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Melatonin improves the ability of spermatozoa to bind with oocytes in the mouse

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ABSTRACT

Context and aims. Melatonin is a powerful antioxidant regulating various biological functions, including alleviating male reproductive damage under pathological conditions. Here, we aim to analyse the effect of melatonin on normal male reproduction in mice. **Methods.** Male mice received an intraperitoneal injection of melatonin (10 mg/kg body weight) for 35 consecutive days. The testis and epididymis morphology, and epididymal sperm parameters were examined. PCNA, HSPA2, SYCP3, ZO-1 and CYP11A1 expressions in epididymis or testis were detected by immunohistochemistry or Western blotting. Male fertility was determined by *in vivo* and *in vitro* fertilisation (IVF) experiments. The differentially expressed sperm proteins were identified by proteomics. **Key results.** No visible structural changes and oxidative damage in the testis and epididymis, and no significant side effects on testis weight, testosterone levels, sperm motility, and sperm morphology were observed in the melatonin-treatment group compared with the control group. Spermatogenesis-related molecules of PCNA, SYCP3, ZO-1, and CYP11A1 showed no significant differences in melatonin-treated testis. However, PCNA and HSPA2 increased their expressions in the epididymal initial segments in the melatonin-treatment group. Normal sperm fertilisation, two-cell and blastocyst development were observed in the melatonin-treated group, but melatonin significantly enhanced the sperm binding ability characterised as more sperm binding to one oocyte (control 7.2 ± 1.3 versus melatonin 11.8 ± 1.5). Sperm proteomics demonstrated that melatonin treatment enhanced the biological process of cell adhesion in sperm. **Conclusions and implications.** This study suggests that melatonin can promote sperm maturation and sperm function, providing important information for further research on the physiological function and protective effect of melatonin in male reproduction.

Keywords: epididymis, male fertility, melatonin, proteomics, reactive oxygen species, spermatogenesis, sperm maturation, sperm quality.

Introduction

Melatonin is an endogenous neurohormone mainly synthesised by the pineal glands, which regulates sleep cycles and circadian rhythms (Sun *et al.* 2020). It also performs antioxidant, anti-inflammatory, free radical removal and other functions, and participates in the regulation of various biological functions through these roles (Bruni *et al.* 2015). These major properties make it useful in the regulation of pathophysiological mechanisms of neurological diseases, respiratory diseases, older adult disorders, and so on (Alamdari *et al.* 2021; Mao *et al.* 2021; Wang *et al.* 2021).

As a natural health product and supplement, melatonin is readily purchased from drugstores and pharmacies, and is commonly used for sleep-related disorders treatment such as anxiety, insomnia and jet lag (Erland and Saxena 2017). However, the exact pharmacological dose, duration, and differences of melatonin in normal physiological functions are not clear (Claustrat and Leston 2015).

Melatonin is synthesised not only by the pineal gland, but also by reproductive organs (Huai *et al.* 2012). It is also thought to play an important role in reproductive development and regulation (Zi *et al.* 2022). Due to living habits, work pressure, environmental pollution and other factors, more and more men have sub-health status, and the age of marriage and child bearing is getting older (Whiteford *et al.* 2013). All these factors lead to an increase in male infertility year by year. Male infertility is now a global problem that attracts increasing social attention (Barratt *et al.* 2017). Oxidative stress (OS) plays a main role in most sub- or infertility cases caused by male factors (Gibb *et al.* 2020). Reactive oxygen species (ROS) are derived from cell metabolism or other biological processes, and they can act as mediators and regulators of cell metabolism and apoptosis (Huang *et al.* 2020). Appropriate production of ROS is crucial for sperm function, such as sperm capacitation and sperm-egg recognition, and binding (Gualtieri *et al.* 2021). However, excessive production of ROS will cause sperm damage leading to defective sperm function, which may result from the effects of ROS on spermatogenesis in the testis, sperm maturation in the epididymis, and sperm itself (Gibb *et al.* 2020). Melatonin could ameliorate reproductive dysfunctions in males associated with pathological conditions and toxicant exposure (Rocha *et al.* 2015). Melatonin also has been used to regulate sleep cycles and the other systemic diseases, and it also serves as a promising antioxidant for improving male sub-fertility (Tordjman *et al.* 2017; Zi *et al.* 2022). As a commonly used antioxidant, the effects of melatonin in reproductively normal males have received less attention.

In the present study, we investigate the effects of melatonin application on spermatogenesis and sperm maturation, and explore its effect on male fertility by evaluating sperm quality. The application of melatonin in the physiological state can promote mice sperm quality to a certain extent, which provides useful information for in-depth study of the molecular mechanism of melatonin in male reproduction, and the role of melatonin application in male reproductive protection related to various oxidative stress.

Methods

Animal experiments

Six-week-old male C57 mice (about 20–22 g) were purchased from Beijing Vital River Laboratory Animal Technology company and raised in specific pathogen free (SPF) environment under stable temperature 22 (± 2)°C, and humidity 45 (± 5) % in a 12 h/12 h light/dark cycle. All mice were given free access to food and drinking water. All experiments were approved by the Medical Ethics Committee of Yantai Yuhuangding Hospital.

Twenty mice were randomly divided into control and melatonin-treated groups with 10 mice in each group. Mice (n = 5) were kept in one cage. Mice in the control group received an intraperitoneal injection of 100 μL normal saline (0.9% NaCl with 0.5% ethanol) daily for 35 days, while mice in the melatonin-treated group received an intraperitoneal injection of melatonin (10 mg/kg body weight, dissolved in 0.9% NaCl + 0.5% ethanol) daily for 35 days. The dosage and duration of melatonin treatment referred to our previous report (Wang *et al.* 2022*a*; Zi *et al.* 2022), and the administration of melatonin was performed at 9 am every day. On the 35th day, all mice were killed (1.25% 2,2,2-tribromoethanol sterile anesthetic, 0.2mL/kg, intraperitoneal injection), one of the testes and epididymides of each mouse were collected for fixation in Bouin's solution (HT10132, Sigma, St. Louis, MO, USA) for immunochemistry staining and microscopic evaluation, and the other testis and epididymis were collected for sperm preparation and mRNA and protein extraction. Blood was collected for serum testosterone detection. The serum testosterone levels were measured using Unicel DXI800 automatic chemiluminescence immunoassay analyser (Beckman Coulter, Brea, CA, USA). Testosterone reagent kit was used according to manufacturer's instructions (Elecsys Testosterone, Roche Diagnostics GmbH, Mannheim, Germany). The relative weight of the testis was indicated as g/g relative to body weight. The cauda epididymal spermatozoa was collected by cutting the cauda epididymis tubules into small pieces to release the spermatozoa into pre-warmed sperm washing buffer (PSW-100, Nidacon, Molndal, Sweden). The sperm parameters were evaluated by computer-assisted sperm analysis (CASA) (Hamilton Thorne, Beverly, MA, USA). The abnormal sperm including curved back sperm head and bent sperm midpiece were averagely counted from five control and five melatonin-treated mice, respectively. The observations were made by two researchers under a light microscope (DM LB2, Leica, Nussloch, Germany) at the magnification of 40×. About 400 sperm were counted for each mouse.

