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Expression of NDUFA13 in asthenozoospermia and possible pathogenesis

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KEY MESSAGE

The etiology and pathogenesis of asthenozoospermia are still not fully understood. This study showed that NDUFA13 deficiency may be associated with asthenozoospermia via disturbance of spermatozoa mitochondrial membrane potential and by increasing apoptosis and intracellular reactive oxygen species. Our study revealed a new molecular mechanism underlying NDUFA13-mediated motility of human spermatozoa.

A B S T R A C T

Asthenozoospermia is a common cause of male infertility, which is characterized by reduced forward motility of spermatozoa. The cause and pathogenesis of asthenozoospermia are not fully understood. The purpose of this study was to investigate the expression of nicotinamide adenine dinucleotide (NADH) dehydrogenase (ubiquinone) 1 alpha subcomplex, 13 (NDUFA13) in the spermatozoa of men with asthenozoospermia and its possible pathogenesis. Protein content of NDUFA13 in spermatozoa was measured by Western blot analysis. The results showed that NDUFA13 expression in spermatozoa was significantly lower in men with asthenozoospermic than in men with normozoospermia ($P < 0.01$). Immunofluorescence experiments showed that NDUFA13 was expressed predominantly in the sperm mid-piece. A lower mitochondrial membrane potential, a higher intracellular reactive oxygen species (ROS) level and more apoptotic cells were also detected in men with asthenozoospermia. NDUFA13-specific small interfering RNA was used in the mouse spermatocyte GC2-spd cell line to down-regulate the expression of NDUFA13. The knockdown of NDUFA13 in the GC2-spd cells caused a collapse of mitochondrial membrane potential, an increase in ROS level and more apoptotic cells. Our study showed that NDUFA13 deficiency may be associated with asthenozoospermia through the disturbance of spermatozoa mitochondrial membrane potential and by increasing apoptosis and intracellular ROS.

Introduction

Infertility refers to failure of a couple to conceive after 12 months of unprotected regular intercourse, and male factor infertility is partially or fully responsible for about 30–55% of cases of infertility (Hamada et al., 2013). Asthenozoospermia is a common cause of male infertility, which is characterized by reduced spermatozoa forward motility (progressive motility <32%) (World Health Organization, 2010). A series of theories that attempt to explain the genesis of

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asthenozoospermia has been established, including disturbances of mitochondrial sheath and axonemal complex formation during spermiogenesis, impaired function of accessory sex glands providing compounds required for movements or epididymis responsible for maturation of spermatozoa, genetic defects and hormonal disturbances. All of these could induce the occurrence of asthenozoospermia (Chemes et al., 1999; Gonzales and Villena, 2001; Piasecka and Kawiak, 2003; Yeung et al., 1997). The cause and pathogenesis of asthenozoospermia are still not fully understood.

Mitochondria are best known as the eukaryotic cell powerhouses, and these organelles also participate in various cellular functions besides adenosine triphosphate (ATP) production, such as intrinsic apoptotic pathways and the generation of reactive oxygen species (ROS) (Amaral et al., 2013). In addition, it has been established that sperm motility depends on mitochondrial respiratory function (Ruiz-Pesini et al., 1998). It has been suggested by many investigators that, although glycolysis forms the major source of ATP along the flagellum, the energy required for sperm motility is mainly produced during mitochondrial respiration (du Plessis et al., 2015). Defects in the sperm mitochondrial ultrastructure are associated with decreased sperm motility in humans (Pelliccione et al., 2011). Additionally, comparative proteomic outcomes suggest that the expression of several sperm mitochondrial proteins may be altered in men with asthenozoospermia (Amaral et al., 2013; Parte et al., 2012).

Nicotinamide adenine dinucleotide (NADH) dehydrogenase [ubiquinone] 1 alpha subcomplex, 13 (NDUFA13) was originally isolated by Angell et al. (2000) as a death-associated protein. Later, it was shown to be a tumour suppressor, and it is involved in apoptosis and growth inhibition (Alchanati et al., 2006; Zhang et al., 2008). Further studies on the bovine heart to characterize NDUFA13 showed that it could be copurified with mitochondrial NADH: ubiquinone oxidoreductase (complex I) (Fearnley et al., 2001). The study of mice by Lu and Cao (2008) showed that NDUFA13 plays an essential role in complex I assembly and electron transfer activities, whereas the disruption of mitochondrial membrane potential (MMP) by NDUFA13 mutants increases cells sensitivity to apoptotic stimuli. Although research has recently been conducted into NDUFA13, the role of NDUFA13 in the pathogenesis of asthenozoospermia is still unknown.

In this study, the expression of NDUFA13 in seminal samples from men with asthenozoospermia was compared with control patients. The MMP, the generation of ROS and apoptotic rate in the two groups were also detected. The NDUFA13 level in mouse spermatocyte cell line GC2-spd was knocked down, and the cellular biological differences were investigated to understand the possible pathogenesis of asthenozoospermia.

