Melatonin rescues impaired penetration ability of human spermatozoa induced by mitochondrial dysfunction

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Abstract

Fertilization failure often occurs during *in vitro* fertilization (IVF) cycles despite apparently normal sperm and oocytes. Accumulating evidence suggests that mitochondria play crucial roles in the regulation of sperm function and male fertility. 3-Nitrophthalic acid (3-NPA) can induce oxidative stress in mitochondria, and melatonin, as an antioxidant, can improve mitochondrial function by reducing mitochondrial oxidative stress. The role of sperm mitochondrial dysfunction in fertilization failure during IVF is unclear. The present study revealed that spermatozoa with low, or poor, fertilization rates had swollen mitochondria, increased mitochondria-derived ROS, and attenuated mitochondrial respiratory capacity. 3-NPA treatment enhanced mitochondrial dysfunction in sperm. Spermatozoa with poor fertilization rates, and spermatozoa treated with 3-NPA, had reduced penetration ability. The concentration of melatonin was decreased in semen samples with low and poor fertilization rates. Melatonin, not only decreased excessive mitochondria-derived ROS, but also 'rescued' the reduced penetration capacity of spermatozoa treated with 3-NPA. Taken together, the study suggested that mitochondria-derived ROS and mitochondrial respiratory capacity are independent bio-markers for sperm dysfunction, and melatonin may be useful in improving sperm quality and overall male fertility.

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Introduction

Infertility is defined as the inability to achieve conception after 12 months of regular, unprotected, sexual intercourse, and currently affects approximately 10–15% of couples around the world (Dyer 2009). Previous studies confirm that 30–40% of these cases can be explained by male-only factor, 30–40% by female factors, 10–20% by combined male and female factors, and another 10–20% by unexplained factors (Boivin *et al.* 2007, Gelbaya *et al.* 2014). In recent years, stress, unhealthy lifestyles and atmospheric pollution have contributed to rising male infertility rates worldwide (Boivin *et al.* 2007, Dyer 2009, Yang *et al.* 2017). *In vitro* fertilization and embryo transfer (IVF-ET) is one of the most effective, assisted reproductive technologies (ARTs) for treatment of these couples (Lutjen *et al.* 1984, Steer *et al.* 1992, Van Voorhis 2007). IVF co-cultures spermatozoa with oocytes, which are obtained from both spouses respectively, and creates a fertilized ovum prior to implantation into the uterus

(Lutjen *et al.* 1984). However, some IVF couples with tubal infertility and normal semen analyses (Tocci & Lucchini 2010, Barratt *et al.* 2011) have poor fertilization rates with IVF treatment. The mechanisms underlying these poor fertilization rates are unclear.

Even though the origins and frequency of infertility vary worldwide, 'male factor' infertility contributes to about 50% of infertile cases overall (Agarwal *et al.* 2015, Kumar & Singh 2015). The 'tadpole'-like shape of spermatozoa determines their structural and functional specificity; the head of the spermatozoa includes the acrosome, some cytoplasm, and a nucleus with compacted DNA, the middle-piece contains ordered mitochondria, and the end-piece comprises many longitudinal microfilaments (Stefanini *et al.* 1967, Egeberg Palme *et al.* 2018). Abnormalities in cellular structures may cause sperm dysfunction and subsequent male infertility (Ollero *et al.* 2001, Zhou *et al.* 2013).

In eukaryotic cells, mitochondria are important for ATP synthesis by oxidative phosphorylation

(Balaban *et al.* 2005) and a number of other key processes including ROS generation (Balaban *et al.* 2005), intracellular calcium recycling and homeostasis (Lim *et al.* 2015), the intrinsic apoptotic pathways (Green & Reed 1998), and sex steroid hormones biosynthesis (Stocco 2001). Mitochondria play pivotal roles during spermatogenesis (Rajender *et al.* 2010), spermatozoa survival and motility (Piomboni *et al.* 2012), the acrosome reaction and successful fertilization (Gallon *et al.* 2006), although paternal mitochondria do not play a role in mitochondrial heredity (Luo & Sun 2013). Any defects in the mitochondrial genome or ultra-structural integrity, as well as abnormalities in mitochondrial membrane potential or energy metabolism, may contribute to impaired sperm function, such as capacitation, the acrosome reaction, oocyte interaction and sperm motility (Amaral *et al.* 2013). Moreover, increasing evidence indicates that mitochondria-derived ROS is crucial for structural integrity and normal functions of spermatozoa (Clyne 2012). In physiological conditions, spermatozoa generate basal levels of ROS for intracellular signaling activation, capacitation, acrosome reaction and fusion with the oocyte (Clyne 2012). However, uncontrolled and excessive accumulation of ROS in spermatozoa may lead to oxidative stress, which in turn, leads to damage of DNA, or, loss of plasma membrane integrity, or inactivation of enzymes; all of which may further impair sperm function (Tremellen 2008, Clyne 2012).