Sperm capacitation and acrosome reaction assay

The sperm capacitation status was evaluated by using chlortetracycline (CTC) staining assay. Briefly, the cauda epididymal sperm (10^6 sperm/mL) from five control or five melatonin-treated mice were collected and incubated in capacitation medium (C-TYH medium, 72021, SUDGEN, Nanjing, China) at 37°C, 5% CO_2 for 60 min for sperm capacitation. The percentage of hyperactivated sperm were evaluated by using CASA detection between control and melatonin-treatment mice. The criteria that define hyperactivated sperm was that: curvilinear velocity (VCL) >150 μm/s, lateral head displacement (ALH) >7.0 μm, and linearity coefficient (LIN) <50%. Then 100 μL sperm suspension were incubated with CTC solution (750 mmol/L CTC in 130 mmol/L NaCl, 5 mmol/L cysteine, 20 mmol/L Tris-HCl, pH 7.8) for 10 min, and the nuclei were stained by DAPI solution. Then the sperm suspension was fixed by 4% paraformaldehyde and CTC staining patterns were observed by using an Axio Observer.Z1/7 fluorescence

microscope (Carl Zeiss, Inc., Oberkochen, Germany). At least 200 spermatozoa were evaluated in each slide and classified into three staining patterns: pattern F of non-capacitated sperm with green fluorescence staining over the sperm head; pattern B of capacitated sperm with no staining in post-acrosomal region; pattern AR of acrosome reacted sperm with no acrosome staining. The capacitation status was displayed as the percentage of pattern B sperm.

For acrosome reaction assay, the capacitated sperm suspension was treated with 10 μm A23187 ionophore in 0.1% (v/v) dimethyl sulphoxide at 37°C, 5% CO_2 for 30 min, then stained by FITC-PSA (L0770, Sigma, St. Louis, MO, USA) for 30 min and the nuclei were stained by propidium iodide (PI), and washed with phosphate buffered saline (PBS) before mounting on slides for evaluation under Axio Observer.Z1/7 fluorescence microscope. Average 200 spermatozoa were calculated for each side.

Western blotting

Protein extractions were performed by grinding the testis tissues with liquid nitrogen, and dissolving by Radio Immunoprecipitation Assay (RIPA, 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) lysis buffer. After centrifugation and protein concentration measurement by bicinchoninic acid (BCA) protein quantification kit (D131, Shandong Cellgene Technology Co., Yantai, China). A total of 50 μg proteins from each sample were loaded in 12% gels for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyvinylidene difluoride (PVDF) membranes were used for protein transfer at 100 V for 1 h, then was blocked with 5% (w/v) skimmed milk for 1 h at room temperature (RT) and incubated with primary antibody (anti-PCNA, ab92552; anti-SYCP3, anti-ab97672; anti-CYP11A1, ab272494; anti-ZO-1, ab221547, Abcam, Cambridge, UK; HSPA2, DF8101; anti-ACTB, AF7018, Affinity Biosciences, JiangSu, China) at 4°C overnight. The membranes were washed three times with 0.5% (v/v) Tween-20 in Tris-buffered saline (TBS) and then incubated with appropriate HRP-conjugated secondary antibody (ZB2305, Zhong-Shan Golden Bridge, Beijing, China; 1:5000) at RT for 1 h. After being washed by Tris-buffered saline with Tween-20 (TBST), the protein bands were detected by an ECL kit (KF8001, Affinity Biosciences) using ChemiScope 6200 Touch (CLINX Science Instruments, Shanghai, China) and quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA) using ACTB as the loading control. The relative expression of each protein was calculated by recording its average grey values normalised to the value of ACTB. The experiments were repeated at least three times.

Histological and immunohistochemical (IHC) assay

Paraffin embedded epididymis specimens which were obtained from five mice in the control group and five mice in the melatonin-treated group were cut into 5 μm thick sections, then were placed in xylene and ethanol for dewaxing and dehydration. For histological examination, the sections were stained with hematoxylin and eosin (H&E), and the morphological structures were observed under a light microscope (DM LB2, Leica). For immunohistochemical staining, after antigen repair in citrate buffer solution (0.01 M, pH 6.0) using a microwave under medium heat for 20 min, the sections were blocked with 3% bovine serum albumin (BSA). Then the sections were incubated with primary antibody (anti-PCNA, ab92552, Abcam; HSPA2, DF8101, Affinity Biosciences) at 4°C overnight. After washing in PBS buffer for three times, the sections were incubated with HRP-conjugated secondary antibody (ZB2305, Zhong-Shan Golden Bridge; 1:400) for 1 h at 37°C. 3,3′-diaminobenzidine (DAB) kit (ZLI-9018, Zhong-Shan Biotechnology, Beijing, China) was used to display the peroxidase active sites, and hematoxylin counterstain was performed to stain nuclei. The sections were dehydrated and examined under light microscopy (DM LB2, Leica). Pre-immune IgG was used as the negative control. The staining was performed on two epididymis sections from each mouse. The average intensity of positive staining in initial segment epididymis was recorded by ImageJ software and statistically compared by GraphPad Prism 8 (GraphPad Prism, La Jolla, CA, USA).

Measurements of oxidative enzymes

The homogenates of mouse testis, caput and cauda epididymis were prepared according the manufacturer's instructions (Beyotime, Shanghai, China). The total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) levels were examined. Optical density (OD) values were read using microplate reader (Varioskan, Thermo Scientific, Shanghai, China) and analysed by using GraphPad Prism 8 software.

Male fertility assay

Male mice fertility was assessed by mice mating experiment and *in vitro* fertility (IVF) assay. At the endpoint of melatonin treatment, five mice were randomly selected from each group, and each male mouse mated with two female mice in oestrus at 5 pm and were examined whether there was a vaginal plug formation the next morning. Female mice with vaginal plugs were kept alone. The ratio of pregnancy in female mice was assessed by comparing the number of pregnant females with the number of females having vaginal plug, and the number of offspring produced by pregnant females was recorded.

Normal female mice were super-ovulated via intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) at 48 h intervals. Cumulus oocyte complexes (COCs) were obtained after 14–17 h of injection of hCG. After cervical dislocation of female mice, COCs were transferred to microscopic droplets of 200 μL HTF medium (72002, SUDGEN, Nanjing,

China), which were incubated at 37°C with saturated humidity, and 5% CO_2. Caudal epididymis obtained sperm were transferred to pre-incubated C-TYH medium (72021, SUDGEN) for 30 min, which were incubated at 37°C with saturated humidity, and 5% CO_2. About 10^4 sperm were transferred into HTF medium containing oocytes. After incubation for 4 h, fertilised oocytes were washed and transferred into a KSOM medium (M1430, AIBEI, Nanjing, China). The average numbers of sperm binding to oocyte were counted. About 35 oocytes were obtained from each female mouse. Fertilisation rate was displayed as the percentage of the number of pronucleus formation oocytes/M II oocytes. The percentage of the two-cell embryos/the number of pronucleus formation oocytes was considered the embryo development rate, and the percentage of the blastocyst/ the number of the two cell embryos was considered the blastocyst formation rate.