Materials and methods

Participants

This study was reviewed and approved by the Institutional Review Board of Qilu Hospital of Shandong University on 21 February 2013 (reference number KYLL-2013-013). Written informed consent was obtained from all participants. Any patients affected by leukocytospermia, varicocele, hypogonadism, cryptorchidism, past or present use of drugs potentially affecting spermatogenesis, a genital tract infection in the last 3 months, smoking history, wet heat exposure, or recent fever, were excluded. The study population consisted of 98 men with asthenozoospermia (progressive motility [grade A + B sperm] < 32%, sperm vitality >58%) and 45 control participants with normal semen parameters from the Infertility Center, Qilu Hospital of Shandong University, Jinan, China.

Semen collection and semen analysis

Seminal samples were collected by masturbation after 3–7 days of sexual abstinence. After liquefaction for at least 30 min, a routine semen analysis was carried out by a computer-assisted semen analysis system (WLJY-9000, China).

Preparation of human spermatozoa

After at least 30 min of liquefaction, the motile fraction of sperm was separated from the seminal fluid by a density gradient medium (IrvineScientific, Santa Ana, CA, USA). The spermatozoa from the density gradient medium were ultimately washed with Biggers–Whitten–Whittingham medium (GenMeD, Shanghai, China), centrifuged at 600 g for 15 min, and finally resuspended in Biggers–Whitten–Whittingham at a final cell concentration of 5×10^6 /ml.

Cell lines and culture

Mouse spermatocyte cell line GC2-spd was obtained from American type culture collection (ATCC, Manassas, Virginia, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratories, Utah, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 100 U/ml penicillin, 100 µg/ml streptomycin, and 4 mM glutamine (Invitrogen, Waltham, Massachusetts, USA) at 37°C in a humidified atmosphere of 5% CO_2–95% air. Lipofectamine 2000 (Invitrogen) was used for the downregulation of NDUFA13 by small interfering RNA (siRNA) in accordance with the manufacturer's instructions. The following sequences were used: NDUFA13, 5'-GGAUUGGAACCCUGAUCUATT-3', and scramble, 5'-UUCUCCGAACGUGUCACGUTT-3' for the control.

Western blot analysis

Western blotting was used to detect the protein level of NDUFA13 in men with asthenozoospermia and controls. The total protein concentration was evaluated by a bicinchonininc acid protein assay kit (Beyotime, China). The total protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then it was transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat dry milk and subsequently incubated with primary antibody against NDUFA13 (Abcam, monoclonal, 1:1000) overnight at 4°C. Immunoreactivity was detected by an appropriate peroxidase-linked secondary antibody (Zhongshan, 1:5000) and an enhanced chemiluminescence (Millipore) detection reagent.

Immunofluorescence

The subcellular localization of NDUFA13 in the spermatozoa was investigated by immunofluorescent staining. An aliquot (50 µl) of the spermatozoa (2×10^4) resuspended with phosphate-buffered saline (PBS) was spotted on glass slides and air-dried. These preparations were fixed with 4% paraformaldehyde for 30 min at room temperature. Then, they were permeabilized with 0.5% Triton X-100 for 20 min. After being blocked with 10% goat serum (Zhongshan, Beijing, China), the preparations were exposed to primary antibod-

ies (rabbit polyclonal antibody against NDUFA13, Santa Cruz Biotechnology, USA, 1:100) for 2 h followed by washing in PBS three times for 10 min each. The preparations were then incubated for 2 h at room temperature in the presence of 1:100 diluted secondary antibodies (Fluorescein-Conjugated AffiniPure Goat Anti-Rabbit IgG, Zhongshan, Beijing, China). For the negative controls, the preparations were incubated with only the secondary antibody or with an irrelevant primary antibody (normal rabbit IgG, Santa Cruz Biotechnology, USA, 1:100).

Detection of mitochondrial membrane potential

The MMP was estimated by an MMP assay kit with 5,50,6,60-Tetrachloro-1,10,3,30-tetraethyl-imidacarbocyanine iodide (JC-1) (Beyotime, Haimen, China). Briefly, the spermatozoa were resuspended at a final cell concentration of 5×10^6/ml, and then incubated with an equal volume of JC-1 staining working solution (5 mg/ml) at 37°C for 20 min, and rinsed twice with a staining buffer. The stained samples were then analysed by flow cytometry. The JC-1 was excited by a 490-nm argon laser, and the results were recorded using green fluorescence (GRN-HLog) and red fluorescence (RED-HLog) channels. Forward and side scatter measurements were taken to generate a scatter plot, which was used to gate for sperm cells only, excluding dead cells and debris. A minimum of 10,000 spermatozoa were analysed for each sample.