Melatonin is a hormone that is principally secreted by the pineal gland in a circadian manner in mammals (Manchester *et al.* 2015), which maintains circadian rhythmicity, regulates aging and reproduction as well as regulating antioxidant activities (Reiter 1980, Manchester *et al.* 2015, Reiter *et al.* 2016). Our previous study suggested that melatonin supplementation during diabetic pregnancy improves myocardial ischemic tolerance in diabetic offspring (Gao *et al.* 2016). Other studies demonstrate that melatonin improves mitochondrial function by reducing mitochondria-derived oxidative stress (Absi *et al.* 2000, Jimenez-Aranda *et al.* 2014, Agil *et al.* 2015, Xu *et al.* 2016). On the other hand, 3-NPA is an inhibitor of succinate dehydrogenase (SDH) of complex II in the mitochondrial electron transfer chain, which can induce oxidative stress in mitochondria (Cheng *et al.* 2016).

Because of the crucial roles of mitochondria in sperm function, we propose that mitochondrial dysfunction may be significant in reduced IVF rates. In the present study, we collected sperm specimens from the male partners of 400 infertile couples. The semen parameters of these male partners were normal; however, the *in vitro* fertilization (IVF) rates of oocytes inseminated by these sperm were different. We examined possible associations between sperm mitochondria status and IVF rates. Furthermore, we explored the potential mechanisms as to how mitochondrial dysfunction impairs fertilization

of human sperm, and whether treatment with melatonin might 'rescue' any such impairment.

Materials and methods

Media and reagents

We used the standard, human spermatozoa culture media throughout the present studies (IVF-PLUS and G-MOPS, Vitrolife, Gothenburg, VastraGotaland, Sweden) and a spermatozoa preparation medium for purification (ISolate Irvine Scientific, Santa Ana, CA, USA). IVF-PLUS is a bicarbonate buffered medium containing human serum albumin, and G-MOPS is a MOPS buffered medium free of protein. 3-nitropropionic (3-NPA) and melatonin were provided by Sigma-Aldrich. Mitotracker Green FM, MitoSOX and wheat germ agglutinin (WGA) were obtained from Invitrogen. Human melatonin ELISA kits were provided by IBL international (Flughafenstr, Hamburg, Germany).

Samples collection

Human semen samples were obtained from the male partners of 400 infertile couples in the reproductive center at International Peace Maternity and Child Health Hospital, Shanghai, China. These patients underwent IVF-ET. Among 400 infertile couples, 390 couples (97.5%) had tubal infertility in the female partners and 10 couples (2.5%) were diagnosed as unexplained infertility. Male partners with abnormal semen parameters, or female partners with polycystic ovary syndrome (PCOS), were excluded (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). Based on IVF rates (FR, the ratio of oocytes fertilized to oocytes inseminated in IVF), the infertile couples were divided into three groups: FR1 (FR > 65%, $n=299$), FR2 (FR > 30–≤65%, $n=79$) and FR3 (FR ≤ 30%, $n=22$).

Ethical approval

The study was approved by the ethics committee on human research at International Peace Maternity and Child Health Hospital, Shanghai, China. All patients provided written informed consent. All animal experiments were approved by the Institute's Animal Ethics Committee of Shanghai Jiao Tong University, and were performed in accordance with the guidelines on the use of laboratory animals.

Human spermatozoa preparation and culture

Human sperm were obtained from freshly ejaculated semen from male partners with normal, low and poor IVF rates, respectively. After 3 days of abstinence, morphology and motility were assessed by Papanicolaou and computer-assisted sperm analyzer (CASA) (HTM-IVOS system, version 12, Hamilton-Thorne Research, Beverly, CA, USA), respectively according to WHO standards. Semen samples were liquefied at 37°C in a shaker for 30 min and purified using Isolate (Irvine Scientific) by discontinuous density centrifugation. A two-layer gradient was prepared by using 'ready-to-use' solutions

of upper and lower isolate (Irvine Scientific). Semen samples were placed on top of the upper layer in a 15 mL centrifuge tube and centrifuged at 254g for 20 min. Pellets of motile sperm at the bottom of the lower isolate (Irvine Scientific) were aspirated and washed once with IVF-PLUS (Vitrolife). Purified sperm were incubated in IVF-PLUS (Vitrolife) at concentrations of 3×10^7 mL with or without treatment with different concentrations of 3-NPA (0, 0.5 or 1 mM) (Rosenstock *et al.* 2004, Perez-De La Cruz *et al.* 2006) and/or melatonin and incubated in one cell culture incubator (Thermo Fisher Scientific) for 3 h before further investigation. For melatonin treatment, melatonin (1 M) in sterile anhydrous DMSO solution was diluted with IVF-PLUS (Vitrolife) to a final concentration of 0.3 mM or 0.5 mM, and sperm were cultured in IVF-PLUS (Vitrolife) containing melatonin for 3 h before further assays. Treated sperm were used to analyze the roles of 3-NPA and melatonin in mitochondrial respiratory capacity, mitochondria-derived ROS, kinematic parameters and penetration ability of sperm.

