DJ-1 deficiency causes metabolic abnormality in ornidazole-induced asthenozoospermia

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Abstract

Asthenozoospermia (AS), defined as low-motility spermatozoa in the ejaculate, is a frequent cause of human male infertility. DJ-1 (also known as PARK7), a protein highly associated with male sterility, binds to the mitochondrial complex I subunit to protect mitochondrial function. However, its involvement in spermatogenesis has not been fully elucidated. Previously, the levels of DJ-1 were shown to be significantly decreased in testicular tissues of rats with ornidazole (ORN)-induced AS. Here, we used a rat model to investigate the localization and expression levels of DJ-1 and its interacting NDUFS3 and NDUFA4 mitochondrial complex I subunits, as well as AS-induced metabolic alterations in testicular tissues. ORN significantly reduced the levels of DJ-1 in the nucleus of secondary spermatocytes, while increasing the expression of NDUFS3 in the cytoplasm of primary spermatocytes. Further, NDUFA4 showed higher expression after treatment with ORN. The principal ORN-induced changes in metabolic small molecules related to the accumulation of glucose, glutamine, and N-acetyl aspartate, enhancement of purine pathway, increase of the phosphatidic acid (PA) (18:0/18:1), phosphatidylethanolamine (PE) (16:0/18:1), and PA (18:0/20:4) lipid metabolites, and imbalance in the concentrations of Na⁺ and K⁺. However, we did not observe any abnormalities of certain small metabolic molecules and metal ions in semen samples from patients with AS. In conclusion, these results suggest that DJ-1 deficiency in testicular tissues might be closely related to the localization of NDUFS3 and content of NDUFA4, thus causing abnormalities in the mitochondrial energy metabolism and multiple other metabolic pathways.

Reproduction (2020) **160** 931–941

Introduction

Asthenozoospermia (AS), a common cause of male infertility in humans (Curi *et al.* 2003), is characterized by decreased sperm motility (sperm motility < 50% or progressive motility < 25%) (WHO 2010), and has been associated with many cellular and molecular defects in the production and maturation of sperm. During natural maturation, sperm gains its motility during epididymal transit. The causes of poor sperm motility include abnormal metabolism of testicular tissues or epididymis, defects in the structure of the sperm tail, and defects in the functions of the epididymis or other accessory gonadal structures (Infante & Huszagh 1985, Ryder *et al.* 1990, Comhaire *et al.* 1999). However, the molecular mechanism of AS has not been fully elucidated.

The DJ-1 (also known as PARK7) protein, encoded by the parkinsonism-associated deglycase (*PARK7*) gene, is highly correlated with male infertility and was first identified as a possible oncogenic product in 1997 (Nagakubo *et al.* 1997). There was a high degree of homology noted between rat contraceptive related protein 1 (CAP-1), mouse sperm protein 22 (SP22), and human DJ-1 (Wagenfeld *et al.* 1998a). After treatment with ornidazole (ORN), epichlorohydrin, or other toxins of the reproductive tract, male rats presented reversible sterility with reduced motility of sperm and decreased expression of DJ-1 in the sperm and epididymis (Wagenfeld *et al.* 1998a,b). Similarly, the amount of SP22 in the cauda epididymal sperm was shown to decrease in mice treated with toxins of the reproductive system, and sperm motility was reported to be subsequently reduced (Klinefelter *et al.* 1997).

It was also reported that DJ-1 was significantly correlated with important sperm parameters in bulls (Boe-Hansen *et al.* 2015). Therefore, it is possible that DJ-1 deficiency in spermatozoa might cause failures in the process of fertilization and thus it is necessary to identify its role in the pathways involved in the production and maturation of sperm.