Quantitative proteomics analysis of melatonin-treated mice sperm

The Isobaric Tags for Relative and Absolute Quantification (iTRAQ) procedures were performed according to our previous publication (Liu *et al.* 2019). Briefly, sperm proteins from five control or melatonin-treated mice were extracted and pooled, respectively. For each mouse, 200 μg sperm proteins were collected and pooled in each group. Finally, 400 μg pooled sperm proteins in each group was used for labelling and identification experiments. After treatment with 20 mM dithiothreitol (DTT) at 56°C for 1 h and 50 mM iodoacetamide in the dark for 30 min, the samples were digested with 3 μg trypsin (sequencing grade; Promega, Madison, WI, USA) at 37°C overnight, and the obtained peptides were labelled with iTRAQ isobaric tags. The first dimensional separation by microLC was conducted by using Durashell RP column (5 μm, 150 Å, 250 mm × 4.6 mm i.d., Agela, Tianjin, China). A total of 10 fractions were collected for further LC-MS/MS analysis by using nanoflow HPLC instrument (EASY-nLC 1000 system, Thermo Fisher Scientific, Waltham, MA, USA) linked with an online Q Exactive mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific) was used. The raw data were processed by using the proteomic workflow of Proteome Discoverer 2.1 and the Mascot search engine (ver. 2.6) with the precursor and fragment mass tolerances set to 15 ppm and 20 milli-mass units (mmu), respectively. The algorithm was set to use trypsin as the enzyme, allowing for two missed cleavage sites. The fixed modification was carbamidomethylation (cysteine), and the variable modifications were oxidation (methionine), acety-lation (protein N terminus), and iTRAQ labelling (tyrosine and lysine, N-terminal residues). Peptide ions were filtered from the cut-off scores of Percolator based on $P < 0.01$. The false discovery rate was set to 1% for peptide identifications. The iTRAQ quantitative values were automatically calculated, and exported to an Excel file, the Gaussian distribution of ratios was recalculated manually, and all ratios were transformed to base 10 logarithm values. A confidence fold of 1.5 was used to determine the cut-off values for statistically significant changes. Technical MS replicates were performed.

Broad bioinformatics analysis was performed on the identified proteins. The Gene Ontology (GO) analysis including molecular function and biological process was conducted by using online bioinformatics tools of the Database for Annotation, Visualisation and Integrated Discovery (DAVID) tools (https://david.ncifcrf.gov/). A *P*-value of less than 0.01 was selected as significant.

Statistical analysis

Data were presented as mean ± s.d. for triple repeats. All statistical analyses were performed by GraphPad Prism 8. The mean values were determined by Student's *t*-test, and *P*-value less than 0.05 was considered significant.

Results

Melatonin treatment had no significant effects on mice testis and epididymis function

C57BL/6 mice were intraperitoneally injected with melatonin for 35 days, and the reproductive phenotypes were characterised. The values of body weight, testosterone levels, percentage of motile sperm and sperm motility showed no significant changes between the two groups (Fig. 1). Melatonin could increase the sperm progressive motility but not significantly. Sperm capacitation status was evaluated by observing the percentages of CTC-stained pattern B sperm and the hyperactivated sperm calculated by CASA. After sperm incubation in capacitation medium, the percentage of capacitated sperm in melatonin-treatment increased, but not significantly. There were no significant differences in the average VCL, ALH values, and the percentage of hyperactivated and acrosome reacted sperm between two groups (Fig. 2). Melatonin treatment displayed no obvious effects on the testis and epididymis morphology. Although melatonin-treatment increased the proportion of stage VIII seminiferous tubules, there was no significant difference (Fig. 3). The results indicated that melatonin treatment had no significant adverse effects on male reproductive organs and their functions.

Oxidative stress is the main marker of testicular and epididymal injury induced by various adverse factors. The testicular and epididymal superoxide dismutase (SOD), glutathione peroxidase (GSH) and MDA levels between melatonin treatment and control group were determined, which showed no significant alterations (Fig. 4). The results indicated that melatonin did not alter the testicular and epididymal marker enzyme activities.

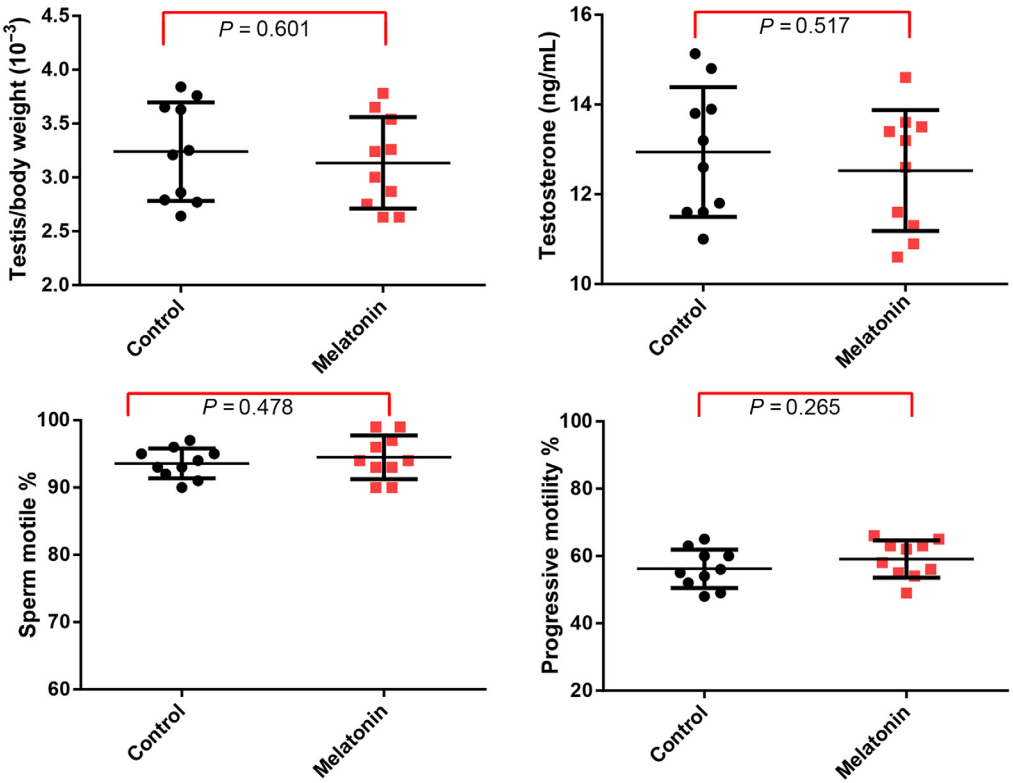

Fig. 1. Characteristics of testis and sperm quality in the control and melatonin treatment groups. Data were presented as the mean ± s.d. of the 10 mice in each group (control and melatonin treated). *P*-value less than 0.05 was considered significance.