Measurement of kinematic parameters of sperm

Sperm motility, progressive motility and other kinematic parameters were assayed by CASA (HTM-IVOS system, version 12, Hamilton-Thorne Research, Beverly, CA, USA) including straight line velocity (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), average path velocity (VAP), amplitude of lateral head displacement (ALH, μm), straightness of trajectory (STR, %) expressed as VSL/VCL, linearity of trajectory (LIN, %) expressed as VSL/VAP, beat cross frequency (BCF, Hz) and wobble of trajectory (WOB, %) expressed as VAP/VCL. The settings of CASA parameters were listed in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article). Sperm suspension ($3 \mu\text{L}$) was added to a four-chamber slide (Leja, Nieuw-Vennep, Haarlemmermeer, Netherlands) for analysis. Four different fields per ejaculate containing 200–300 sperm were analyzed. Triplicate determinations for each ejaculate were performed. All measurements of spermatozoa samples were performed at 37°C.

Measurement of mitochondrial mass

Purified human sperm cultured in IVF-PLUS (Vitrolife) were harvested by centrifugation and washed twice with Hanks buffer. 2×10^6 spermatozoa were incubated in 200 μL Hanks buffer containing 200 nM Mitotracker Green FM (Cottet-Rousselle *et al.* 2011, Yamamori *et al.* 2012) and probed at 37°C for 20 min in dark. Then, spermatozoa were pelleted by centrifugation at 800g for 5 min at room temperature and washed three times with warm Hanks buffer. Mitochondrial mass (Cottet-Rousselle *et al.* 2011, Yamamori *et al.* 2012) of spermatozoa was analyzed by flow cytometry (Beckman Coulter) using 419 nm excitation laser and 516 nm emission laser, respectively. 7-Amino-Actinomycin D (7-AAD) was used to exclude dead sperm. In single, alive sperm population, the mean fluorescence intensity (MFI) of Mitotracker Green FM was used to quantify mitochondrial mass in sperm. FlowJo 10.0 software was used to analyze the data of flow cytometry.

Measurement of mitochondrial ROS

Mitochondria-derived ROS in spermatozoa was studied with mitochondrial superoxide indicator, MitoSOX (Cottet-Rousselle *et al.* 2011, Yamamori *et al.* 2012). Purified human sperm cultured in IVF-PLUS (Vitrolife) were pelleted by centrifugation and washed twice with warm Hanks buffer. Then, 2×10^6 spermatozoa were resuspended with 200 μL Hanks buffer containing 3 μM MitoSOX and incubated at 37°C for 15 min in the dark. Spermatozoa were gently washed three times with warm Hanks buffer by centrifugation at 800g for 5 min. Flow cytometry (Beckman Coulter) and fluorescence microscopy (Zeiss) were used to evaluate mitochondrial ROS in spermatozoa at excitation and emission of approximately 510 nm and 580 nm. 7-AAD was used to exclude dead sperm. In the population of alive sperm, the MFI of MitoSOX was used to quantify mitochondrial ROS in sperm. FlowJo 10.0 software was used to analyze the data of flow cytometry.

Transmission electron microscopy

Purified human spermatozoa cultured in IVF-PLUS (Vitrolife) were collected by centrifugation, and then 1×10^7 sperm were fixed with 2.5% (v/v) glutaraldehyde in phosphate buffered saline (PBS, 0.1 M, pH 7.0) overnight. Spermatozoa were embedded in liquid agarose and solidified for 15 min at 4°C, before being cut into small portions and post-fixed in 1% osmium tetroxide (OsO_4) for 1 h and washed again three times in PBS (0.1 M, pH 7.0). After being dehydrated through a graded series of ethanol, the samples were transferred into absolute acetone for 20 min. Sperm samples were embedded in spur resin and heated at 70°C for more than 9 h, and then sectioned with LEICA EM UC7 ultratome before staining with uranyl acetate and then alkaline lead citrate for 5 and 10 min, respectively. The sections were imaged with Hitachi Code H7650 TEM (Hitachi). Four samples of spermatozoa in each group (FR1, 2 and 3), and 20–40 sperm in each sample, were observed and analyzed.

Measurement of mitochondrial respiratory capacity in live sperm by seahorse XF cell mito stress test system

Seahorse XF cell mito stress test system was adopted to analyze mitochondrial respiratory capacity (Pelgrom *et al.* 2016, Van Der Windt *et al.* 2016). Purified sperm cultured in IVF-PLUS (Vitrolife) were collected by centrifugation, and washed twice in non-carbonate buffered G-MOPS medium (Vitrolife), and resuspended in G-MOPS medium to a density of 3.0×10^7 mL. The sperm suspension (160 μL) was seeded in each well of a seahorse XF96 cell culture microplate and incubated in the non- CO_2 37°C cell incubator for 3 h. During this period, the seahorse sensor cartridge was loaded with 20 μL of 18 μM oligomycin (Penešky 1985), 10 μM FCCP (Benz & McLaughlin 1983), and a mixture of 11 μM rotenone (Li *et al.* 2003) and 11 μM antimycin A (Lai *et al.* 2005), respectively. The final concentrations in each well of sperm suspension were 2 μM oligomycin, 1 μM FCCP, 1 μM rotenone and antimycin A mixture. Seahorse assay was undertaken by monitoring oxygen

consumption rate (OCR) per well of human spermatozoa. Oligomycin, FCCP, and a mixture of rotenone and antimycin A were serially injected into each well of human sperm suspension to measure ATP-linked respiration, maximal respiration and non-mitochondrial respiration, respectively (Pelgrom *et al.* 2016, Van Der Windt *et al.* 2016). The Seahorse Cell Mito Stress Test Report Generator was used to analyze mitochondrial respiratory capacity.

