Original Research

Expression of mitofusin 2 in human sperm and its relationship to sperm motility and cryoprotective potentials

Fang Fang[1,2], Ke Ni[3], Jin Shang[2], Xiaoke Zhang[2], Chengliang Xiong[2,4] and Tianqing Meng[4]

[1]Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China; [2]Family Planning Research Institute, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; [3]Department of Anesthesiology, Tongji Medical College, Huazhong University of Science and Technology, Tongji Hospital, Wuhan 430030, China; [4]Center of Reproductive Medicine, Wuhan Tongji Reproductive Medicine Hospital, Wuhan 430013, China

Corresponding author: Tianqing Meng. Email: tqmengtj@sina.com

Impact statement

The exact function of mitochondria in human sperm before and during fertilization process remains controversial. MFN2 is a kind of mitochondria membrane protein and participates in the regulation of mitochondrial morphology and function. In this study, we discover the relationship of MFN2 expression to human sperm motility and cryoprotective potentials. Our results suggest that MFN2 could be a new target for the mechanism research of asthenozoospermia. MFN2 may also serve as a protein marker predicting the ability of human sperm to sustain cryopreservation.

Abstract

Mitofusin 2 is a kind of mitochondria membrane protein that has been implicated in maintenance of mitochondrial morphology and function. However, the expression and function of mitofusin 2 in human sperm are not well described at present. The aim of this study was to explore the location of mitofusin 2 in human sperm and to discover its relationship to human sperm functions like motility and cryoprotective potentials. Our result showed that mitofusin 2 is specifically localized in the 5–7 μm midpiece between the neck and main part of human sperm tail. The expression level of mitofusin 2 in human sperm was significantly different between the normozoospermia and asthenozoospermia groups ($P < 0.05$); meanwhile, the sperm of the asthenozoospermia group had a lower mitochondrial membrane potential ($P < 0.05$), but the results of TUNEL assay did not show significant difference between the two groups. Furthermore, we found that the expression level of mitofusin 2 in the freeze-resistant group (cryo-survival rate >40%) was significantly higher than that of the freeze-intolerant group (cryo-survival rate ≤40%) ($P < 0.05$). These results demonstrate that the expression level of mitofusin 2 is related to motility and cryoprotective potentials of human sperm. Mitofusin 2 may play a crucial role in the function of human sperm, which needs further research to discover the mechanism.

Keywords: Asthenozoospermia, cryopreservation, human sperm, mitofusin 2, mitochondria, sperm motility

***Experimental Biology and Medicine* 2018; 243: 963–969. DOI: 10.1177/1535370218790919**

Introduction

Mitochondria play an essential function in eukaryotic cells. Mitochondria in human sperm have extensive morphological heterogeneity, and the exact function of them before and during fertilization process remains controversial. Although it is widely accepted that sperm mitochondria are eliminated at the early embryo stages to maintain maternal mitochondrial inheritance,[1] Luo *et al.*[2] found that mitochondria DNA could be transmitted to offspring if it occasionally remained and entered the zygote in mice. Mitochondria not only provide energy for sperm motility, but also participate in various sperm functions, including calcium homoeostasis, generation of reactive oxygen species, and apoptotic pathway.[3–5] Moreover, during different stages of spermatogenesis, the number, morphology, and location of mitochondria in spermatogenic cells are changing obviously with varying expression of mitochondrial marker proteins.[6–8]

Mitofusin 2 (MFN2) is a kind of mitochondria membrane protein that has been demonstrated involved in maintenance of mitochondrial morphology and function, such as energy metabolism, cell apoptosis, and intracellular signaling.[9] All the functions of MFN2 are critical factors influencing the quality of sperm. The expression of MFN2 in sperm has been investigated in animal models. In mice, MFN2 was present in the midpiece of the tail in caput and

cauda epididymal sperm and co-localized with the meiosis-specific nuclear structural 1 (MNS1) to the sperm flagellum, indicating that they may play an integral role in mouse sperm flagellar structure and function.[10] Moreover, the presence of MFN2 in boar sperm was found to be expanded from midpiece to the principal piece after *in vitro* capacitation and *in vitro* acrosome reaction, suggesting that MFN2 participated in the modulation of boar sperm mitochondrial function.[11]

