding, and MHC class II protein complex binding (Fig. 2A). The KEGG pathways included necroptosis, IL-17 signaling pathway, Th17 cell differentiation, NOD-like receptor signaling pathway, antigen processing and presentation, as well as measles, legionellosis, toxoplasmosis, and salmonella infection (Fig. 2B). In Reactome analysis, the terms of the category "Immune System" (e.g., innate immune system, antimicrobial peptides, neutrophil degranulation, Toll-like receptor cascades, and the NLRP3 inflammasome) and "Disease" (e.g., vRNP assembly, influenza infection, infectious disease, purinergic signaling in leishmaniasis infection, and pro-inflammatory response) were highly enriched (Fig. 2C). In addition, there was evidence of direct correlation with male reproductive function, as showed by the enrichment of spermatogenesis (GO: 0007283), spermatid development (GO: 0007286), and spermatid differentiation (GO: 0048515) in biological process (Table S2).

Correlation analysis and interaction network of deps

To explore the relationship among DEPs, we further conducted a Spearman's correlation analysis. As shown in Fig. 3A-C, PTPA and ARL8B had the most significant positive correlation ($r = 0.942$), while UGP2 and TWSG1 had the most negative correlation (r = -0.808). In addition, the expression of CLSTN1 was negatively correlated with sperm concentration, total sperm count, as well as total and progressive sperm motility. We also found a significant correlation of HSP90AB1, PSMA6, and TFF3 levels with one or more semen parameters (Fig 3D).

To identify potential hub proteins, the PPI network was constructed and the top 8 DEPs were filtered through the MCODE and Cytohubba algorithms (Fig. 4A, B). Seven proteins were found to be common in two hub PPI networks, including HSPA1B, HSPA2, HSP90AB1, HSP90B1, CCT8, RUVBL1, and PSMA6. In GO analysis, these proteins had a high enrichment in unfolded protein binding, protein folding chaperone, ATP hydrolysis activity, and response to heat. In KEGG analysis, estrogen signaling pathway, protein processing in endoplasmic reticulum, and lipid and atherosclerosis were significantly enriched (Fig. 4C).

Validation of deps by western blot

To verify the validity of proteomic profiling, three selected proteins were further analyzed by Western blot of seminal plasma from the validation cohort (Fig. 5). Consistently, the results revealed that the DEP GSTM2 was significantly downregulated, whereas ITLN1 was significantly upregulated in asymptomatic COVID-19 patients. As a negative control, the expression of PSAP did not differ significantly between groups, which was similar in proteomic data.

Discussion

The global decline of semen quality continues at an accelerated pace in the 21st century, posing a significant risk to male infertility [35]. A majority of males are asymptomatic after SARS-CoV-2 infection, while the effect on reproductive function and the pathogenic mechanisms remain unclear. In the present study, semen parameters were compared between COVID-19 and control groups as well as before and after infection. Through label-free quantitative proteomic analysis, we then assessed the expression of differential proteins in seminal plasma and verified it using Western blot. Furthermore, we employed functional enrichment analyses to explore potential mechanisms underlying the reduction of semen parameters associated with asymptomatic COVID-19.

Consistent with previous studies [15, 16], a before-and-after comparison of semen parameters showed that the total sperm count decreased significantly following acute asymptomatic SARS-CoV-2 infection. No viral RNA was detected in all semen samples, implying a potential