Determination of reactive oxygen species production

The level of ROS in the spermatozoa was quantified by an oxidation-sensitive fluorescent probe 2',7'- dichlorofluorescein diacetate (DCFH-DA). The spermatozoa were suspended in DCFH-DA diluted to a final concentration of 10 mM at a density of 1×10^6/ml, and were incubated in the dark for 30 min at 37°C. The fluorescence was analysed quantitatively by flow cytometry after being washed three times with PBS. A primary gate based on physical parameters (forward and side light scatter) was set to exclude dead cells and debris. The excitation and emission wavelengths were set at 488 and 525 nm.

Detection of apoptosis

An Annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, china) was used to detect the apoptosis of the spermatozoa samples. Briefly, 5 × 105 spermatozoa were suspended in 500 ml binding buffer after rinsing with PBS. Then, 5 ml annexin V-FITC was mixed with the suspension, and 5 ml propidium iodide was added. Samples were incubated in the dark at room temperature for 15 min. A flow analysis was carried out by Guava easyCyte HT Flow Cytometry equipped with an argon ion laser tuned at 488-nm wavelength, and the results were recorded using GRN-HLog and RED-HLog channels. A primary gate based on physical parameters (forward and side light scatter) was set to exclude dead cells and debris.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 18.0 (SPSS Inc., USA) was used for statistical analysis. All data are expressed as means ± SD. The statistical analyses of variance was used for analysis. $P < 0.05$ was considered to be statistically significant.

Results

Demographic and clinical data of participants

The demographic and semen characteristics of the study population are presented in **Table 1**. No significant difference was found in age, ejaculate volume, pH and sperm concentration in the two groups. The progressive motility (percentage of grade A + B sperm), however, was significantly reduced in the men with asthenozoospermia ($P < 0.01$).

Expression of NDUFA13 in the spermatozoa of men with asthenozoospermia

To investigate the potential role of NDUFA13 in asthenozoospermia development, the location of NDUFA13 in the spermatozoa was first detected. The results showed that, although occasional signals were detected in the sperm head, NDUFA13 is predominantly expressed in the sperm mid-piece where the sperm mitochondria are housed (**Figure 1A**). In addition, the expression of NDUFA13 in the spermatozoa was compared, and it was significantly lower in men with asthenozoospermia than than in men with normozoospermia (NDUFA13/GAPDH: 0.47 ± 0.26 versus 0.87 ± 0.22, $^{**}P < 0.01$) (**Figure 1B and 1C**).

Mitochondrial membrane potential and reactive oxygen species of spermatozoa in men with asthenozoospermia

A previous study showed that a deficiency of NDUFA13 may lead to a collapse in the MMP and increased apoptosis (Chen et al., 2015), so the MMP of the spermatozoa in men with asthenozoospermia was evaluated. JC-1 was used to estimate the MMP of the spermatozoa, and the shift in JC-1 fluorescence from red to green indicated a collapse of the MMP (**Figure 2A**). Flow cytometric analysis showed that the percentage of sperm with bright orange in the asthenozoospermia group ($n = 35$, Q1/Q2: 0.72 ± 0.28) were significantly lower than that in the normozoospermia group ($n = 25$, Q1/Q2: 0.97 ± 0.25), suggesting that the spermatozoa in the asthenozoospermia group had a lower MMP compared with the normozoospermia group ($P < 0.01$) (**Figure 2B**).

Mitochondria are the main source of sperm-produced ROS, notably through the formation of superoxide in the electron transfer chain. As shown in **Figure 2C**, the intracellular ROS production in men with asthenozoospermia increases considerably ($n = 30$, R3/R2: 0.92 ± 0.38) compared with men with normozoospermia ($n = 25$, R3/R2: 0.65 ± 0.34, $^{**}P < 0.01$).

Table 1 – Participant demographic and clinical data.

	Normozoospermia	*Asthenozoospermia*
n	45	98
Age (years)	31.2 ± 3.9	30.6 ± 4.3
Ejaculate volume (ml)	3.0 ± 0.9	2.9 ± 1.1
pH	7.2 ± 0.2	7.3 ± 0.2
Sperm concentration (10^6/ml)	65.7 ± 42.4	58.9 ± 37.2
Progressive motility (%)	52.6 ± 12.2	16.8 ± 4.3[a]

[a] $P < 0.01$.