Melatonin increased PCNA expression in the epididymal initial segment

To further explore the effects of melatonin on mice spermatogenesis by checking the expressions of marker molecules, key proteins related to spermatogenesis processes were detected. PCNA expression could reflect the proliferation of germ cells of spermatogonia and spermatocytes, and it had an increased expression trend in melatonin-treated mice, but was not significant. SYCP3 reflected the meiosis process, ZO-1 reflected the integrity of the blood-testis barrier, and CYP11A1 reflected the androgen secretion capacity in Leydig cells. They were all detected on testis by Western blot, and showed no significant alterations in melatonin-treated mice (Fig. 5). Meanwhile, the expressions of PCNA and HSPA2 in testicular germ cells were identified by immunohistochemistry. The results showed that PCNA was mainly expressed in spermatogonium cells (Fig. 5*a*), while HSPA2 was mainly expressed in spermatocyte and spermatids (Supplementary Fig. S1). There was no significant difference in their expression levels between the control and the melatonin treated group.

To explore the effects of melatonin on mice sperm maturation, PCNA and HSPA2 expression in mice epididymis were detected by immunohistochemistry. PCNA represented the proliferative activity of epithelial cells. HSPA2 was related to sperm maturation and fertility. The results showed that PCNA and HSPA2 were predominantly expressed in epididymal initial segments, and both had increased expressions in melatonin-treated mice (Fig. 6). These results indicated that melatonin-treatment may promote the proliferation of caput epididymal epithelial cells.

Melatonin treatment enhanced sperm binding ability to oocytes

Male fertility was determined by *in vivo* and *in vitro* fertilisation (IVF) experiments. After caging with normal female mice, no significant differences were found in the vaginal plug formation rate and the numbers of offspring between the two groups (Table 1). IVF results showed that melatonin treatment did not affect sperm fertility following normal two-cell and blastocyst development. While melatonin treatment significantly enhanced the sperm-binding ability to oocytes with more sperm-binding with one oocyte (control 7.2 ± 1.3 versus melatonin 11.8 ± 1.5) (Fig. 7). These results indicated that melatonin treatment could enhance the interaction of sperm with oocytes.

Sperm proteome of melatonin-treated mice

To understand the molecular basis of melatonin-treatment enhancing sperm function, we identified differentially

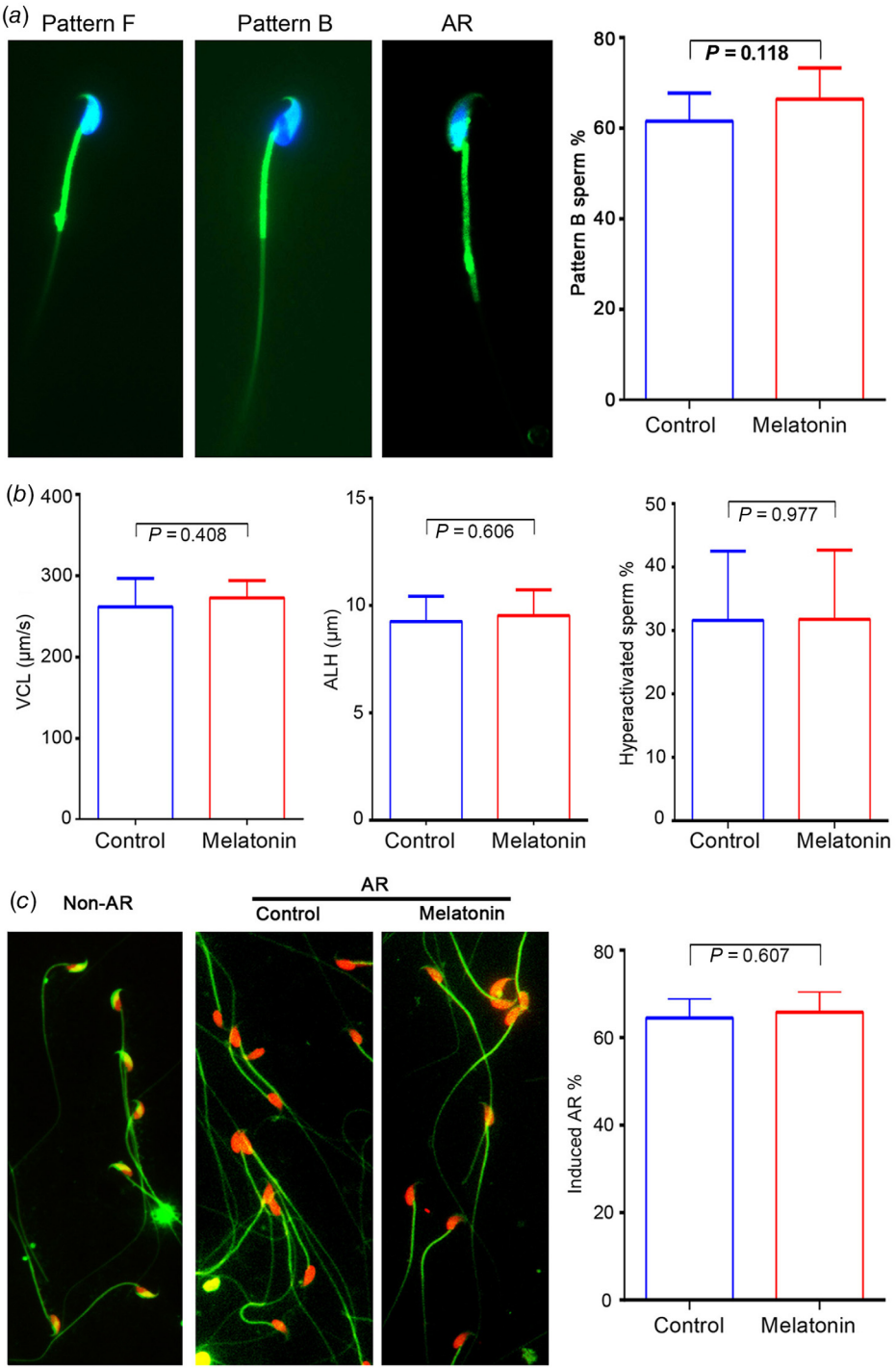

Fig. 2. Characteristics of sperm capacitation and acrosome reaction in the control and melatonin treatment groups. (*a*) CTC fluorescence patterns of sperm with the status of non-capacitation (pattern F), capacitation (pattern B) and acrosome reaction (AR), the percentage of pattern B sperm were calculated; (*b*) the sperm parameters of VCL, ALH and the percentage of hyperactivated sperm were calculated between control and melatonin treatment group; (*c*) the percentage of acrosome reacted sperm were calculated between control and melatonin treatment group; AR, acrosome reaction; Data were presented as the mean ± s.d. of the five mice in each group (control and melatonin treated) and at least 200 spermatozoa were calculated in each mice. *P*-value less than 0.05 was considered significance.