Over the past decade or so, research on DJ-1 has focused on the mechanisms of its involvement in Parkinson's disease. It has been demonstrated that DJ-1 may act as a modulator of mitochondrial metabolic efficiency and a switch between glycolysis and oxidative phosphorylation (Weinert *et al.* 2019). But there have been few reports of male infertility. In recent years, our research group has conducted a series of studies on the pathogenesis of AS involving DJ-1 using samples of patients with AS, and rat models of the disease, in order to find the interacting proteins and potentially involved pathways. In particular, downregulation of the DJ-1 protein was detected in the ejaculated spermatozoa of Chinese patients with AS (An *et al.* 2011). It has also been demonstrated that deficiency of DJ-1 resulted in increased levels of lactoperoxidase (LPO) and reduced antioxidative capacity of spermatozoa. Under conditions of oxidative stress, DJ-1 was shown to be translocated into the mitochondria to maintain normal mitochondrial function (Sun *et al.* 2014). In testicular tissues of a rat model of AS, DJ-1 was reported to maintain mitochondrial function during spermatogenesis by interacting with NADH: ubiquinone oxidoreductase core subunit S3 (NDUFS3), the core component of the mitochondrial complex I (Wang *et al.* 2018). Despite the identification of major mitochondrial proteins interacting with DJ-1, its effect on the main function of mitochondria, and energy metabolism, has not been elucidated.

Continuing our previous research, in this study, we focused on the role of DJ-1 in the mitochondrial metabolism. In particular, we tried to elucidate whether DJ-1 deficiency in rats with ORN-induced AS leads to abnormal mitochondrial metabolism. We first identified changes in the distribution of DJ-1 and its mitochondria-related proteins in testicular tissues of rats with ORN-induced AS. We then detected changes in the content of small molecules related to the metabolism of energy, purines, and lipids. Finally, we detected the concentrations of metal ions and several small metabolic molecules in seminal plasma and spermatozoa lysates from patients with AS.

Materials and methods

Ethics statement

Participants

We recruited 82 males (aged 21–45 years) diagnosed with infertility and 50 age-matched control subjects with normal semen parameters in the Medical Reproduction Center, Peking University International Hospital, Beijing, China. Semen samples of patients with pyospermia, or varicocele, or with a history of smoking were not included in this study. Our study was approved by the Ethics Institutional Review Board of Peking University International Hospital and complies with the Declaration of Helsinki. The subjects gave informed written consent.

Animals

Sexually mature male Sprague Dawley rats weighing 330–370 g at the beginning of the experiment were obtained from the Charles River Laboratories, Inc. (SCXK (Jing) 2012-0001, Beijing, China). Rats were maintained at a controlled temperature ($24 \pm 2^\circ\text{C}$) and housed by groups in separate cages (12 h light:12 h darkness cycle) with access to food and water *ad libitum*. All experimental procedures involving the use of animals were approved by the Animal Care and Use Committee of Peking University.

Experimental procedures with human seminal fluid

Screening procedures for donors and patients

Seminal samples were collected by masturbation after 2–7 days of sexual abstinence. Each semen sample was liquefied for 30 min at 37°C before preparations of samples. The volume, pH, concentration, and motility of spermatozoa in the sample were assessed according to the guidelines of the World Health Organization (2010) by a computer-assisted semen analyzer (Weili Company, Beijing, China). Patients were divided into two groups: patients with mild AS with A-grade spermatozoa percentage $> 10\%$ ($n=47$) and patients with moderate AS with A-grade spermatozoa percentage $< 10\%$ ($n=35$) (Table 1).

Biochemical analyses of semen

Semen was centrifuged at high speed (15,000 g) for 15 min, and the seminal plasma in the supernatant was aspirated. Spermatozoa were washed three times with ice-chilled Tris–HCl buffer, resuspended to 20×10^6 cells/mL, ultrasonically oscillated, and then homogenized for 1 h on ice. Lysates were centrifuged at 12,000 g for 20 min to remove insoluble debris, and supernatant fractions were frozen at -80°C until use. Levels of glucose, urea, creatinine, Na^+ , K^+ , and Ca^{2+} in

Table 1 General seminal parameters.

	Age	pH	Ejaculate volume (mL)	Sperm density ($10^6/\text{mL}$)	Grade A sperm (%)
Ctrl	32.28 ± 3.70	7.48 ± 0.25	3.40 ± 1.52	95.44 ± 57.09	41.95 ± 10.21
MiA	32.84 ± 4.45	7.54 ± 0.32	3.44 ± 1.23	73.78 ± 54.77	$17.30 \pm 3.82^{****}$
MoA	31.89 ± 4.68	7.44 ± 0.27	3.60 ± 1.19	54.36 ± 36.08	$5.82 \pm 2.99^{****}$

**** $P < 0.0001$, one-way ANOVA test, $n=35$ –50 in each group.

seminal plasma and spermatozoa lysates were detected using the Beckman Coulter Chemistry Analyzer AU5800 (Beckman Coulter, Atlanta, GA, USA).