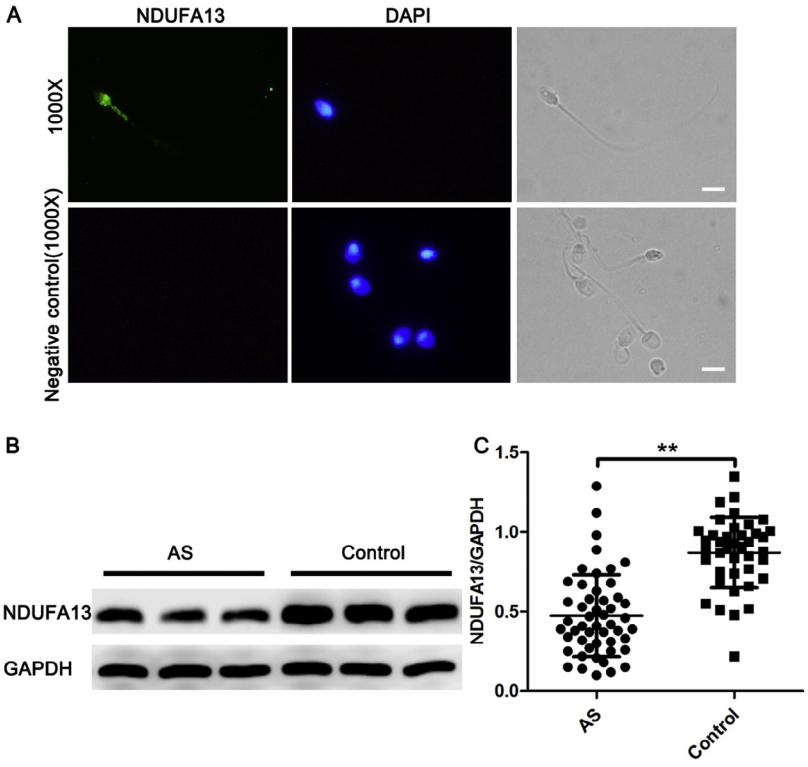

Figure 1 – Expression of nicotinamide adenine dinucleotide (NADH) dehydrogenase (ubiquinone) 1 alpha subcomplex, 13 (NDUFA13) in spermatozoa. (A) Immunofluorescent staining was carried out to investigate the subcellular localization of NDUFA13 in the spermatozoa of men with normozoospermia. In the middle is 4',6-diamidino-2-phenylindole (DAPI) staining. On the right side is light micrograph. Immunofluorescence staining with normal rabbit immunoglobulin G is shown as a negative control. Scale bar = 5 μm; (B, C) a Western blot analysis was conducted to assess the protein levels of NDUFA13 in the spermatozoa of men with asthenozoospermia (n = 50) and men with normozoospermia (n = 40) (NDUFA13/GAPDH: 0.47 ± 0.26 versus 0.87 ± 0.22). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control. The representative examples are shown, and each lane represents a different patient. All data are expressed as mean ± SD. $^{**}P < 0.01$. AS, asthenozoospermia.

Apoptosis of spermatozoa in men with asthenozoospermia

To detect the potential roles of NDUFA13 in asthenozoospermia, the apoptosis of the spermatozoa in men with asthenozoospermia, a cellular event promoted by NDUFA13, were examined. Flow cytometric analyses of annexin V/PI in the men with asthenozoospermia and the controls are presented in **Figure 3**. Significantly more apoptotic cells were found in Q4 in the asthenozoospermia group (n = 25, Q4: 14.36 ± 4.54) than in the normozoospermia group (n = 20, Q4: 7.19 ± 3.27, $P < 0.01$). These results suggest that an increased level of apoptosis may be related to sperm parameters.

Downregulation of NDUFA13 and its effect in mouse spermatocyte cell line GC2-spd

To study the effect of the low level of NDUFA13 in asthenozoospermia, the expression of NDUFA13 was downregulated by specific siRNA in the GC2-spd cell line. As shown in **Figure 4A**, The expression of NDUFA13 was successfully knocked down. The MMP, intracellular ROS production, apoptosis in the NDUFA13 siRNA group and the scramble control group were then analysed. A significant decrease was observed in the MMP after NDUFA13 siRNA transfection (Q1/Q2: 3.93 ± 0.20 versus 6.66 ± 0.14, $P < 0.01$) (**Figure 4B**), and the cells in the NDUFA13 siRNA group had higher levels of ROS than those in the control group (R3/R2: 0.99 ± 0.13 versus 0.26 ± 0.06, $P < 0.01$) (**Figure 4C**). In addition, the apoptotic cells increased significantly in the NDUFA13 siRNA group (Q4: 14.74 ± 1.25 vs. 7.17 ± 1.38, $P < 0.01$) (**Figure 4D**). These results indicated that NDUFA13 may be involved in asthenozoospermia by affecting the MMP, intracellular ROS production and increasing apoptosis.

Discussion

To date, the role of NDUFA13 in asthenozoospermia has not been reported, although it has previously been shown to be essential to mitochondrial respiratory function. In the present study, we revealed a new molecular mechanism underlying NDUFA13 mediated human spermatozoa motility.