Detection of melatonin in semen plasma samples

After liquefying at 37°C for 30 min, human semen samples were centrifuged at 3500g for 10 min. Seminal plasma samples were obtained from the centrifuged supernatant of liquefied semen. Then melatonin concentration in seminal plasma was determined using ELISA Kit (RE54021, IBL international) according to the manufacturer's instructions.

Human spermatozoa penetration of hamster zona-free oocytes

Female, seven-week-old hamsters weighing approximately 110g were superovulated by intraperitoneal (i.p.) injection of 30IU pregnant mare's serum gonadotropin (Lapolt *et al.* 1992) in 100µL saline solution (0.9% NaCl). Fifty-four hours later, each female hamster received an i.p. injection of 40IU of hCG in 100µL saline solution (0.9% NaCl). Seventeen hours later, the hamsters were anesthetized and killed by i.p. injection of 3 mL 0.5% (w/v) pentobarbital sodium in 0.9% NaCl. Oviducts were excised and placed in ice-cold Hanks buffer. Ova enclosed by cumulus were expelled from the bulbous section of the duct with 26 gauge needle and transferred into 2 mL GAMET (Vitrolife) containing 8IU/mL hyaluronidase (Vitrolife) for 3 min at 37°C to remove cumulus mass enclosing ova. The ova were washed three times with GAMET (Vitrolife). Zona pellucida was then removed by transferring zona intact ova into GAMET (Vitrolife) containing 1 mg/mL trypsin (Sigma) for 2 min. The zona-free ova were then washed three times in GAMET (Vitrolife) and transferred into 90µL IVF-PLUS (Vitrolife) drops covered by mineral oil (Vitrolife). Human sperm suspension (10µL) with motile sperm concentration of 3.0×10^7 /mL was added into the droplet containing ova (10 ova per droplet) to arrive at a final concentration of 3.0×10^6 human sperm per milliliter. After 8–10h, ova were washed twice with IVF-PLUS (Vitrolife) and stained with WGA (Life technology) and DAPI (Vectashield). The number of penetrated sperm per ovum was recorded by confocal imaging method, and the sperm penetration index was the average number of penetrated sperm per ovum.

Statistical analysis

All experiments were repeated at least three times with triplicate determination. Data were analyzed using SPSS 16.0 for Windows (SPSS, Inc). The Student *t* test was used to evaluate statistical significance between two groups. One-way analysis of variance (ANOVA) with the Tukey's *post hoc* tests was used to evaluate the statistical significance of the difference between more than two groups. *P* value <0.05 was considered statistically significant.

Results

Basal clinical characteristics and routine semen analysis of unexplained infertile patients

In this study, we examined the semen parameters of males from 400 infertile couples and found that all parameters were in the normal range of reference values defined by WHO fifth edition (Table 1). Compared to FR1 group, the percentages of normal morphology, progressive motility and ALH of sperm in FR2 group were slightly reduced ($8.6 \pm 3.8\%$ vs $10.1 \pm 4.2\%$, $51.3 \pm 10.9\%$ vs $54.6 \pm 11.2\%$ and 4.8 ± 1.3 vs 5.1 ± 1.2 , respectively, $P < 0.05$) (Table 1). However, the average IVF rates in FR2 and FR3 groups were $51.3 \pm 10.9\%$ and $7.2 \pm 9.5\%$, respectively, which were much lower than those in FR1 group ($87.7 \pm 10.9\%$). Because the cause of infertility in these males could not be explained by the parameters from routine semen analysis, we called these 'unexplained' infertile males. Strikingly, after treatment with ICSI in a subsequent cycle with a new oocytes retrieval, the average fertilization rate in FR3 group reached $82.8 \pm 15.5\%$ (Table 1). The results suggest that reduced penetration ability of sperm may be important in these 'unexplained' infertile males.

Impaired ultra-structures and increased mROS were observed in mitochondria of sperm with low or poor IVF rates

To identify whether mitochondrial impairment was involved in sperm dysfunction of unexplained infertile males, firstly we analyzed mitochondrial structures of sperm with transmission electric microscopy (TEM). The data showed that swelling and metamorphosing mitochondria were widely detected in sperm from FR2 and FR3 groups (Fig. 1A). Vacuolation and reduced cristae were also extensively distributed in mitochondria of these sperm from FR2 and FR3 groups (Fig. 1A). We also examined mROS in sperm using MitoSOX staining and found that, compared to FR1 group, the MFI of MitoSOX was significantly higher in mitochondria of sperm from FR2 and FR3 groups than that from FR1 group, which indicated that mROS was much increased in sperm from FR2 and FR3 groups compared to that from FR1 group (Fig. 1B and C). However, there was no significant difference in mitochondrial mass in sperm among groups of FR1, FR2 and FR3 (Fig. 1D).