Experimental procedures with rat samples

Rat model of AS

A rat model of AS was generated by intragastric administration of ORN, according to a previously described method with some modifications (Oberlander *et al.* 1994). Briefly, 20 sexually mature (330–370 g) male Sprague Dawley rats were randomly assigned to two groups (ORN and control) with ten rats per group. In the ORN group, ORN was dissolved in 1% (w/v) sodium carboxymethylcellulose (CMC-Na) (MilliporeSigma) in water, and a dose of 400 mg per kg of body weight was fed to adult male rats once a day by oral gavage for a period of 14 days. Control rats received 1% (w/v) CMC-Na in water without ORN throughout the experiment.

Detection of ATP levels by ELISA

The animals were anesthetized with intraperitoneal (i.p.) administration of 0.6% pentobarbital sodium (10 mL/kg). Spermatozoa in the cauda epididymis were collected and prepared as described elsewhere (Kheirollahi *et al.* 2018). In brief, each cauda epididymis was cut and spermatozoa were released in 5 mL of Ham's F-10 medium (Sigma) containing 0.5% BSA and incubated at 37°C (with 5% CO₂) for 20 min. Spermatozoa were resuspended to 1×10^6 cells/mL. ATP concentrations within spermatozoa were determined using a rat ATP ELISA Kit (Dogesce Biological Technology, Beijing, China) according to the manufacturer's instructions.

Western blotting

Rats were deeply anesthetized with 60 mg/kg pentobarbital sodium and subjected to cardiac perfusion with normal saline. Testicular tissues were placed in ice-chilled lysis RIPA buffer (MilliporeSigma), to which was added an EDTA-free halt protease inhibitor cocktail (Thermo Fisher Scientific), followed by ultrasonic oscillation, and then homogenization for 1 h on ice. Lysates were centrifuged at 12,000 *g* for 20 min to remove insoluble debris, and supernatant fractions were frozen at –80°C until use. Protein concentration was measured using an enhanced bicinchoninic acid assay kit (Beyotime, Shanghai, China). Proteins (60 µg) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 5% non-fat milk in Tris-buffered solution with Tween 20 under agitation. Proteins were sequentially incubated with rabbit anti-NDUFA4 MAB (1:3000, Abcam) and horseradish peroxidase (HRP)-conjugated IgG (1:5000, KPL, Gaithersburg, MD, USA). β-actin was used as an internal standard. Protein bands were visualized using an enhanced chemiluminescence detection kit (ECL; Applygen Technologies Inc., Beijing, China) and a Molecular Imager ChemiDoc XRS+ Imaging System (Bio-Rad). The optical density of each band was quantified using the Quantity One software (Bio-Rad), and the amount of NDUFA4 was normalized to that of β-actin.

Immunofluorescence assay

Rats were deeply anesthetized with 60 mg/kg pentobarbital sodium and fixed by cardiac perfusion with 4% paraformaldehyde (pH 7.4) after flushing out red blood cells with 0.1 M PBS. Then, the testes were harvested and postfixed with 4% paraformaldehyde for 12 h, followed by dehydration in a gradient of 20 and 30% sucrose over a period of 2 days, until testes sank to the bottom of the sucrose solution container. After rapid freezing in dry ice, the testes were embedded in an optimal cutting temperature compound (Leica) and 10-µm-thick successive coronal sections were prepared using a freezing microtome Leica CM 1950 (Leica).

Sections were incubated in PBS containing 10% normal goat serum and 0.3% Triton X-100 for 1 h at 37°C, and then incubated overnight at 4°C in a humidified chamber with rabbit anti-DJ-1 MAB (1:200, Abcam), mouse anti-NDUFS3 MAB (1:200, Abcam), and rabbit anti-NDUFA4 polyclonal antibody (1:200, Abcam), respectively, in a solution containing 2% normal goat serum and 0.3% Triton X-100. After three washes with PBS, sections were incubated with corresponding secondary antibodies (Alexa Fluor 555-conjugated goat anti-rabbit IgG, Alexa Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 488-conjugated goat anti-mouse IgG, 1:200) (Thermo Fisher Scientific) in a solution containing 2% normal goat serum and 0.3% Triton X-100 for 1 h at 37°C. Sections were washed thrice with PBS, and then counterstained with Hoechst 33342 (5 µg/mL) (Thermo Fisher Scientific) for 5 min at 25°C. Finally, sections were mounted with fluorescent mounting medium. All samples were observed using laser scanning confocal microscopy on a TCS SP8-Confocal-MP-FLIM microscope (Leica).