One of the major outcomes of this study is that expression of NDUFA13 was significantly lower in the spermatozoa of men with asthenozoospermia, demonstrating the relevance between a low level of NDUFA13 and an occurrence of asthenozoospermia. Flow cytometric analysis showed that MMP is lower, intracellular ROS production increases considerably, and more apoptotic cells are observed in men with asthenozoospermia than men with normozoospermia. Furthermore, our study shows that downregulation of NDUFA13 leads to a collapse in the MMP, increased ROS production and more apoptosis in the GC2-spd cell line. Therefore, we conclude that NDUFA13

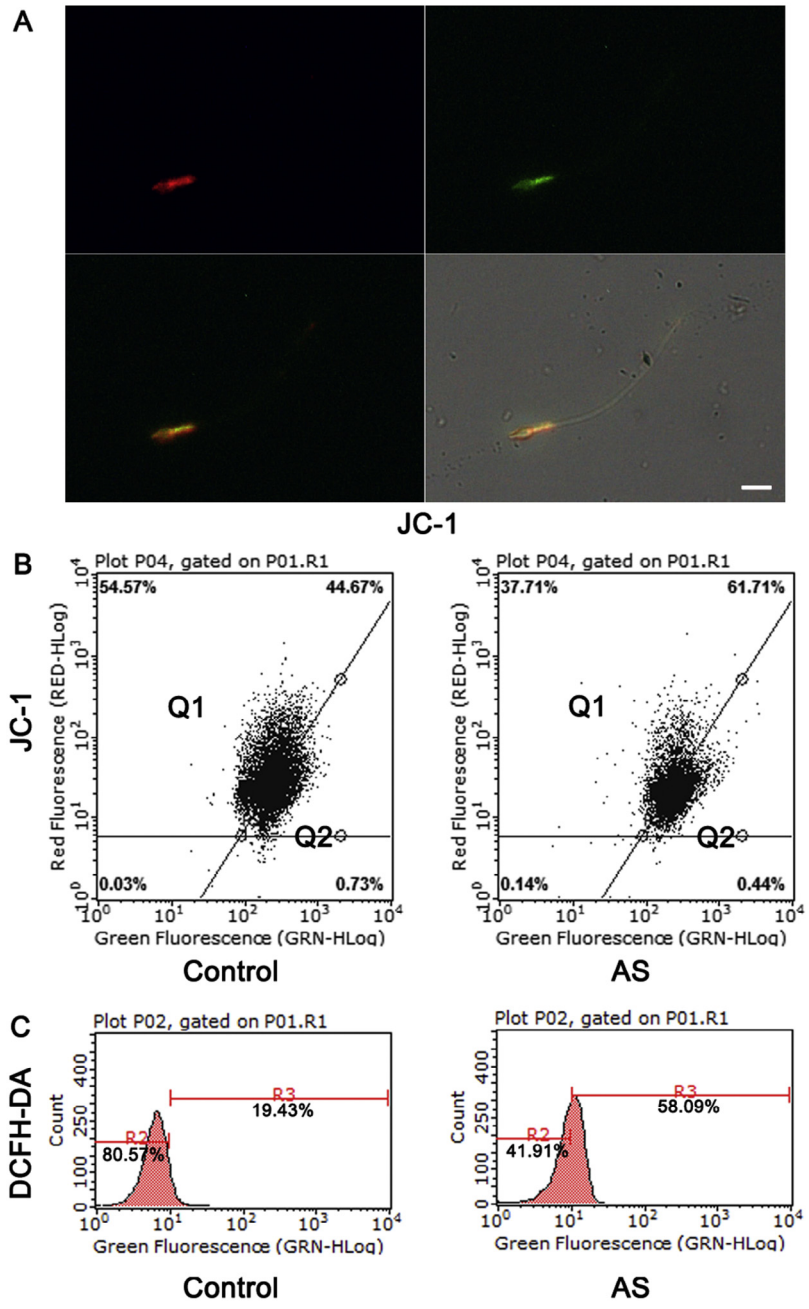

Figure 2 – Mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) of spermatozoa in men with asthenozoospermia. (A) JC-1 was used to estimate the MMP of the spermatozoa, and the shift in the JC-1 fluorescence from red to green indicates a collapse of the MMP. The representative examples of one sperm are shown. The JC-1 aggregate image shows red fluorescence, and the JC-1 monomer image shows green fluorescence. In one sperm, both red fluorescence (upper left) and green fluorescence (upper right) can be seen under a fluorescent microscope. The merged image combines red and green images (lower panel); (B) the representative flow cytometric analyses of the MMP using JC-1 are shown. In the dot plot, the cells with a high MMP were observed in the upper left quadrant (Q1), and the cells with a low MMP were identified in the lower right quadrant (Q2). The percentage of sperm with bright orange in the asthenozoospermia group (n = 35, Q1/Q2: 0.72 ± 0.28) were significantly lower than that in the normozoospermia group (n = 25, Q1/Q2: 0.97 ± 0.25). All data are expressed as mean ± SD. $P < 0.01$; (C) the level of ROS in the spermatozoa was quantified by dichlorofluorescein diacetate. A representative flow cytometric analysis showed that there was a considerable increase in the intracellular ROS production in the asthenozoospermia patients (n = 30, R3/R2: 0.92 ± 0.38) compared with men with normozoospermia (n = 25, R3/R2: 0.65 ± 0.34). All data are expressed as mean ± SD. $P < 0.01$. AS, asthenozoospermia; DCFH-DA, dichlorofluorescein diacetate.