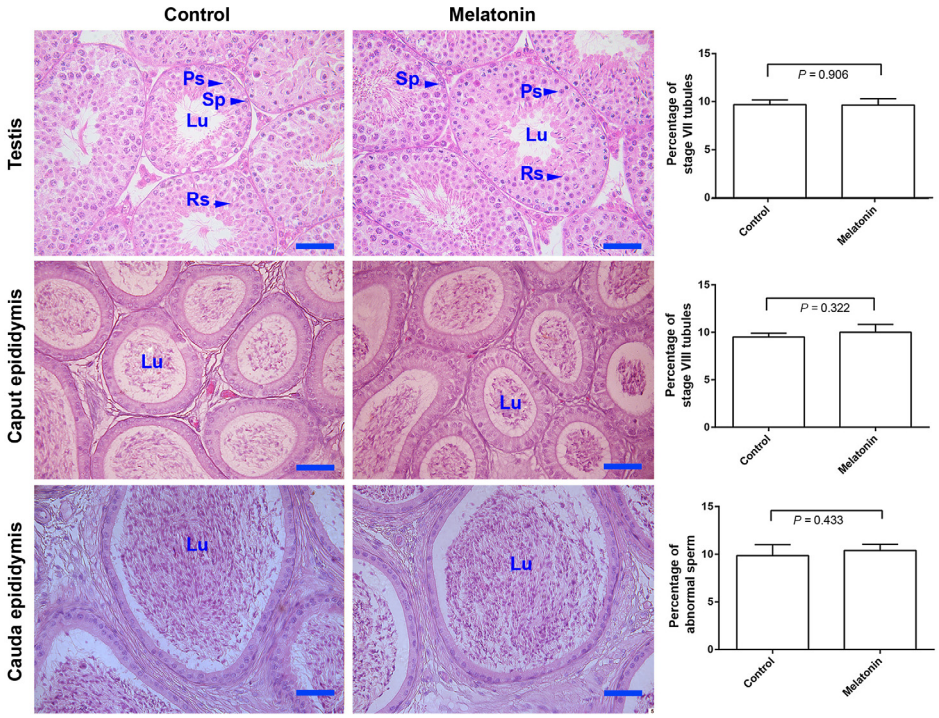

Fig. 3. The effects of melatonin treatment on the morphology of mouse testis and epididymis stained with H&E. Percentages of testicular stage VII and VIII tubules, and abnormal sperm in control and melatonin-treated mice were calculated. Sp, spermatogonia; Ps, pachytene spermatocyte; Rs, round spermatid; Lu, luminal; The data were analysed by *t* test. Data were presented as the mean ± s.d. of the five mice in each group (control and melatonin treated). *P*-value less than 0.05 was considered significance. Scale bars = 50 μm.

expressed sperm proteins between control and melatonin-treated mice by iTRAQ proteomics, and screened 126 differentially expressed proteins, including 43 up-regulated proteins by melatonin treatment (Table S1). A broad functional classification showed that these differentially expressed proteins were mainly related to cell adhesion molecules, defense/immunity, extracellular matrix, protein-binding modulator, translation, transmembrane signal receptor, and transporter. Enriched bioinformatics analysis indicated that up-regulated proteins were mainly involved in the biological processes of cell adhesion, while the biological processes of NF-kb and IL-1 signalling were enriched in the down-regulated proteins (Fig. 8).

Discussion

Melatonin, known as a circadian rhythm regulator, is synthesised in the pineal gland of mammals. It has the capacity for proliferation, anti-inflammatory, and anti-apoptotic properties (Frungieri *et al.* 2017). Melatonin is now used in many pathophysiological fields because of its ability to interact with cells from the immune and cardiovascular systems and its significant role in the regulation of glucose and metabolic disorders (Navarro-Alarcón *et al.* 2014; Cho *et al.* 2021; Wang *et al.* 2022*b*). Because of its ability to cross physiological barriers, such as the blood–testis barrier (Venditti *et al.* 2021), exogenous melatonin can reach the testis in a certain concentration (Álvarez-Fernández *et al.* 2023). Thus, melatonin is now considered an excellent candidate in the field of preventing and/or treating male reproductive dysfunctions caused by oxidative stress (Rocha *et al.* 2015). Although melatonin has low toxicity, there are few reports about their specific effects on normal reproduction functions compared with other treatments of systemic diseases. Here, key molecules associated with spermatogenesis, sperm maturation and sperm functions were analysed to add to the understanding of the molecular basis and mechanisms of melatonin in male reproduction.

In this paper, the effects of melatonin on male reproduction in normal mice were evaluated from three aspects of spermatogenesis, sperm maturation and sperm function. Testis and epididymis maintained the normal morphology in the melatonin-treated mice, indicating that melatonin may have no obvious side effects on the male reproductive system. This was reflected in the cauda epididymal sperm analysis that was not changed significantly by melatonin treatment, including sperm motility, capacitation and acrosome. The spermatogenesis processes were evaluated at the molecular