Mitochondrial respiratory capacity was inhibited in sperm with low, or poor, IVF rates

In this study, mitochondrial respiratory capacity was analyzed using the seahorse system. The data showed that mitochondrial respiratory capacity of sperm in FR2 and FR3 groups were significantly reduced compared to that in FR1 group (Fig. 2A). We also found that basal OCR, oligomycin-induced OCR, FCCP-induced OCR as

Table 1 Basal clinical characteristics of infertile couples and routine semen analysis.

Characteristics	FR1 (>65%)	FR2 (>30–≤65%)	FR3 (≤30%)
<i>n</i>	299	79	22
Primary infertility, <i>n</i> (%)	146 (48.8)	45 (57.0)	11 (50.0)
Secondary infertility, <i>n</i> (%)	153 (51.2)	34 (43.0)	11 (50.0)
Infertility duration, years	3.8±2.9 (1–20)	3.8±2.4 (1–13)	4.3±2.3 (1–9)
Tubal infertility, <i>n</i> (%)	291 (97.3)	78 (98.7)	21 (95.5)
Unexplained infertility, <i>n</i> (%)	8 (2.7)	1 (1.3)	1 (4.5)
No. of oocytes inseminated	10.8±5.3 (3–28)	10.6±4.7 (3–24)	13.4±7.4 (3–30)
No. of oocytes fertilized	9.4±4.6 (2–26)	5.5±2.7 (1–14)	1.1±1.8 (0–7)
FR after IVF, %	87.7±10.9 (66.7–100)	51.3±10.9 (31.3–65.0)	7.2±9.5 (0–28.6)
FR after ICSI, %	–	–	82.8±15.5 (50.0–100)
Data on females			
Age, years	32±4 (21–43)	31±4 (22–40)	32±4 (25–39)
Menarche, years	13.8±1.1 (11–18)	13.6±1.1 (12–18)	14.1±0.8 (12–16)
Menstrual period, days	30.2±3.2 (23–40)	29.8±3.1 (22–40)	30.2±3.2 (25–40)
Abortion history, <i>n</i> (%)	133 (44.5)	27 (34.2)	10 (45.5)
Data on males			
Age, years	33±5 (23–50)	32±4 (25–43)	34±3 (27–39)
Semen analysis			
% of normal morphology of sperms	10.1±4.2 (4.0–26.0)	8.5±3.7 (4.0–23.0)**	9.8±3.1 (4.0–16.0)
Sperm concentration, 10 ⁶ /L	61.3±37.5 (15.1–236.2)	53.9±37.9 (15.2–295.9)	59.0±44.9 (19.9–242.0)
Progressive motile sperms, %	54.6±11.2 (32.4–82.9)	51.2±11.4 (32.3–79.7)*	53.4±11.9 (33.8–78.2)
Motile sperms, %	65.4±11.2 (42.3–94.1)	62.9±12.3 (42.9–87.8)	62.2±12.7 (40.0–86.0)
VCL, um/s	96.9±20.4 (47.3–160.6)	92.2±20.0 (50.5–146.4)	95.9±15.3 (64.0–121.0)
VSL, um/s	49.5±11.2 (20.0–88.1)	48.0±12.2 (24.1–84.2)	55.6±9.1 (34.1–68.4)
VAP, um/sec	60.3±11.2 (32.0–101.2)	58.0±11.7 (33.2–91.1)	60.6±8.8 (43.8–78.0)
LIN, %	52.4±8.1 (33.7–70.5)	53.3±8.9 (31.5–69.6)	53.7±5.9 (44.6–62.7)
ALH, um	5.1±1.2 (2.3–8.8)	4.8±1.2 (2.4–8.2)*	4.9±0.9 (3.2–6.2)
STR, %	79.7±6.8 (58.5–91.3)	80.0±7.5 (60.7–91.3)	81.4±5.3 (69.9–89.2)
BCF, Hz	24.2±3.3 (16.5–34.1)	24.7±4.2 (16.4–35.2)	24.3±3.3 (17.7–30.4)
WOB, %	63.8±5.4 (50.7–84.8)	64.5±5.6 (51.4–74.9)	64.2±3.6 (58.8–71.2)
BMI (kg/m ²)	24.6±3.6 (16.0–39.2)	24.4±3.4 (19.2–35.9)	24.0±3.1 (17.3–30.1)
BMI category, <i>n</i> (%)			
Underweight: <18.5	8 (2.7)	0 (0)	1 (4.5)
Normal weight: 18.5–24.9	157 (52.5)	41 (51.9)	12 (54.5)
Overweight: 25.0–29.9	108 (36.1)	33 (41.8)	8 (36.4)
Obese: ≥30.00	26 (8.7)	5 (6.3)	1 (4.5)

Data = mean ± S.E.M.