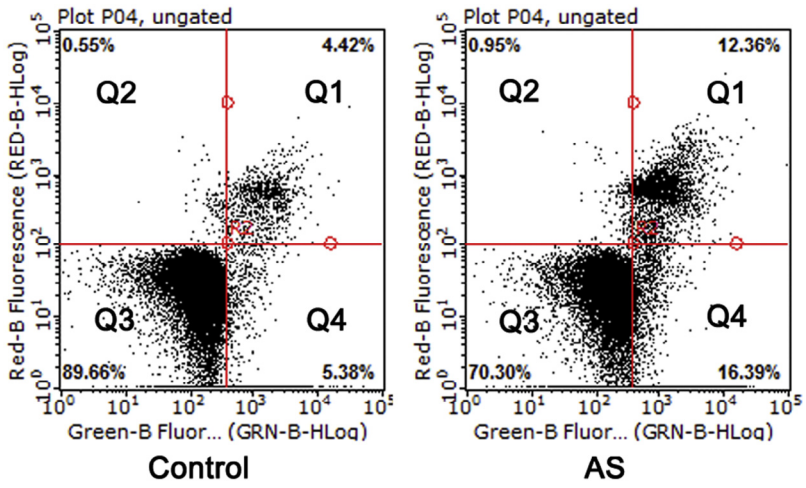

Figure 3 – Apoptosis of the spermatozoa in men with asthenozoospermia. Annexin V-FITC was used to detect the apoptosis of the spermatozoa samples. The representative flow cytograms are shown. The numbers in the upper right quadrant (Q1), upper left quadrant (Q2), lower left quadrant (Q3) and lower right quadrant (Q4) represent the relative amount of necrotic, damaged and live, and relative number of apoptotic cells, respectively. Men with asthenozoospermia had significantly more apoptotic cells (n = 25, Q4: 14.36 ± 4.54) than in men with normozoospermia (n = 20, Q4: 7.19 ± 3.27). All data are expressed as mean ± SD. $P < 0.01$. AS, asthenozoospermia.

deficiency may play a role in asthenozoospermia via regulating the MMP, intracellular ROS production and apoptosis.

One cause of male infertility is reduced sperm motility. It seems that a reduction in the efficiency of mitochondrial respiratory activity may contribute to the lower motility (Cedikova et al., 2014). Mitochondria are powerhouse organelles involved in ATP synthesis, calcium signalling, oxidative stress from the production of ROS, cell cycle arrest caused by apoptosis and sex steroid hormones biosynthesis. Mitochondrial regulation of ROS concentrations is related to the improvement of sperm parameters, including motility, capacitation, acrosome reaction and oocyte interaction (Benkhalifa et al., 2014). Previous studies have shown that mitochondria play an important role in human spermatozoa motility (Ferramosca et al., 2012). A positive correlation between mitochondrial function and human sperm fertilization potential and quality has recently been reported (Buzadzic et al., 2015; Gallon et al., 2006; Otasevic et al., 2013). Mitochondrial respiratory efficiency is correlated with spermatozoa motility, and many different electron transfer chain inhibitors have been shown to negatively affect sperm motility (Amaral et al., 2013; Ruiz-Pesini et al., 2000; St John et al., 2005). An increased leakage of protons from the mitochondrial matrix leads to a reduction in the efficiency of phosphorylation. Cedikova et al. (2014) confirmed the activity of complex I is reduced in men with asthenozoospermia. These changes may be involved in the reduced sperm motility. NDUFA13 is a functional subunit of mitochondrial respiratory chain complex I and plays an essential role in the assembly and enzymatic activity of complex I (Lu and Cao, 2008). Another important outcome of our study is that NDUFA13 is predominantly localized to the sperm mid-piece, where the sperm mitochondria are housed. Therefore, our study indicates that NDUFA13 may play an important role in human spermatozoa motility by affecting the mitochondrial function.