Matrix-assisted laser desorption/ionization–mass spectrometry imaging (MALDI–MSI)

Tissue preparation, mass spectrometry imaging of small molecules and data analysis were performed according to a previous study (Liu *et al.* 2014). In brief, rats were deeply anesthetized with 60 mg/kg pentobarbital sodium, and testes were quickly excised, immediately frozen in liquid nitrogen, and stored at –80°C. Ten-µm-thick successive coronal sections were prepared using a freezing Leica CM 1950 microtome (Leica). Tissue sections were transferred by thaw mounting onto conductive indium tin oxide glass slides (Bruker Daltonics, Billerica, MA, USA), which were then stored in a vacuum dryer for approximately 1 h before application of the matrix. The matrix solution, that is 1,5-diaminonaphthalene (1,5-DAN) hydrochloride (MilliporeSigma) in 50% ethanol/water, was prepared and sprayed on tissue sections, ensuring uniform matrix coverage over the entire surface of the tissues (Wu *et al.* 2019).

MALDI-MSI was performed using an Autoflex Speed™ MALDI TOF (TOF) system with a 2-kHz Smartbeam-II laser (Bruker Daltonics). Data were acquired in negative ionization mode at 200 µm spatial resolution (200 laser shots), and signals between *m/z* 80 and 1000 Da were collected. All MSI data were normalized according to the total ion current, and the signal intensity of each image in the figure is represented as normalized intensity.

Statistical analysis

Calculation of the nucleus-to-cytoplasm ratio of the DJ-1 protein fluorescence intensity was performed using the Leica Application Suite Advanced Fluorescence (LAS AF) software (Leica). The nucleus and cytoplasm regions of primary spermatocytes and secondary spermatocytes in the images were outlined, and the average fluorescence intensities of the two regions were calculated respectively. The ratio of the two fluorescence intensities represented the nucleus-to-cytoplasm ratio of the DJ-1 protein fluorescence intensity in primary spermatocytes and secondary spermatocytes. Analysis of MALDI-MSI data were performed using the SCI LS Lab software (Bruker Daltonics) based on the normalization of the total ion chromatography data.

Data are expressed as means $\pm$ S.E.M. All data were analyzed using GraphPad Prism 7.0 (GraphPad Prism). All results were

compared using a one-way ANOVA. $P < 0.05$ was considered significant.

Results

Changes in the localization and expression levels of DJ-1 and mitochondrial proteins

In previous studies, we demonstrated a significant decrease in the expression levels of DJ-1 and mitochondrial complex I subunit NDUFS3 in spermatozoa and testes in patients with AS and in ORN-induced AS animal models (Wang *et al.* 2018). In this study, we further obtained information regarding the localization of DJ-1, NDUFS3, and NDUFA4 in the testis. Compared with primary spermatocytes, the nucleus-to-cytoplasm ratio of the

Figure 1 Changes in the localization and expression levels of DJ-1 and mitochondrial proteins. (A and B) Cellular localization of the DJ-1 protein in testicular tissues. (A) Immunohistological staining and confocal images showing the localization of the DJ-1 protein. According to the shape of the nucleus, primary spermatocytes can be seen between the two white dotted lines, whereas secondary spermatocytes are noted above them. White arrows indicate secondary spermatocytes with the expression of DJ-1 in the nucleus. Scale bar=25 μ m. (B) Statistical analysis of the nucleus to cytoplasm ratio of fluorescence intensity of the DJ-1 protein in primary and secondary spermatocytes. In normal testicular tissue, this ratio is significantly higher in secondary than in primary spermatocytes. No significant difference was observed in this ratio in testicular tissues of AS. Compared with the control group, this ratio in secondary spermatocytes was significantly reduced in the AS group. Data are presented as mean $\pm$ S.E.M. *** $P < 0.001$; $n=51$ cells; three animals per group. (C) Cellular localization of NDUFS3 in testicular tissues. (a and b) Immunohistological staining and confocal images of cross-sections of seminiferous tubules in testicular tissues. Scale bar=75 μ m. (c–e) and (i–k) Enlarged images of the left white box areas. (f–h) and (l–n) z-stack scans of the position shown by the white dotted lines. Scale bar=10 μ m. $n=54$ fields of view, three animals per group. (D, E and F) Cellular localization of NDUFA4 and Western blot analysis in testicular tissues. (D) Immunohistological staining and confocal images showing the localization of NDUFA4 in testicular tissues. Scale bar=100 μ m. (E) All the immunoblot results of NDUFA4 used for quantification (as presented in F) are shown. (F) Quantification of 'E'. Results show that the expression level of NDUFA4 increased significantly in the AS group. Data are presented as mean $\pm$ S.E.M. ** $P < 0.01$; $n=6$ per group. ORN, ornidazole; PSC, primary spermatocyte; SSC, secondary spermatocyte.