* and ** $P < 0.05$ and $P < 0.01$, respectively, compared to FR1 group.

well as rotenone plus antimycin-induced OCR of sperm from males in FR2 and FR3 groups were significantly lower than that in FR1 group, as indicated by an evident impairment of mitochondrial respiratory capacity (Fig. 2B). Furthermore, the significant decrement of FCCP-induced OCR also suggested a reduction of mitochondrial reserve respiratory capacity in sperm from unexplained infertile males in FR2 and FR3 groups (Fig. 2B).

3-NPA treatment reduced mitochondrial respiratory capacity and the penetration capacity of sperm from infertile males of FR1 group

In order to analyze the impact of 3-NPA on sperm mitochondria, mitochondrial respiratory capacity and mitochondria-derived ROS were determined by Seahorse system and MitoSOX staining. Compared to the control (0 mM 3-NPA treatment), treatment of sperm with 0.5 and 1 mM 3-NPA significantly attenuated

the mitochondrial respiratory capacity ($P < 0.05$, Fig. 3A). Basal, oligomycin-induced, FCCP-induced, and rotenone plus antimycin-induced OCR were significantly lower in sperm treated with 0.5 mM and 1 mM 3-NPA, respectively, than controls ($P < 0.05$, Fig. 3B). The patterns of reduced mitochondrial respiratory capacity of sperm treated with 0.5 mM and 1 mM 3-NPA, respectively, were similar to those of sperm in the FR2 or FR3 group (Fig. 2A and B). 3-NPA treatment also significantly increased MitoSOX staining intensities ($P < 0.05$, Fig. 3C), indicating excessive ROS production in the mitochondria. We evaluated kinematic parameters of sperm treated with 0.5 and 1 mM 3-NPA, respectively, and found that there was no significant difference in the parameters including motility, VCL, VSL, VAP, LIN, STR, BCF and WOB between sperm treated with or without 3-NPA (Fig. 3D, E, F, G, H, I, J and K). However, progressive motility and ALH were slightly depressed in the sperm treated with 1 mM 3-NPA (Fig. 3L and M).

Figure 1 Impaired mitochondrial ultra-structure and increased mitochondria-derived ROS are observed in sperm of unexplained infertile male patients. (A) Mitochondrial ultra-structures in sperm from FR1, FR2 and FR3 groups were observed by TEM. The red arrows indicate impaired mitochondrial ultra-structure (such as metamorphosis, black bulk, phyalides and decreased cristae) in sperm from group FR2 and FR3. Four samples in each group (FR1, 2 and 3), and 20–40 sperm in each sample, were analyzed. Scale bar=0.2 μ m. (B) The distribution and content of mitochondria-derived ROS in sperm from FR1, FR2 and FR3 groups were analyzed by MitoSOX staining and fluorescence microscopy. Red color indicates MitoSOX staining. The middle and lower panels showed the overlapped pictures of fluorescence and bright fields. Scale bar=50 μ m in the upper and middle panels, scale bar=5 μ m in the lower panel. (C) The MFI of MitoSOX staining in sperm from FR1, FR2 and FR3 groups in the absence or presence of 0.3 mM melatonin was assessed by flow cytometry and shown as bar graph (mean $\pm$ S.E.M.). *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, determined by ANOVA with the Tukey's *post hoc* test. Data are representative of three independent experiments. (D) Mitochondrial mass in sperm was assessed by Mitotracker Green FM staining and flow cytometry and shown as bar graph (mean $\pm$ S.E.M.). *n*, number of samples. Data are representative of three independent experiments.

Figure 2 Mitochondrial respiratory capacity is reduced in sperm of FR2 and FR3 groups compared to that of FR1 group. (A) Mitochondrial respiratory curves of sperm were assessed with Seahorse system. '*' or '**' indicates significant difference when compared FR3 with FR1, *P* values were determined by the Student *t* test. (B) Basal OCR, oligomycin-induced OCR, FCCP-induced OCR and rotenone plus antimycin-induced OCR of mitochondria in sperm from FR1, FR2 and FR3 groups were shown as bar graph (mean $\pm$ S.E.M.). *P* values were determined by ANOVA with the Tukey's *post hoc* test. * and **, *P* < 0.05 and *P* < 0.01, respectively. *n*, number of samples. Data are representative of three independent experiments.

In order to confirm whether oxidative stress could lead to fertilization failure, we evaluated the fertilization potential of the sperm treated with, or without, 3-NPA by determining penetration ability of sperm into hamster zona-free oocytes. Sperm with normal fertilization rate were collected from the normal men in FR1 group. After treatment of sperm with 1 mM 3-NPA, the penetration ability of sperm into hamster zona-free oocytes was reduced by 50% compared to 0 mM 3-NPA treatment (Fig. 4A and B).