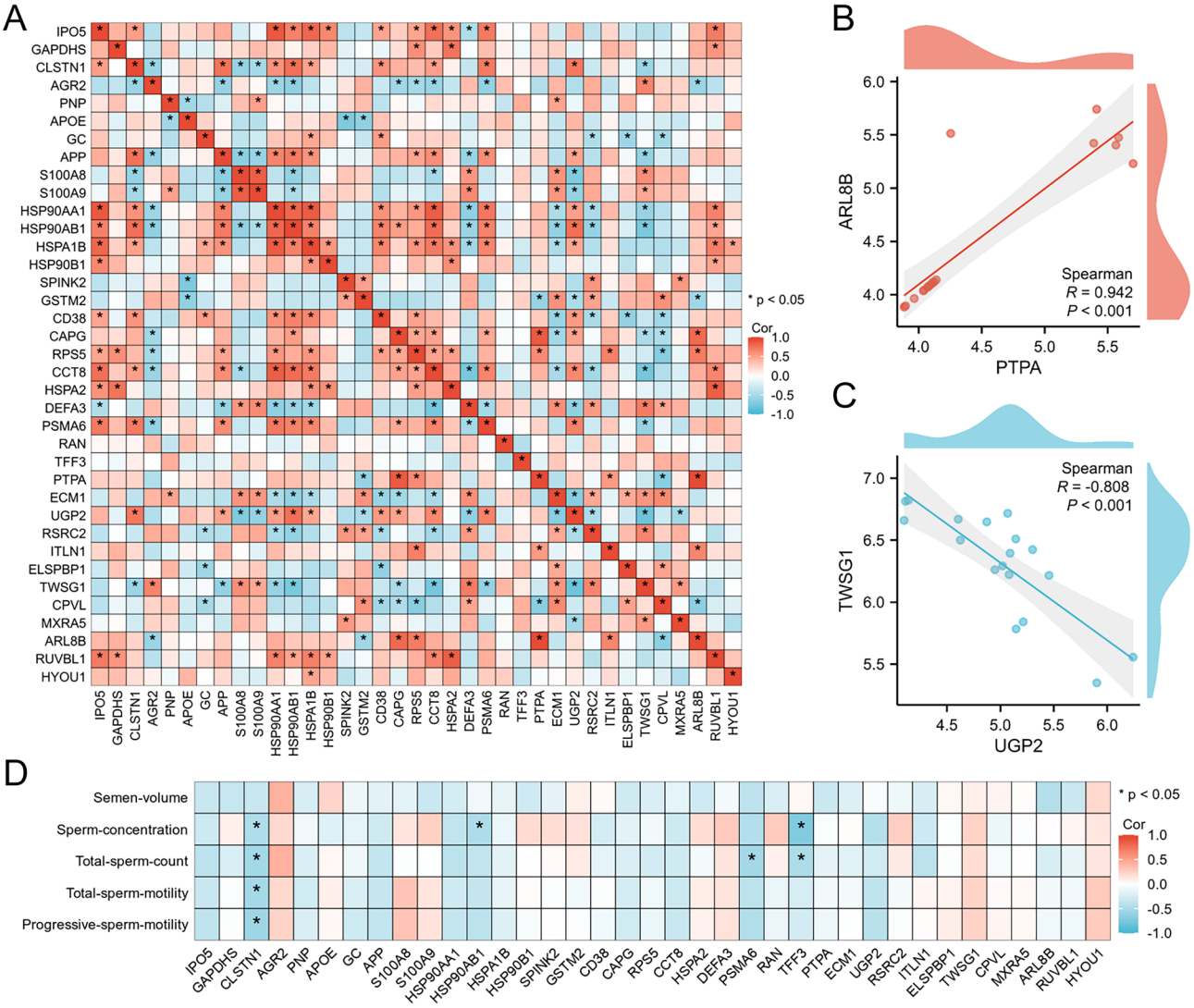

Fig. 3 Correlation analyses of differentially expressed proteins (DEPs) and semen parameters. (**A**) Correlation heatmap among the 34 DEPs. (**B**) Scatterplot of PTPA and ARL8B with the most significant positive correlation. (**C**) Scatterplot of UGP2 and TWSG1 with the most significant negative correlation. (**D**) Correlation heatmap between the 34 DEPs and five semen parameters

protective role of the blood-testis barrier [16]. Nonetheless, the functional analyses of DEPs via GO, KEGG and Reactome consistently demonstrated a high enrichment in infectious, inflammatory, and immune signaling pathways. The findings indicate that COVID-19, as a systemic disease, may still alter the seminal plasma microenvironment in the absence of direct testicular infection and consequently lead to the sperm quality change.

In a previous semen proteomic study, Ghosh et al. [10] focused on COVID-19 convalescent men after mild to moderate infection. Compared with the control group, 21 proteins were detected to be downregulated and 27 were upregulated in recovered males. Further bioinformatics analysis revealed that the pathways related to reproductive functions, such as sperm-oocyte recognition, testosterone response, cell motility regulation, adhesion regulation, and endopeptidase activity, were all downregulated in recovered patients. While the present study was conducted on asymptomatic patients during acute infection, we also found that the biological processes of spermatogenesis, spermatid development, and spermatid differentiation were impaired in GO analysis. Additionally, several DEPs, especially CLSTN1, were observed to have negative correlations with semen parameters, further strengthening the adverse effect of COVID-19 on male fertility.