Figure 2 Effects of treatment with ORN on small molecules associated with aerobic oxidation and metabolism of energy in testis. *In situ* matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI MSI) of (A) glucose, (B) glutamine, (C) N-acetylaspartate, (D) GMP, (E) AMP, (F) ADP, and (G) ATP in testicular tissues. Among them, the contents of glucose, glutamine, and N-acetyl aspartate are demonstrated to be obviously increased. Scale bar=5 mm; Spatial resolution=200 μ m. Relative intensity ratios of these molecules were normalized and analyzed using the SCiLS Lab software. Data are presented as mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$; $n=3$ per group. ORN, ornidazole; m/z , mass-to-charge ratio.

expression level of DJ-1 in secondary spermatocytes was observed to be significantly increased ($P < 0.001$), whereas administration of ORN led to a decrease in this ratio ($P < 0.001$) (Fig. 1A and B), and had little effect on primary spermatocytes. ORN was shown to cause an increase in the expression of NDUFS3 in the cytoplasm of these cells, whereas there was no significant change observed in secondary spermatocytes (Fig. 1C). In the testes, NDUFA4 was shown to be more highly expressed in spermatozoa than in cells from each developmental stage before the formation of spermatozoa. The

expression of NDUFA4 was also significantly increased after treatment with ORN ($P < 0.01$, Fig. 1D, E and F).

MALDI-MSI analysis of metabolic small molecules in testis after treatment with ORN

Effects of treatment with ORN on small molecules associated with aerobic oxidation and energy metabolism in testis

After treatment with ORN, we detected a significant increase in the content of glucose using MALDI-MSI

Figure 3 Effects of treatment with ORN on small molecules associated with the metabolism of purines in testis. *In situ* matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI MSI) of (A) inosine, (B) hypoxanthine, and (C) xanthine in testicular tissues. Scale bar=5 mm; spatial resolution=200 μ m. Relative intensity ratios of these molecules were normalized and analyzed using the SCiLS Lab software. Data are presented as mean $\pm$ S.E.M. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; $n = 3$ per group. ORN, ornidazole; m/z , mass-to-charge ratio.

($P < 0.05$, Fig. 2A). Similar results were noted for glutamine and N-acetyl-L-aspartate, the intermediate downstream metabolites of α -ketoglutarate and oxaloacetate, respectively ($P < 0.01$, Fig. 2B and C). Although our findings showed an increase in the content of these three small molecules associated with the tricarboxylic acid (TCA) cycle, this change did not seem to have much effect on the downstream generation of energy. No significant difference was observed in the content of GMP, AMP, ADP, and ATP with or without treatment with ORN ($P > 0.05$, Fig. 2D, E, F and G).

Effects of treatment with ORN on small molecules associated with the metabolism of purines and lipids, and metal ions in testis

Treatment with ORN was demonstrated to disturb the normal process of the metabolism of purines and lipids. The levels of inosine, hypoxanthine, and xanthine, which are important intermediates in the metabolism of purines, were significantly increased after treatment with ORN compared with the normal (untreated) group ($P < 0.01$ to 0.0001 , Fig. 3). At the same time, we also detected significant changes in the levels of several lipid metabolites, including phosphatidic acid (PA) (18:0/18:1), phosphatidylethanolamine (PE) (16:0/18:1),

and PA (18:0/20:4), all of which were higher relative to the non-ORN group ($P < 0.01$ to 0.001 , Fig. 4A).