Melatonin treatment rescues mitochondrial oxidative stress and improves kinematic parameters as well as the penetration ability of sperm

The role of melatonin in sperm and male infertility is undefined. In the study, the concentrations of melatonin in semen plasma samples of infertile males from three groups were determined by ELISA. The data showed that the concentrations of melatonin in semen samples from FR2 and FR3 groups were evidently decreased compared to that of FR1 (Fig. 4A), which suggested that melatonin might play a significant role in unexplained infertility. First, we considered whether melatonin has an antioxidant effect in sperm; our data show that melatonin treatment reversed abnormal increases of mitochondria-derived ROS in spermatozoa from FR2 and FR3 groups (Fig. 1C) as well as 3-NPA-treated sperm (*P* < 0.05, Fig. 3C). Second, treatment of sperm with 0.3 mM melatonin significantly improved not only progressive motility and ALH, but also penetration ability of sperm into hamster zona-free oocytes in the presence of 1 mM 3-NPA (Figs 3L and M, 4B and C). Finally, we evaluated the role of melatonin in sperm penetration ability from unexplained infertile males with poor FR. Strikingly, following treatment of sperm from unexplained infertile males in FR3 group with 0.3 and 0.5 mM melatonin, respectively, the penetration index of sperm into hamster zona-free oocytes was increased from 1.2 ± 0.3 (control, without melatonin treatment) to 4.0 ± 0.8 (0.3 mM melatonin treatment) and 7.3 ± 0.6 (0.5 mM melatonin treatment), respectively (Fig. 4D and E).

Discussion

Although the development of assisted reproduction technologies has helped millions of infertile couples have their own babies (Lutjen *et al.* 1984, Van Voorhis 2007), the etiology of male infertility during natural reproduction or assisted reproduction is still not fully understood. In this study, we focused on one pattern of atypical, male infertility, that we termed 'unexplained' male infertility, and explored its possible pathogenesis. Our clinical investigation demonstrated that 20% (79/400) infertile couples had low IVF rate ($51.3 \pm 10.9\%$) and 6% (22/400) infertile couples had poor IVF rate ($7.2 \pm 9.5\%$). Strikingly, the clinical investigation found that ICSI (intracytoplasmic sperm injection), an IVF procedure in which a single sperm cell is injected directly into the cytoplasm of an egg, could dramatically improve IVF rates in infertile couples from 7.2 to 82.8%. These data suggested that reduced penetration ability of spermatozoa probably contributes to decreased IVF rates in these infertile couples undergoing conventional IVF-ET, which could not be detected by routine semen analysis.

Mitochondria play essential roles in the regulation of sperm functions (Rajender *et al.* 2010, Amaral *et al.* 2013).

Figure 3 Effects of 3-NPA on mitochondrial respiratory capacity, mitochondria-derived ROS and kinematic parameters of sperm with normal IVF rate from FR1 group. (A) Mitochondrial respiratory curves were assessed by Seahorse system for sperm treated without or with 3-NPA (0.5 mM or 1 mM), respectively. *, ** or *** indicates significant differences when compared 3-NPA 1 mM group with 3-NPA 0 mM group. *P* values were determined by the Student *t* test. (B) Basal OCR, oligomycin-induced OCR, FCCP-induced OCR and rotenone plus antimycin-induced OCR of sperm in the absence or presence of 3-NPA (0.5 mM or 1 mM) were shown as bar graph (mean $\pm$ S.E.M.). *P* values were determined by ANOVA with the Tukey's *post hoc* test. (C) The MFI of MitoSOX staining in sperm treated without or with 3-NPA (0.5 mM or 1 mM) in the absence or presence of 0.3 mM melatonin was assessed by flow cytometry and shown as bar graph (mean $\pm$ S.E.M.). *P* values were determined by ANOVA with the Tukey's *post hoc* test. (D, E, F, G, H, I, J, K, L and M) The kinematic parameters of sperm treated without or with 3-NPA (0.5 mM or 1 mM) in the absence or presence of 0.3 mM melatonin were assessed by CASA and shown as bar graph (mean $\pm$ S.E.M.). *P* values were determined by ANOVA with the Tukey's *post hoc* test. *, ** and *** *P* < 0.05, *P* < 0.01 and *P* < 0.001, respectively. *n*, number of samples. Data are representative of three independent experiments.

In this study, we examined the mitochondrial status of sperm from males with 'unexplained' infertility with several strategies. We found that the ultra-structures of sperm mitochondria from these unexplained infertile males were impaired. Moreover, mitochondria-derived oxidative stress was increased, while the mitochondrial respiratory capacity was reduced in their sperm, which indicated that dysfunctional mitochondria probably led to this type of unexplained male infertility. Previous studies have shown that mitochondrial dysfunction might impair the motility of sperm (Piomboni *et al.* 2012, Amaral *et al.* 2013). However, we did not find significantly impaired mobility in sperm of unexplained infertile males. Overall, our results indicate that although sperm with poor IVF rates looked normal in the routine semen analysis, their mitochondria might be impaired and dysfunctional.

3-NPA is an inhibitor of succinate dehydrogenase (SDH) of complex II in mitochondrial electron transfer chain (Cheng *et al.* 2016), and 3-NPA treatment

induces oxidative stress in mitochondria and leads to mitochondrial dysfunction (Cheng *et al.* 2016). In this study, we treated normal sperm with 3-NPA to simulate impaired mitochondria in sperm of unexplained infertile males. We found that 3-NPA had little impact on the parameters of routine analysis; however, 3-NPA dramatically decreased oxygen consumption and increased the production of mitochondria-derived ROS in sperm. These results were consistent with our clinical investigations and suggested that impaired mitochondrial respiratory capacity and increased ROS might play important roles in sperm with poor IVF rate from 'unexplained' infertile males.