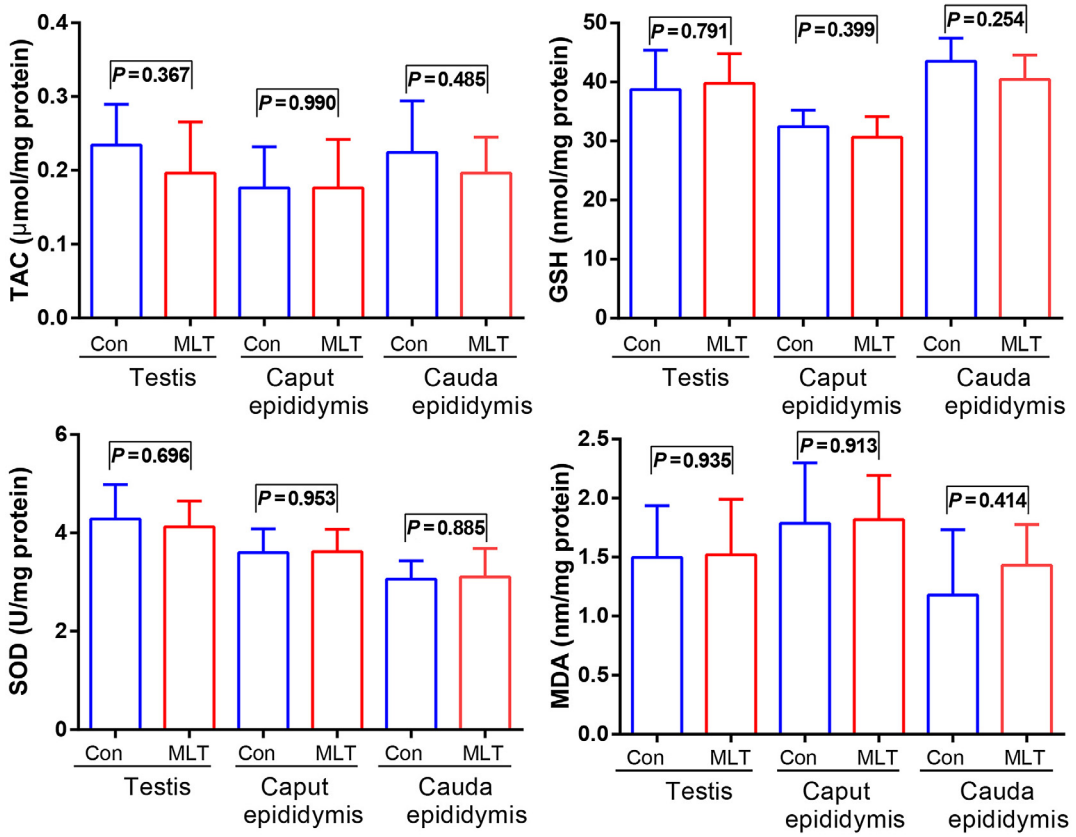

Fig. 4. Detection of antioxidant capacity in mice testis from control and melatonin-treatment groups. Con, control; MLT, melatonin. Data were presented as the mean ± s.d. of the five mice in each group (control and melatonin treated). *P*-value less than 0.05 was considered significance.

level. PCNA, an indicator of germ cell proliferation, was mainly located in spermatogonia and spermatocytes. Up-regulated PCNA expression in spermatogonia and spermatocytes was found in melatonin-treated mice, but with no significant difference. SYCP3 reflects the meiosis process, and ZO-1 reflects the integrity of the blood–testis barrier (Liu *et al.* 2021). Neither SYCP3 nor ZO-1 was affected by melatonin treatment, indicating that melatonin has no adverse influence on spermatogenesis. CYP11A1 is a key enzyme for steroidogenesis in Leydig cells (Liu *et al.* 2021), whose expression was not changed by melatonin treatment which was consistent with the determination of testosterone levels. However, some *in vitro* experiments have shown that melatonin can affect testosterone production in Leydig cells in a dose-dependent manner, the lack of *in vivo* effect of melatonin in the present study may be somewhat attributed to inappropriate dosage used (Deng *et al.* 2018; Li *et al.* 2020; Yang *et al.* 2021). Overall, melatonin treatment had no adverse effect on testis function, as there was no excess ROS production in physiological status, which was measured in testis and epididymis.

The epididymis is an organ for sperm maturation, and spermatozoa produced in the testis acquire the ability of motility and fertilisation during its transit in the epididymal tubule, including the interaction with the sperm maturation microenvironment in a region-dependent manner (James *et al.* 2020). The epididymis is also an organ that is mainly under androgen regulation (Robaire and Hamzeh 2002). No androgen alteration was found in melatonin treatment, suggesting that melatonin may not have a significant effect on epididymis development. However, the epididymal initial segment (IS) is an active segment of epididymis, whose cellular changes could not be reversed by circulating testosterone (Krutskikh *et al.* 2011). The IS, the most proximal part of the epididymis, plays a critical role in sperm maturation. If the IS was not fully developed, sperm showed defective maturation with flagellar angulation/hairpin and reduced motility, which may lead to male infertility (Jun *et al.* 2014). The IS can absorb approximately 90% of the fluid that leaves the rete testis (James *et al.* 2020). Fluid absorption resulted in the increase of sperm concentration, an important factor for male fertility. PCNA was an indicator of cell proliferation, and its high expression was important for the function of the initial segment. As a male fertility marker, HSPA2 plays a key role in the germ cell differentiation, participates in the reconstruction of special sperm domains, and plays an

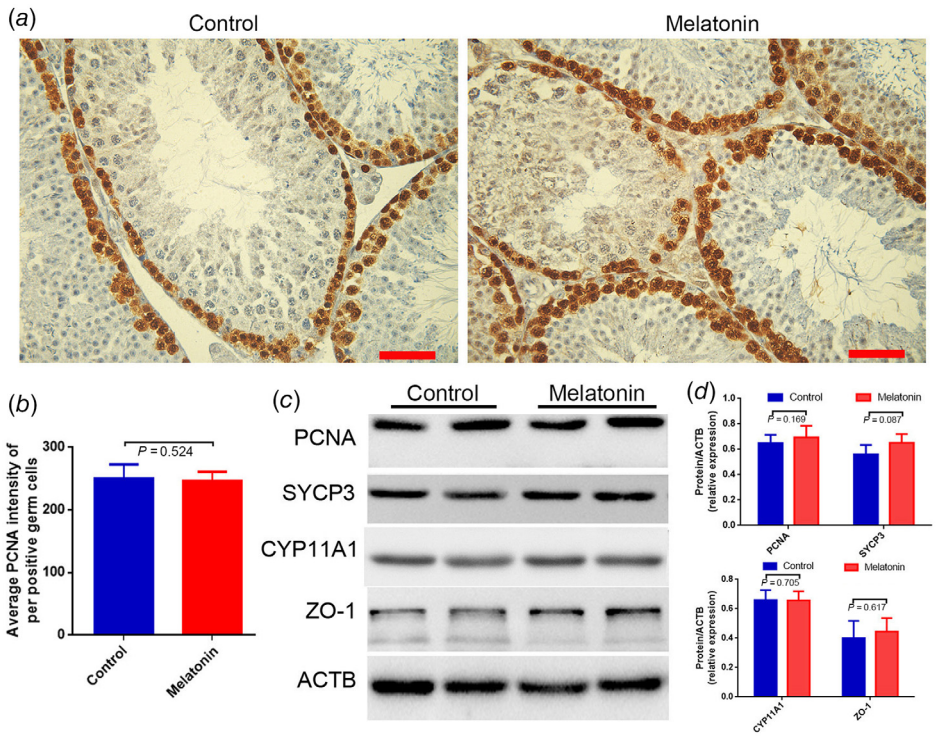

Fig. 5. The effects of melatonin treatment on testicular expressions of PCNA, SYCP3, CYP11A1 and ZO-1. (*a*) Expression of PCNA; (*b*) Quantification of PCNA; (*c*) Western blot analysis of PCNA, SYCP3, CYP11A1 and ZO-1 in triple replication; (*d*) quantification of Western blot results. Data are presented as the mean ± s.d. of the five mice in each group (control and melatonin treated). *P*-value less than 0.05 was considered significance. Scale bars = 50 μm.