We also found abnormalities in the levels of sodium and potassium ions in the testis. The levels of both metal ions were significantly increased after treatment with ORN ($P < 0.01$, Fig. 4B).

ORN reduced intracellular ATP levels of rat spermatozoa

Further, we sought to understand whether ORN affected cellular ATP levels in rat spermatozoa. We observed significantly lower ATP levels in the ORN-treated group than in the control group ($P < 0.05$) (Fig. 5).

No significant changes in the concentration of small molecules in the semen of patients with AS

Finally, we used samples from patients with AS to detect changes in certain small metabolic molecules. Because of the difficulty in obtaining testicular tissue samples from patients with AS, we measured the concentrations of the related small molecules, including glucose, urea, creatinine, and metal ions (Na^+ , K^+ , and Ca^{2+}) in the seminal plasma and spermatozoa lysates. However, we could not

Figure 4 Effects of treatment with ORN on small molecules associated with the metabolism of lipids and metal ions in testis. *In situ* matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI MSI) of (A) the PA (18:0/18:1), PE (16:0/18:1), and PA (18:0/20:4) small molecules associated with the metabolism of lipids, and (B) Na⁺ and K⁺ metal ions, in testicular tissues. Scale bar=5 mm; Spatial resolution=200 μ m. The concentrations of these small molecules are demonstrated to be significantly increased. Relative intensity ratios of these molecules were normalized and analyzed using the SCiLS Lab software. Data are presented as mean $\pm$ S.E.M. ** $P < 0.01$, *** $P < 0.001$, $n=3$ per group. ORN, ornidazole; PA, phosphatidic acid; PE, phosphatidylethanolamine; m/z , mass-to-charge ratio.

observe any significant change in the levels of any of these small molecules and metal ions ($P > 0.05$, Fig. 6).

Discussion

This study is the first to map the distribution of DJ-1 and its interacting mitochondrial complex I protein subunits NDUFS3 and NDUFA4 in the spermatid ducts of rats with AS. Accordingly, we showed that the distribution of DJ-1 and NDUFS3 between the nucleus and cytoplasm differed significantly in primary and secondary spermatocytes of rats with AS. AS resulted in a reduced amount of DJ-1 in secondary spermatocytes of the testicular tissue, whereas abnormalities of NDUFS3 appear to mainly occur in primary spermatocytes. Moreover, NDUFA4 was more highly expressed in spermatocytes than in cells at other stages of spermatogenesis and its expression was significantly elevated after treatment with ORN. Regarding metabolic effects, we noted that ORN caused the accumulation of glucose with little effect on the quantity of ATP, accelerated the metabolism of purines and lipids, and destroyed the homeostasis of metal ions. Results of tests on seminal plasma and spermatozoa lysates in patients with AS showed that there were no abnormalities in the small molecular indicators commonly used in clinical tests.

Abnormal localization of DJ-1, NDUFS3, and NDUFA4 destroyed mitochondrial function

In rats, DJ-1 is first expressed in the developing germ cells and transferred to the equatorial segment of the

sperm head during epididymal transit (Welch *et al.* 1998). Protein expression gradually increases during the maturation of sperm, possibly due to the interaction with epididymal proteins (Klinefelter *et al.* 2002). In a previous study, we demonstrated a significant reduction in the expression of DJ-1 in testicular tissues of rats with AS (Wang *et al.* 2018), whereas, in this study, we aimed to clarify the changes in the localization of DJ-1. Treatment with ORN resulted in the significantly decreased expression of DJ-1 in the nucleus of secondary spermatocytes. Notably, DJ-1 has been shown to be localized both in the cytoplasm and nucleus, and to be translocated from the cytoplasm to nucleus during the S phase of the cell cycle in a mitogen-dependent manner (Nagakubo *et al.* 1997). The translocation of DJ-1 during the cell cycle suggested that it might be a regulatory protein, for example a protein kinase or phosphatase, such as a MAP or cyclin-dependent kinase (Nagakubo *et al.* 1997), as treatment with ORN inhibited the translocation of DJ-1 into the nucleus, thereby disrupting the cell cycle of secondary spermatocytes.