Functional GO enrichment results suggested that the differential proteins were significantly associated with vesicle-dependent transport. Based on precious reports, the transfer of versatile proteins by extracellular vesicles (EVs) to sperm could stabilize sperm plasma membrane, mediate maturation and motility acquisition, regulate

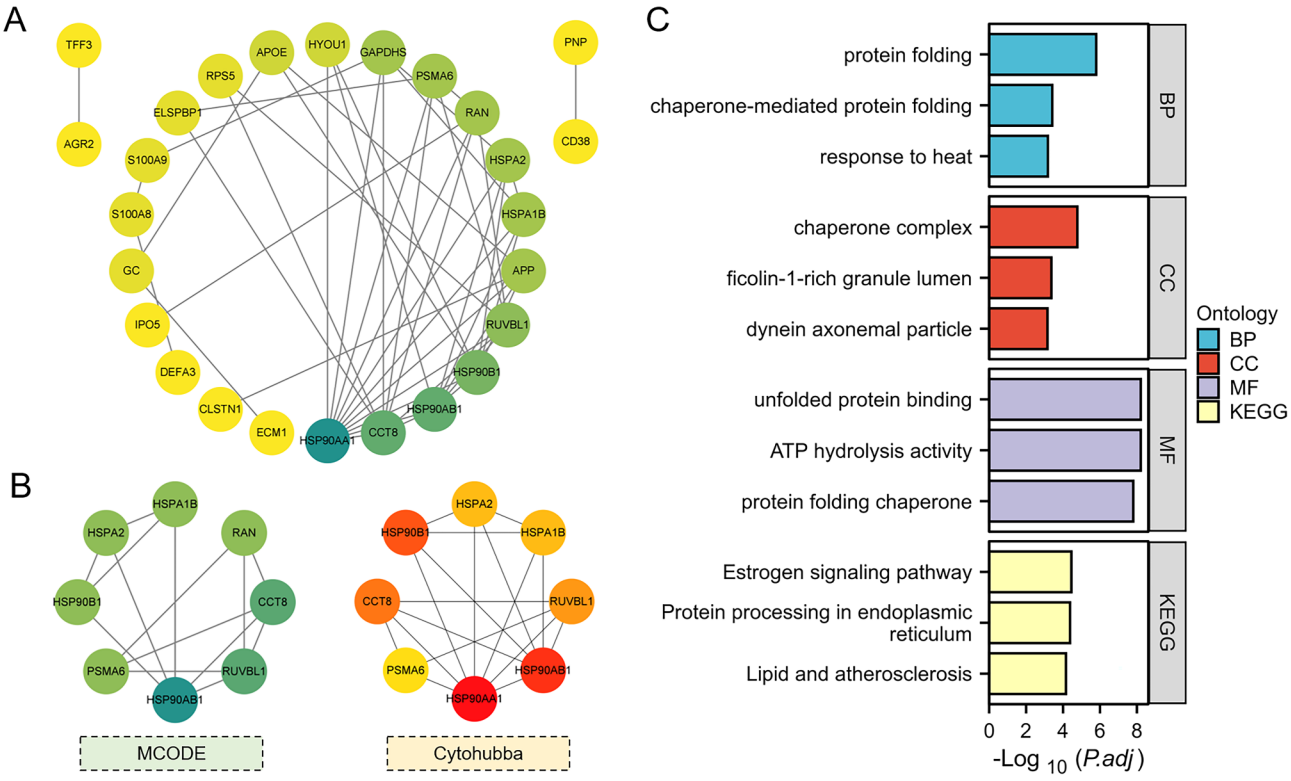

Fig. 4 Interaction network analysis of differentially expressed proteins in seminal plasma. (**A**) Construction of the protein-protein interaction network. (**B**) Screening of hub proteins by MCODE and Cytohubba algorithms. (**C**) Functional enrichment analysis of common hub proteins

capacitation and acrosome reaction, as well as protect damage from inflammation and oxidative stress [36, 37]. Beyond EVs, intracellular vesicular transport, a fundamental process to maintain the homeostasis of membrane-enclosed organelles in eukaryotic cells, is also crucial for spermatogenesis [38]. For example, the acrosome is an organelle that develops from the Golgi apparatus, in which vesicle trafficking facilitates the assembly and secretion of acrosomal enzymes and proteins [39]. Therefore, the disruption may lead to impaired sperm development and fertility issues. However, the detailed mechanisms remain to be investigated how COVID-19 affects this expression change.