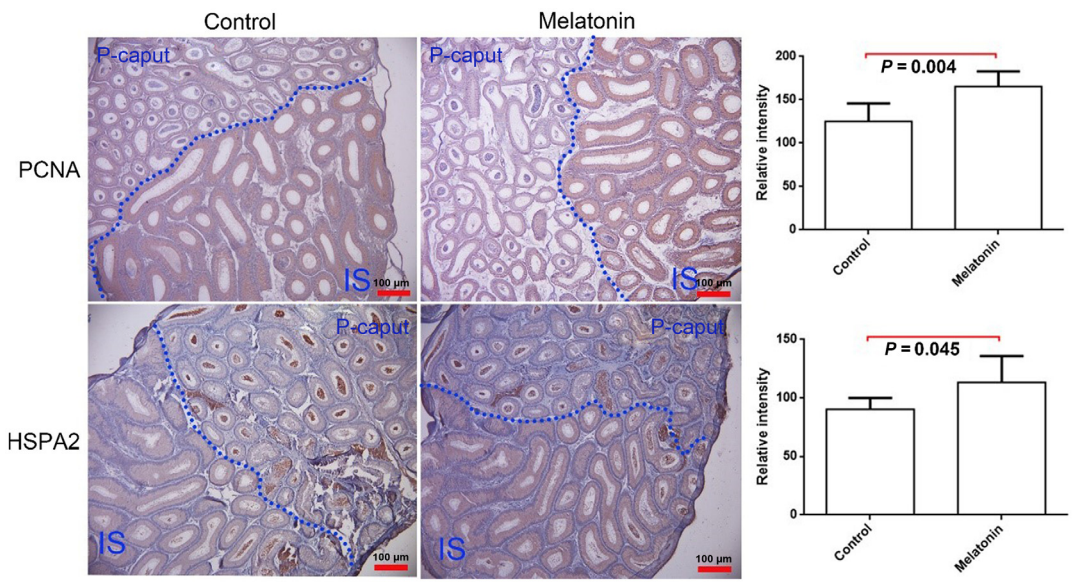

Fig. 6. PCNA and HSPA2 expression in mouse caput epididymis. IS, initial segment; P-caput, proximal caput. The average positive intensity of each tubule at the initial segment were analysed by *t*-test. Data were presented as the mean ± s.d. of the five mice in each group (control and melatonin treated). *P*-value less than 0.05 was considered significance. Scale bars = 100 μm.

Table 1. Fertility and fecundity of normal control and melatonin treated mice.

Group	Male fertility	Litter numbers
Control	10/10	8.46 ± 1.85
Melatonin treated	10/10	8.60 ± 1.72

Each male mouse from the indicated group was caged with two normal female mice, respectively; Male fertility was indicated as the number of pregnant female mice/the number of female mice with vaginal plugs; no significance was observed in the two groups.

important role in sperm and egg recognition (Jannatifar *et al.* 2021). As one important component of epididymal milieu, HSPA2 was identified in caput epididymosomes, which may participate in post-testicular sperm maturation (Nixon *et al.* 2019). Oxidative stress can affect the HSPA2 expression in germ cells and spermatozoa (Bromfield *et al.* 2017). PCNA and HSPA2 showed high expressions in IS in melatonin-treated mice, suggesting that melatonin could enhance the IS development. An *in vitro* sperm function experiment was conducted to study whether melatonin treatment had an

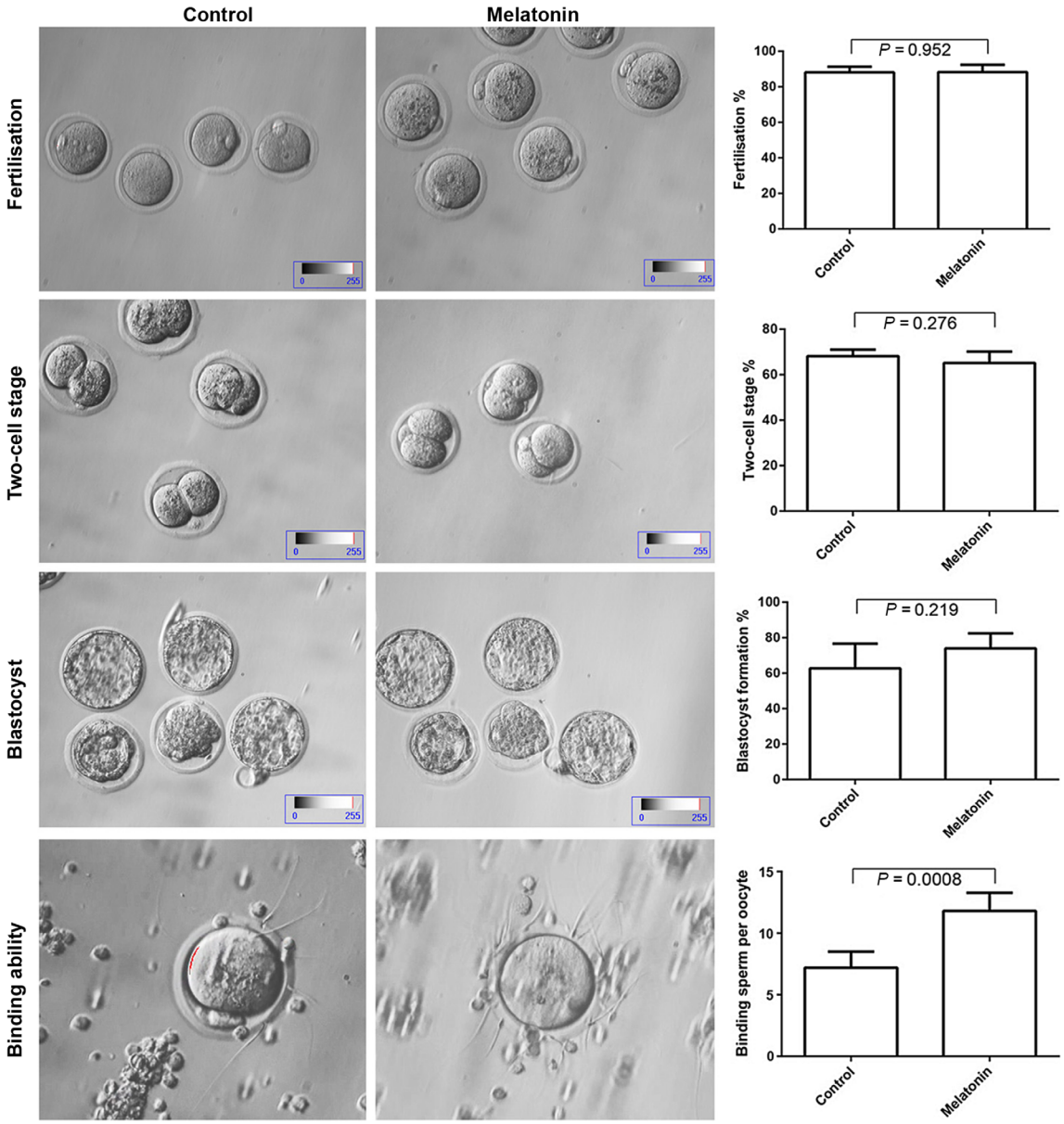

Fig. 7. The effects of melatonin treatment on male fertility ability by IVF analysis. Embryo development rate = the two-cell embryos/the number of pronucleus formation oocyte; blastocyte rate = the blastocyst/the number of the two cell embryos; sperm binding ability was displayed as the average number of sperm binding with one oocyte. Data were presented as the mean ± s.d. of the five mice in each group (control and melatonin treated). *P*-value less than 0.05 was considered significance.