In this study, we showed that the levels of both the NDUFS3 and NDUFA4 mitochondrial complex I subunits interacting with DJ-1, were also changed. At present, very little research has been done on the properties and functionality of NDUFS3 and NDUFA4 in male germ cells. NDUFS3 is known to be assembled as part of the hydrogenase domain (Q module) of complex I, and constitutes an important subunit involved in the preassembly phase of the hydrogenase domain, so its introduction, folding, and eventual binding with other subunits to form the initial precomplex is critical

Figure 5 Effects of treatment with ORN on ATP level in spermatozoa in the cauda epididymis. ORN induced a significant decrease in ATP levels of spermatozoa in the cauda epididymis. Data are presented as mean $\pm$ S.E.M. * $P < 0.05$, $n = 10$ per group. ORN, ornidazole.

(Jaokar *et al.* 2013). The expression of NDUFS3 has been reported to be significantly higher in hypoxic regions of breast cancer specimens (Suhane *et al.* 2011). In addition, the abnormal increase in the levels of NDUFS3 was shown to be accompanied by the disappearance of the entire assembly complex (Vogel *et al.* 2007). ORN-induced accumulation of NDUFS3 in the cytoplasm of primary spermatocytes might have led to the breakdown of complex I and thereby impaired mitochondrial function. Further, NDUFA4 was first identified as a subunit of complex I and later as a subunit of complex IV (cytochrome c oxidase). Recent studies have strongly suggested that NDUFA4 is not a subunit of cytochrome c oxidase, but might represent an assembly factor for cytochrome c oxidase or supercomplexes (respirasomes) in mitochondria of growing cells and cancer tissues (Kadenbach 2017, Lei *et al.* 2017). We here showed that treatment with ORN resulted in the increased expression of NDUFA4 in spermatozoa in testicular tissues. This result, however, appeared to contradict the findings regarding the motility of spermatozoa. We could not obtain enough evidence to suggest that the increase in the levels of NDUFA4 promoted the electron transfer of complex IV, or the production of ATP. Based on our previous results, which showed a significant decrease in spermatozoa motility, one possible explanation is that the imbalance of NDUFA4 disrupts electron transfer and function of complex IV.

Based on the above results, we speculated that due to the complexity and diversity of cell types in testicular tissues, DJ-1, NDUFS3, and NDUFA4 might play different roles at various stages of differentiation.

Abnormal metabolism of small molecules

Since ORN was shown to cause abnormalities in the localization and content of mitochondrial proteins, we explored changes in the TCA cycle- and energy metabolism-related small molecules in testicular tissues using MALDI-MSI. This technique has been widely used for the analysis of small molecules in biological samples by providing direct two-dimensional visualization of metabolism. Our results showed that treatment with ORN led to the accumulation of glucose and increased levels of glutamine and N-acetyl aspartate, whereas the levels of ATP and its metabolites were shown to not change significantly. Although glucose is the common energy substrate used by most cells, the metabolism of energy in the testis is known to exhibit some specificity. In particular, lactate is the main energy metabolite used by germ cells. This metabolite is provided, and transported to germ cells where it is used, by Sertoli

Figure 6 No significant changes in the concentrations of small molecules in the seminal plasma and spermatozoa of patients with AS. The biochemical test of seminal plasma (A) and spermatozoa (B) showed no significant differences in the levels of glucose, urea, creatinine, Na⁺, K⁺, or Ca²⁺ between the normal control and AS groups. Data are presented as mean $\pm$ S.E.M. $n = 35$ –50 per group. MiA, mild asthenozoospermia; MoA, moderate asthenozoospermia; GLU, glucose; UREA, urea; CREA-S, creatinine.

cells, which are key cells in the development and maintenance of stem cell spermatogenesis (Boussouar & Benahmed 2004). Glucose homeostasis is critical for the process of spermatogenesis (Assisi *et al.* 2019), as high concentrations of glucose might lead to the impaired function of Sertoli cells and consecutive failure to provide lactate to germ cells, thus affecting male fertility (Tavares *et al.* 2017). Another important metabolic pathway used by Sertoli cells is the oxidation of glutamine, which has been reported to contribute to the final pool of lactate (Mateus *et al.* 2018). Hence, the accumulation of glutamine in testicular tissues after treatment with ORN might be due to the decreased consumption of glutamine by Sertoli cells. On another note, the molecular regulation of the metabolism of N-acetyl aspartate in testicular cells is poorly understood. Nonetheless, we can assume that an abnormal content of N-acetyl aspartic acid might also reflect the ORN-induced abnormal mitochondrial TCA cycle in testicular tissues. Although these abnormal results did not cause any changes in the production and content of ATP and its metabolites, the level of ATP in spermatozoa in the cauda epididymis decreased significantly. The motility of spermatozoa, which is an essential predictor of male fertility, depends on ATP synthesized by mitochondrial respiration. This low level of ATP may explain the low motility of sperm in the ORN induced AS model (Wang *et al.* 2018).