KEGG and Reactome analyses revealed that the post-translational protein phosphorylation and PI3K-AKT pathway were enriched. The PI3K-AKT signaling is involved in many stages of male reproduction, including the proliferation and differentiation of spermatogonia and somatic cells, the regulation of sperm autophagy, as well as testicular endocrine function [40]. Sperm phosphoproteome profiling in asthenozoospermic patients also demonstrated that the PI3K-AKT pathway is one of the most important pathways in sperm motility [41], and targeted treatment with granulocyte-macrophage colony-stimulating factor treatment improves sperm parameters [42]. In addition, prior proteomic study showed that around 12.5% of the ~12 000 screened phosphorylation sites were significantly changed in response to SARS-CoV-2 infection, suggesting the active involvement of a variety of kinases including AKT [43]. Li et al. [44] also demonstrated that SARS-CoV-2 could upregulate the intracellular reactive oxygen species and subsequently inhibit PI3K-AKT-mTOR axis, leading to the inflammatory response and apoptosis in infected cells. Hence, the disrupted PI3K-AKT pathway may be a link between COVID-19 and sperm dysfunction as well.

Among the 14 downregulated DEPs in COVID-19 seminal plasma, some have been reported to play a crucial role in reproductive function, such as ECM1 and IPO5. ECM1 is a ubiquitous extracellular matrix protein that interacts with a variety of structural proteins to maintain tissue integrity [45, 46]. In the male reproductive tract, ECM1 is highly expressed in epididymis and has been used a biomarker for the differential diagnosis of obstructive and nonobstructive azoospermia [47]. In addition, ECM1 level in mouse epididymal adipose tissue was shown to be altered after high fat diet, which could result in a decreased sperm motility [48]. Another example is the importin protein IPO5, which is easily detected in postnatal mouse testis and responsible for the transport of cytoplasmic cargo proteins into the nucleus [49]. During early spermatogenesis, IPO5 facilitates the nuclear translocation and further promotes the release of RNA-binding targets of Musashi-1, a critical regulator