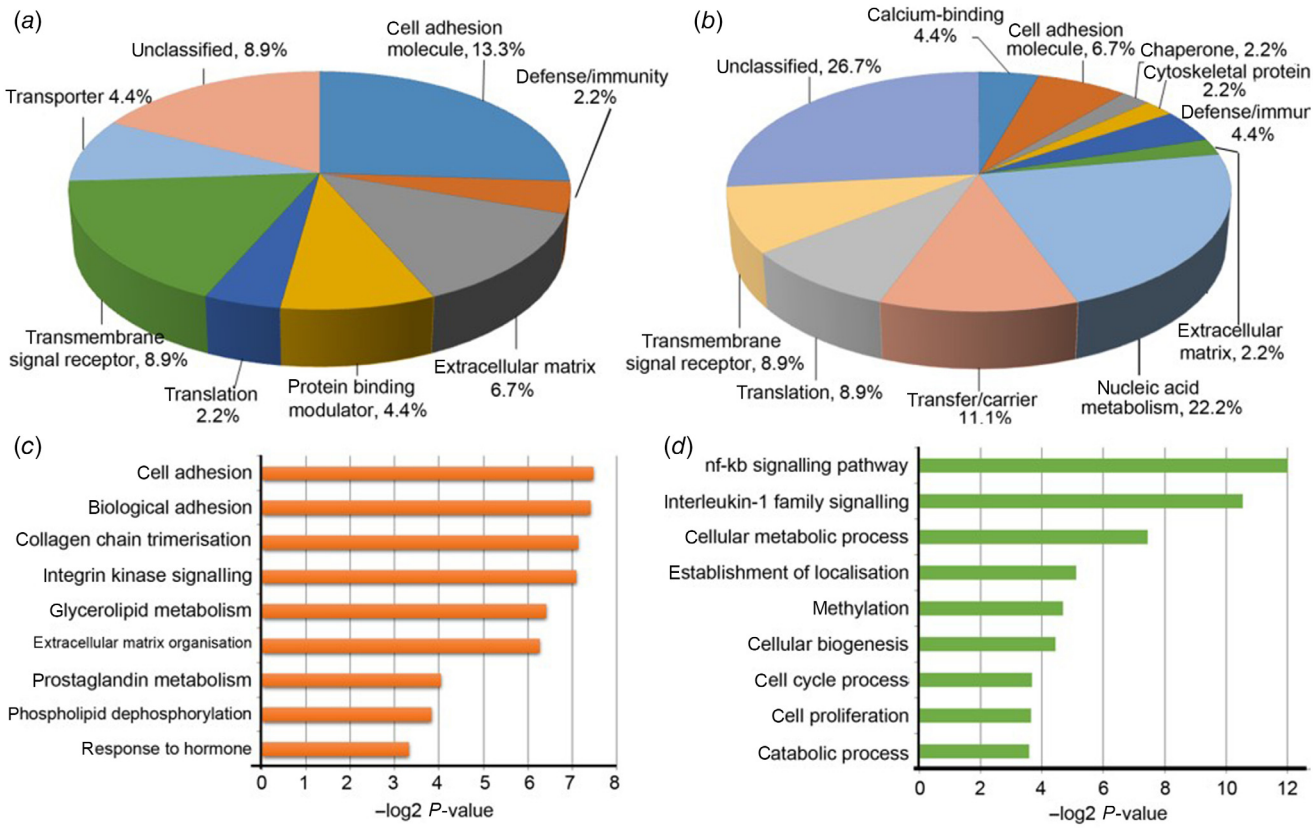

Fig. 8. Bioinformatics analysis differentially expressed sperm proteins in melatonin-treated mice. Functional classification of up-regulated (*a*) and down-regulated (*b*) sperm proteins in melatonin-treated mice ($n = 5$); Over-representative analysis of biology processes of up-regulated (*c*) and down-regulated (*d*) sperm proteins in melatonin-treated mice ($n = 5$).

effect on sperm function. IVF results showed that melatonin treatment had no significant effects on the development of the two-cell stage and blastocyst, but the sperm-egg binding ability was significantly enhanced. This phenomenon was consistent with high expression of HSPA2 and suggested melatonin treatment may enhance the expression of some sperm functional proteins, and thus enhance the sperm-egg binding ability.

In order to further understand the effect of melatonin on sperm function, we conducted a quantitative proteomic analysis on the sperm of melatonin-treated mice. Bioinformatics analysis indicated that the up-regulated proteins in melatonin-treated mice were mainly related to cell adhesion function, which could explain the increased sperm-egg binding ability of melatonin-treated mice. Adhesion molecules on sperm play vital roles for sperm–oocyte interactions. Melatonin significantly enhanced the expressions of collagen family molecules COL1A1, COL1A2, COL5A2, COL6A5, COL12A1, COL14A1 on sperm, which may promote the sperm binding ability to oocyte (Zhou *et al.* 2004; He *et al.* 2005).

Melatonin can play an important role in testis and sperm function either by interacting with its receptors or as a free radical scavenger (Zhao *et al.* 2019). It can protect testis against oxidative damages, chemotherapy drug and environmental toxicants, and also improve sperm quality by affecting Leydig cells and Sertoli cells (Heidarizadi *et al.* 2022). The function of melatonin against oxidative damage, inflammation and apoptosis may be related to the oxidative stress status faced by cells and tissues. This paper studied the effects of melatonin on spermatogenesis, sperm maturation and sperm function in normal mice. The results suggest that melatonin does not have significant effects on testis and epididymis function, but can be helpful for improving the sperm–egg binding function and promoting sperm function, which may be attributed to post-translational modification of sperm that was completed in the epididymis. As for humans, many studies have used therapeutic melatonin to mediate circadian function. Although previous studies have reported exogenous melatonin did not disturb the circadian rhythm or just induced circadian rhythm synchronisation, and only disruption of circadian clock can be directly involved in multiple pathological processes, including male reproduction (Hemadi *et al.* 2012; Laste *et al.* 2013), we will further perform circadian regulation research in the future by adjusting administration time and doses of melatonin to investigate the effects on male reproduction.

Supplementary material

Supplementary material is available online.

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Conflicts of interest. The authors declare that there are no conflicts of interest.

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