Besides energy-related metabolism, other pathways, such as the metabolism of purines and lipids, and the regulation of metal ions, were shown to exhibit abnormalities. For example, inosine, xanthine and hypoxanthine, which are important intermediates of the metabolism of purines, were found to be increased in rat testicular tissues after treatment with ORN, probably reflecting the fact that ORN might promote the catabolism of purines. Although studies on the relationship between the concentration of purines in testicular tissues and male infertility have been scarce, their concentration in the seminal plasma of infertile males has been statistically analyzed. Accordingly, the concentrations of inosine, xanthine, and hypoxanthine were significantly higher than those of the control group with normal fertility, suggesting that they could be potentially used as biomarkers of male infertility (Lazzarino *et al.* 2018).

The metabolism of lipids and oxidative stress has been reported to play a crucial role in testicular growth and the maintenance of testicular health (Datar *et al.* 2017). The results of this study showed that the levels of the PA (18:0/18:1), PE (16:0/18:1), and PA (18:0/20:4) lipid metabolites were increased. These phospholipids have many unsaturated fatty acids, which are vulnerable to oxidative attack, which can easily cause lipid peroxidation of the plasma membrane, resulting in damage to the motility and viability of sperm (Gharagozloo & Aitken 2011).

In addition, our results showed that the concentrations of K^+ and Na^+ significantly increased after treatment with ORN. In fact, the levels of K^+ and Na^+ in testicular tissues have long been found to be somewhat specific. The composition of the seminiferous tubule fluid is known to be distinct from that of blood plasma, with concentrations of K^+ and Na^+ ten-fold higher and lower respectively (Tuck *et al.* 1970). The maintenance of this concentration depends on the $Na^+-K^+-2Cl^-$ cotransporter (NKCC1) expressed in the membranes of Sertoli and germ cells. NKCC1-mediated ion transport has been shown to play a crucial role in spermatogenesis, and in this study treatment with ORN might have impaired the function of this transporter (Pace *et al.* 2000).

Finally, we detected the levels of glucose, urea, creatinine, and metal ions (Na^+ , K^+ and Ca^{2+}) in the seminal plasma and spermatozoa lysates of patients with AS and found no differences from the normal control group. The inconsistencies between the patient semen and the rat testis may be due to the examination of different tissue samples. In addition, the complex causes of human asthenospermia may affect these indicators differently from a single factor in the rat model.

Conclusion

DJ-1, a protein highly associated with male sterility, was demonstrated to change both in content and cellular localization in ornidazole-induced testicular tissues in rats with AS. Similar changes occurred in the NDUFS3 and NDUFA4 DJ-1-interacting mitochondrial complex I subunits. However, these changes were shown to be inconsistent across different cell types, potentially due to the regulation of cells at various stages of differentiation by different molecular mechanisms. Abnormalities in these mitochondrial proteins might inevitably lead to mitochondrial dysfunction, reflected in changes in the concentrations of small molecules associated with the mitochondrial metabolism of energy; however, treatment with ORN might result in the activation of a compensation mechanism, thereby maintaining normal levels of ATP. In addition, abnormal metabolism of purines and lipids and imbalanced metal ions might contribute to decreased sperm motility. This study provided evidence to suggest that disorders of DJ-1 could lead to mitochondrial dysfunction and supplementation of the pathogenesis of AS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Natural Science Foundation of China (no. 81401259), and the central government's special fund for basic scientific research in universities (no. BMU2018MX019).

Author contribution statement

Y S and X P S conceived the study, analyzed data and wrote the paper. Y S, X P S, L M Z, Z Z, Y P W and Z F D performed the experiments. X Z and X P P performed the final editing of the text.

Acknowledgement

The authors thank Yandong Zhang from Clinical Laboratory, Peking University International Hospital, Beijing, PR China for collecting semen samples.

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Received 18 February 2020

First decision 14 April 2020

Revised Manuscript received 9 September 2020

Accepted 28 September 2020