Previous studies have shown a lack of NDUFA13 leads to the abnormality of the mitochondrion structure, morphology and cellular distribution (Chao et al., 2015; Huang et al., 2004). A deficiency of NDUFA13 leads to a collapse of MMP and an increased apoptosis in HTR-8/SVneo cell line (Chen et al., 2015). In our study, a collapse of the MMP was observed in the GC2-spd cell line after a knockdown of NDUFA13. These results confirmed that the decreased expression of NDUFA13 was consistent with the lower level of the MMP in asthenozoospermia.

It has been reported that oxidative stress is one of the main issues associated with asthenozoospermia (Sun et al., 2014). Ample evidence now shows that increased ROS levels may be implicated in spermatozoa DNA fragmentation, lipoperoxidation damage, increased apoptosis and decreased motility (Agarwal et al., 2008). Previous studies indicated that mitochondria are the main source of sperm produced ROS, notably through the formation of superoxide in the electron transfer chain (Koppers et al., 2008). In our study, a considerable increase in the intracellular ROS production in men with asthenozoospermia was observed. To study the correlation between NDUFA13 and intracellular ROS production in spermatozoa, ROS production in the GC2-spd cell line was examined after NDUFA13 knockdown. We found that the ROS level in the NDUFA13 siRNA group was significantly higher than in the control group. These results indicated that a deficiency of NDUFA13 in asthenozoospermia is related to intracellular ROS production.

The presence of activated caspase 9 (activated by the mitochondrial pathway of apoptosis) and caspase 3 (activated by both apoptotic pathways) has been related to poor sperm quality, although the capacity of mature sperm to undergo real apoptosis has been questioned owing to the paucity of cytoplasm (Almeida et al., 2011; Amaral et al., 2013; Grunewald et al., 2008; Kotwicka et al., 2008; Sakkas et al., 2002). Shen et al. (2002) indicated that Annexin-V staining for the identification of phosphatidylserine exposure is a valid tool for the assay of apoptosis in sperm (Shen et al., 2002). Our study found that there is more apoptosis in asthenozoospermia. A number of apoptosis markers have been shown in spermatozoa from infertile men, such as various degrees of plasma membrane translocation of phosphatidylserine, active capsase-3 (the main executor of apoptosis) with an apparent exclusive cellular location in the mid-piece, and DNA fragmentation (Cavallini, 2006; Oehninger et al., 2003). Increased ROS in tubules and seminal plasma and increased apoptosis are thought to affect sperm concentration, motility and morphology (Cavallini, 2006). Other studies also revealed more viable cells in men with normozoospermia, and further demonstrates the adverse functional effect of sperm apoptosis on sperm quality such as motility, vitality and sperm defects (Shen et al., 2002).