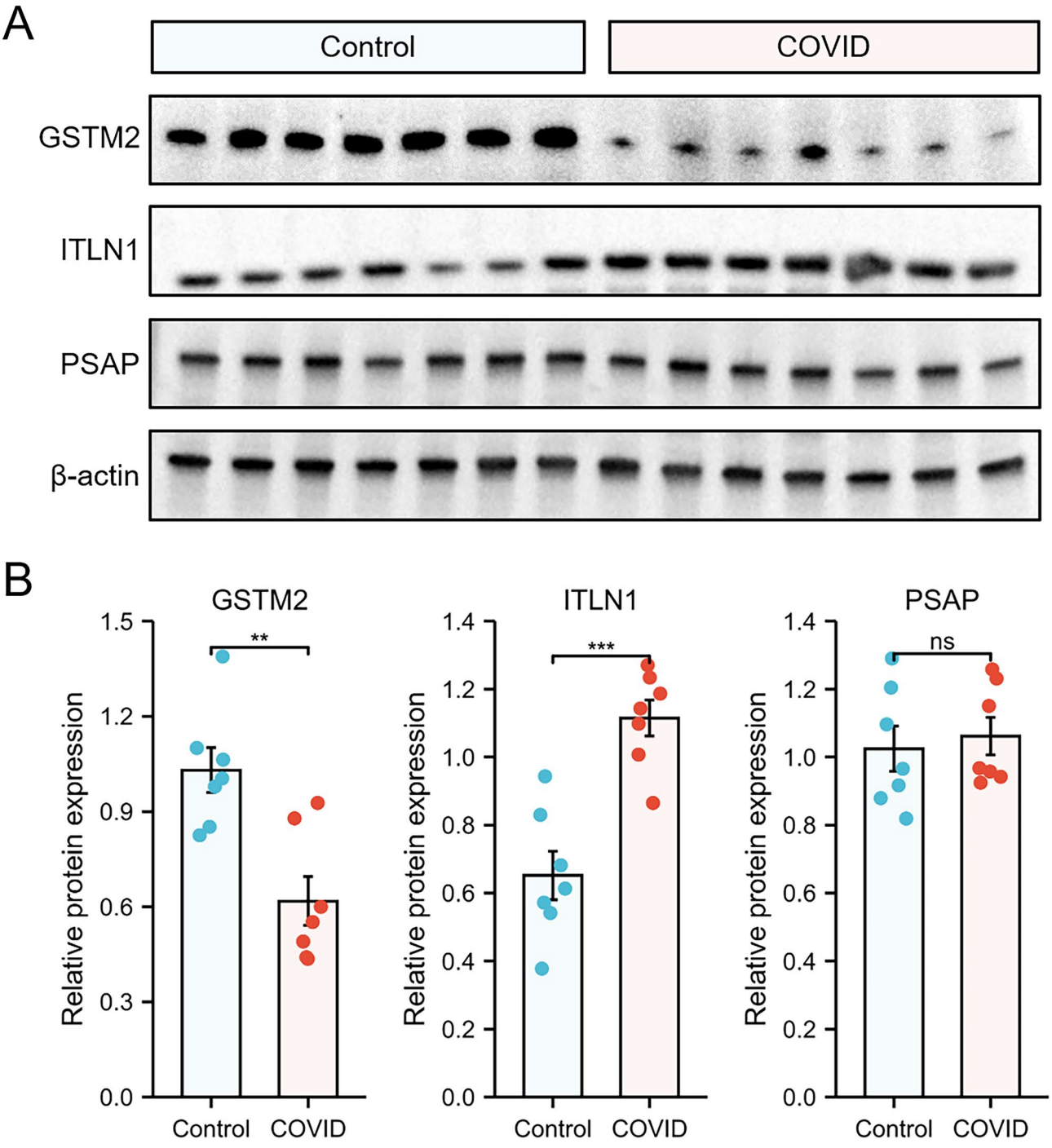

Fig. 5 Verification of protein expression in the validation cohort. (**A**) Western blot showing protein abundances of GSTM2, ITLN1, PSAP, and β-actin in seminal plasma of COVID-19 ($n=7$) and control ($n=7$) groups. (**B**) Relative protein quantification of GSTM2, ITLN1, and PSAP levels normalized to β-actin. ** $p<0.01$, *** $p<0.001$

of post-transcriptional control [50]. It also increases the BMP4 signaling activity and responsiveness, thus modulating the survival, proliferation, migration, and differentiation of germ cells [51]. These results indicate that the decrease of ECM1 and IPO5 may adversely affect sperm parameters in case of acute asymptomatic SARS-CoV-2 infection.

In hub protein analysis, we found that a series of heat shock protein (HSP) were clustered with significantly upregulated expression, including HSPA1B, HSPA2, HSP90AB1, and HSP90B1. As a highly conserved

molecular chaperone, HSP is effectively expressed under the stimulative conditions of trauma, infection and tumor [52]. It functions primarily through interacting with substrate proteins, thereby promoting their folding and activity [52]. Under different physiological and pathological conditions, mounting evidences have demonstrated its pivotal role in regulating various cellular processes, such as DNA damage, cell cycle progression, and gene expression [53]. Similarly, HSP is involved in different stages of spermatogenesis [54]. Clinical studies have shown that HSPA1B rs1061581 gene polymorphism is associated with susceptibility to idiopathic male infertility in the Iranian population [55], while HSPA2 expression is negatively correlated with sperm concentration [56, 57]. After acute heat stress (43 °C for 0.5 h), the elevation of HSP90AB1 protein could reduce viability, promote apoptosis, and affect metabolite production of Sertoli cells, thus possibly affecting sperm development [58]. Therefore, HSP may play an important role in the decline of male fertility induced by COVID-19.

The other three hub proteins (ITLN1, CCT8, and RUVBL1) also increased significantly after infection. ITLN1, or named as omentin-1, is an adipokine with anti-inflammatory and anti-oxidative stress properties. In seminal plasma, ITLN1 mainly originates from seminal vesicles and its levels are negatively correlated with sperm concentration, progressive motility, normal morphology, and vitality. Compared with fertile men, those with genitourinary infection, varicocele and idiopathic infertility also showed elevated ITLN1 concentration [31]. CCT8 is a subunit of the chaperonin containing T-complex protein 1 (CCT), a heterooligomeric complex that ensures proper folding of actin, tubulin, and mitosis regulators [59]. The CCTs are predominantly localized in centrosomes and microtubules during the process of spermatogenesis and discarded in residual bodies at spermiation [60]. Therefore, CCT8 serves as an indicative marker for the identification of immature sperm population. Higher CCT8 levels in semen samples have been reported to be associated with a lower fertility index or conception rate in bulls [61, 62]. RUVBL1 exhibits ATP-dependent DNA helicase activity and plays a role in various transcription complexes and histone modifications [63, 64]. It is located in the sperm flagellum and has been found to interact with the testis/sperm-specific small kinetochore-associated protein (SKAP1) [65]. Moreover, RUVBL1 forms part of the R2TP complex, functioning as a co-chaperone alongside HSP90 [66]. In sperm proteomics, RUVBL1 is positively correlated with sperm count and its abundance level is lower in oligoasthenozoospermic men [67]. Consistently, our study in seminal plasma showed a higher concentration after SARS-CoV-2 infection, suggesting a disruption of sperm structure or apoptotic stimulation.