The core strategy of IVF is to co-culture sperm with oocytes *in vitro* to produce a fertilized ovum (Lutjen *et al.* 1984). Sperm have enough chances to meet oocytes during IVF manipulation. However, poor IVF rates were observed in some patients and led to IVF failure. ICSI can be used to improve fertilization rates in some cases.

Figure 4 Melatonin rescues the impaired penetration potential of sperm treated with 3-NPA or sperm with poor IVF rate. (A) Melatonin concentration in seminal plasma samples from FR1, FR2 and FR3 groups was assessed by ELISA and shown as bar graph (mean $\pm$ S.E.M.). *P* values were determined by the Student *t* test. *n*, number of samples. (B) The penetration of hamster zona-free oocytes by sperm treated without or with 3-NPA (0.5 mM or 1 mM) in the absence or presence of 0.3 mM melatonin were observed by confocal fluorescence microscopy. Green color indicates wheat germ agglutinin (WGA) staining of the membrane of the oocyte. Blue color indicates DAPI staining of the nucleus. The red arrow indicates the nucleus of the oocyte, the other DAPI + nucleus indicate the nucleus of sperm that entered into the oocyte. Scale bar = 20 μ m. (C) The penetration index of sperm treated without or with 3-NPA (0.5 mM or 1 mM) in the absence or presence of 0.3 mM melatonin was shown as bar graph (mean $\pm$ S.E.M.). *P* values were determined by ANOVA with the Tukey's *post hoc* test. (D) The penetration of hamster zona-free oocytes by sperm with poor IVF rate in the absence or presence of melatonin (0.3 mM or 0.5 mM) was observed by confocal fluorescence microscopy. Green color indicates WGA staining of the membrane of the oocyte. Blue color indicates DAPI staining of the nucleus. The red arrow indicates the nucleus of the oocyte, the other DAPI + nucleus indicate the nucleus of sperm that entered the oocyte. (E) The penetration index of sperm with poor IVF rates, in the absence or presence of melatonin (0.3 mM or 0.5 mM) shown as bar graph (mean $\pm$ S.E.M.). *P* values were determined by ANOVA with the Tukey's *post hoc* test. *, ** and ***, *P* < 0.05, *P* < 0.01 and *P* < 0.001, respectively. *n*, number of oocytes. Data are representative of three independent experiments.

In the present study, we adopted the strategy of hamster, zona-free, oocyte penetration by human sperm to examine whether sperm with poor IVF fertilization rates have reduced penetration ability. Indeed, we found that sperm with poor IVF fertilization rates had significantly attenuated capacity for penetration. Moreover, we found that 3-NPA treatment significantly reduced the penetration ability of human sperm with normal IVF rate. These data suggest that dysfunctional mitochondria in sperm probably lead to unexplained male infertility by impairing the penetration ability of sperm.

Melatonin may increase the activities of mitochondrial complex I/IV, and further improve cellular respiratory capacity (Jimenez-Aranda *et al.* 2014). Several other studies demonstrate that melatonin enhances mitochondrial functions through reducing oxidative stress (Manchester *et al.* 2015, Reiter *et al.* 2016). The impact of melatonin on sperm and male infertility is still unknown. In the present study, we found that concentrations of melatonin in semen were negatively correlated with IVF rates and less than half of unexplained infertile males

from FR2 + FR3 groups as many as that from FR1 group. Melatonin notably reduced mitochondria-derived ROS in sperm with low/poor IVF rates or 3-NPA-treated sperm. Furthermore, melatonin not only improved progressive motility and ALH, but also enhanced the penetration ability into hamster zona-free oocytes of 3-NPA-treated sperm. We also found that melatonin increased the penetration index of sperm with poor IVF rates. Overall, the study supplied evidence that oxidative stress and increased ROS in mitochondria of sperm might lead to IVF failure, but melatonin may improve oxidative stress in mitochondria of sperm as well as the penetration capacity of sperm into oocytes, which further suggests that melatonin might improve IVF outcomes.

In summary, even though the etiology of sperm dysfunction and male infertility is multifactorial and complex, this study provides new insight into male factor infertility during IVF cycles. Mitochondria-derived ROS is an independent biomarker for atypical, male factor infertility, and melatonin has the clinical potential to improve sperm quality.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0231>.

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.

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Author contribution statement

H-F H had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. H-F H coordinated the study. H-F H, X-Y Z, Y-M X co-designed the studies. X-Y Z, Y-M X contributed equally to this study. X-Y Z, Y-M X, L W, R L, L J were responsible for enrolment of participants and arranged informed consent from the participants. X-Y Z, Y-M X, L W, Y-T H, Z-H T, Z-M W, F-H Y, Z-Q W, Y X provided patient care and/or took samples. X-Y Z, Y-M X, Y-J T, X-H L, X-M L, Y Z carried out or supported data analysis, including the statistical analyses. J-Z S helped with the manuscript writing. All authors critically reviewed the report.

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