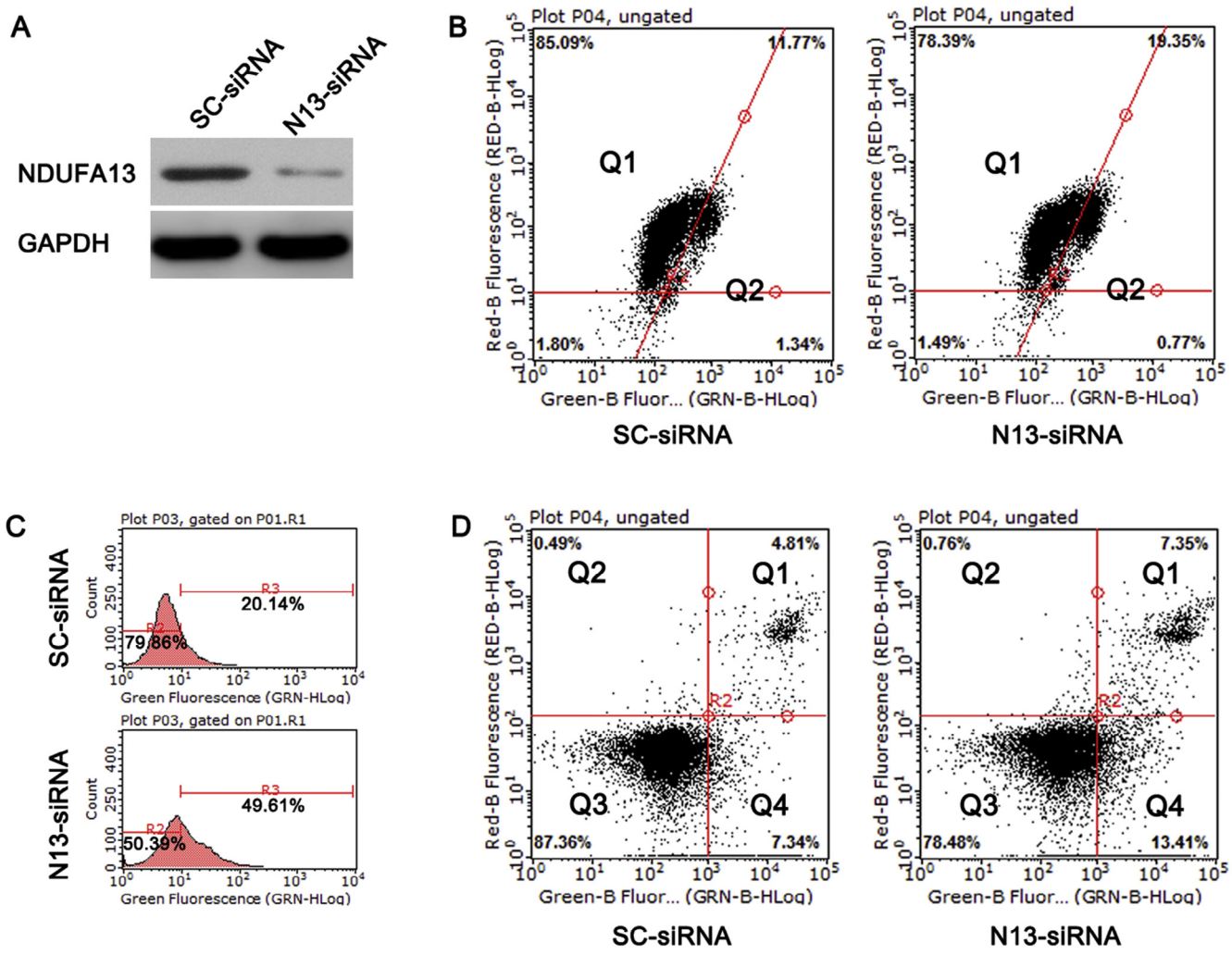

Figure 4 – Downregulation of nicotinamide adenine dinucleotide (NADH) dehydrogenase (ubiquinone) 1 alpha subcomplex, 13 (NDUFA13) and its effect in mouse spermatocyte cell line GC2-spd. (A) A Western blot analysis indicated notable depletion of NDUFA13 in the NDUFA13 small interfering RNA (siRNA) transfected cells. (B) JC-1 was used to estimate the mitochondrial membrane potential (MMP) of the cells. In the dot plot, the cells with a high MMP were observed in the upper left quadrant (Q1), and the cells with a low MMP were identified in the lower right quadrant (Q2). The representative examples showed that there was a significant decrease in the MMP after NDUFA13 siRNA transfection (Q1/Q2: 3.93 ± 0.20 versus 6.66 ± 0.14). Three independent experiments were carried out. All data are expressed as mean ± SD. $P < 0.01$; (C) dichlorofluorescein diacetate was used to determine the intracellular reactive oxygen species level. The representative examples showed that cells in the NDUFA13 siRNA group had higher levels of reactive oxygen species than those in the control group (R3/R2: 0.99 ± 0.13 vs. 0.26 ± 0.06). Three independent experiments were carried out. All data are expressed as mean ± SD. $P < 0.01$; (D) annexin V-FITC was used to detect the apoptosis of cells. The numbers in the upper right quadrant (Q1), upper left quadrant (Q2), lower left quadrant (Q3), and lower right quadrant (Q4) represent the relative amount of necrotic, damaged, and live, and relative number of apoptotic cells, respectively. A representative flow cytometric analysis showed that apoptotic cells increased significantly in the NDUFA13 small interfering RNA group (Q4: 14.74 ± 1.25 versus 7.17 ± 1.38). Three independent experiments were carried out. All data are expressed as mean ± SD. $P < 0.01$. SC, scramble control, N13: NDUFA13.

Mammalian cell line studies have shown that mitochondria play an important role in apoptosis, and various apoptogenic factors are released with mitochondrial electron transport chain disruption (Chen et al., 2015; Huang et al., 2013; Liu et al., 1996; Verhagen et al., 2000). NDUFA13 is a component of complex I, functions as an essential part of complex I, and integrates the entire mitochondrial electron transfer chain. It is speculated that the destruction of mitochondrial function resulting from NDUFA13 deficiency could be an important mechanism underlying more sperm apoptosis. Recent studies have also implicated mitochondrial ROS generation in human sperm apoptosis, with resultant ROS-derived DNA damage (Aitken et al., 2012; Amaral et al., 2013). On the other hand, a previous study found that the number of sperm with Fas expression was low in men with normal sperm parameters but high in men with abnormal sperm parameters (Sakkas et al., 1999; Shen et al., 2002). Therefore, further studies are necessary to confirm the mechanisms underlying sperm apoptosis. In our study, we found that the knockdown of NDUFA13 could lead to more apoptotic cells, suggesting that NDUFA13 might be one of the molecules involved in sperm apoptosis.

In conclusion, our data support the original hypothesis that there may be an association between asthenozoospermia and low levels of NDUFA13. NDUFA13 may be involved in the pathogenesis of asthenozoospermia via spermatozoa MMP, intracellular ROS and cell apoptosis.

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