To our knowledge, this is the first study to assess the seminal plasma proteome in acute asymptomatic COVID-19 patients, yet several limitations should be acknowledged. Firstly, as a preliminary study, our sample size is relatively small and should be expanded with additional multi-center cohorts. Long-term follow-up should also be conducted to assess the fertility outcomes of infected patients after recovery from COVID-19. Moreover, parameters beyond semen quality are not fully considered for male reproductive health, such as scrotal pathology by Doppler ultrasound, comprehensive sex hormone profile, and sperm DNA fragmentation. Secondly, no in-depth functional investigation was conducted on the novel proteins identified. For example, we first reported the significant correlation of CLSTN1 with semen parameters, while the detailed mechanisms remain unknown. Thirdly, over 4200 proteins have been identified in human seminal EVs [68, 69], contributing to around 3% of the total associated seminal plasma proteins [36]. However, we did not distinguish them from other free proteins. Given the importance of EVs in modulating sperm function via cargo transfer [36], future studies should simultaneously isolate seminal EVs for a more specialized proteomic analysis. Lastly, seminal plasma also contains a variety of metabolites (e.g., lipids, amino acids, and carbohydrates) involved in sperm physiology and fertility disorders. In this regard, integrated analyses of proteomics and metabolomics may provide a more comprehensive overview.

Conclusion

In conclusion, our study showed that acute asymptomatic COVID-19 could impair semen quality and induce proteomic dysregulation of seminal plasma without direct viral infection. A total of 37 differential proteins were identified, which were functionally enriched in infection, inflammation, and immunity-related pathways as well as spermatogenesis-associated biological process. These findings should enlighten the physicians about the adverse effects of SARS-CoV-2 infection on male reproduction, and provide valuable resources for the reproductive biologists to further decipher the molecular pathomechanisms.

Abbreviations

ACE2	Angiotensin-converting enzyme 2
AGC	Automatic gain control
CASA	Computer-assisted semen analysis
CCT	Chaperonin containing T-complex protein 1
COVID-19	Coronavirus disease 2019
DEP	Differentially expressed protein
EV	Extracellular vesicle
GO	Gene Ontology (GO)
GSTM2	Glutathione S-transferase mu 2
HSP	Heat shock protein
ITLN1	Intelectin-1
KEGG	Kyoto Encyclopedia of Genes and Genomes

MIT	Maximum ion injection time
PPI	Protein-protein network
PSAP	Prosaposin
SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
TMPRSS2	Transmembrane protease serine 2

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions
N.S., H.Y.C., and X.W.W. were responsible for study conceptualization and design. J.L.H., Y.W.Z., H.C., and X.X.W. collected and handled semen samples. J.L.H. and J.W.W. conducted the patient data curation and statistical analyses. J.L.H., Y.F.S., and J.W.W. analyzed the proteomic data and wrote the original draft. J.L.H. and Y.F.S. performed the Western blot validation experiment. Z.F. and Y.H.X. contributed to results interpretation and discussion. N.S., H.Y.C., and X.W.W. supervised the project administration and edited the manuscript. All authors read and approved the final manuscript after critical review and revision for intellectual content.

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Data availability
The dataset analyzed in the present study has been submitted to the Integrated Proteome Resources (iProX; https://www.iprox.cn/) with Project ID (IPX0008343000).

Declarations

Ethics approval and consent to participate
The study was approved by the Reproductive Medicine Ethics Committee of Jiangxi Maternal and Child Health Hospital, and conducted in accordance with the Declaration of Helsinki. All patients signed informed contents before participation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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