Sperm cryopreservation has been widely used in assisted reproductive technology (ART). Preservation of sperm vitality for longer periods is achieved by cooling and freezing. However, freezing and thawing have obvious damage on the structure and function of sperm, including sperm motility, vitality, and fertilizing ability.[12,13] Speculatively, this impairment may be related to changes in the expression and location of sperm MFN2. In this regard, it was reported that cryopreservation-induced alterations in boar sperm mitochondrial function were concomitant with a progressive extension of MFN2 location from the apical zone of the midpiece to the whole midpiece.[14] Furthermore, HEK293 cells were protected against cold stress-induced injury with increased expression of MFN2 and mitochondrial fusion.[15]

However, the expression and function of MFN2 in human sperm are not well described at present. In this study, we explored the location of MFN2 in human sperm and found that the expression level of MFN2 was related to human sperm motility and cryoprotective potentials, suggesting that MFN2 may play a crucial role in the function of human sperm.

Materials and methods

Semen collection

Semen samples were obtained from patients undergoing routine semen analysis for couple infertility at the reproductive center of Tongji Medical College or consenting donors at Hubei human sperm bank of China, with written informed consent to allow use of the remaining semen for research purposes. Semen samples were collected by masturbation after three days of sexual abstinence. Ethical approval for human study was obtained from the institutional review board of Tongji medical college.

Sperm motility analysis

Semen analysis was evaluated according to World Health Organization guidelines.[16] Immediately after receiving the semen samples, an aliquot was taken to perform the sperm motility analysis (fresh semen sample, M_F). After the semen sample was liquefied completely by a water bath at 37°C, 10 μL of semen sample was put on a Makler counting chamber (Sefi Medical Instruments, Israel) and observed using a microscope (XSZ-D2, Chongqing Optical Instruments, China). Sperm motility was classified as percentage of motile sperm. Two independent observers counted the percentage of motile sperm for each sample with three replicates, and we took the average as the final result.

Semen cryopreservation

In addition of cryoprotectant, the semen cryopreservation was processed with programmed freezing protocol. Frozen samples were stored in liquid N_2 for 24 h. After this, the samples were thawed by a water bath at 37°C for 10 min. Then, samples were immediately analyzed to determine the sperm motility (frozen-thawed semen sample, M_T). Comparing the sperm motility of frozen-thawed semen sample with fresh sample, we calculated the cryo-survival rates (CSRs) of the semen sample by the following equation

$$\mathrm{CSR}(\%) = \frac{M_T}{M_F} \times 100$$

We performed strict quality control once a week to ensure the accuracy of the results.

Immunofluorescence

The expression and location of MFN2 were determined by immunofluorescence; 50 μL of the normal semen samples were spread onto polylysine-coated microscope slides and left to air-dry for 15 min. Then the samples were fixed in 4% paraformaldehyde. After being washed with phosphate-buffered saline (PBS) (Hyclone, Utah, USA), the samples were permeabilized for 20 min with 0.2% Triton X-100 (Sigma, St. Louis, USA) in PBS at 37°C. Afterward, the slides were washed three times with PBS and were blocked with 1% BSA for 30 min at room temperature. Then sperm sample were incubated with polyclonal rabbit anti-MFN2 (1:200, Santa Cruz, TX, USA) primary antibodies overnight at 4°C. After being washed thoroughly with PBS the following day, the samples were incubated with FITC-conjugated goat anti-rabbit (1:100, Boster, Wuhan, China) secondary antibodies for 1 h at 37°C. As negative controls, samples were incubated with rabbit serum instead of primary antibodies. Nuclei were stained with 1% PI. Finally, the slides were mounted in an antifading mounting solution and observed with a confocal scanning microscope (FV500, Olympus, Tokyo, Japan).

Sperm protein extraction

The samples were washed with ice-cold PBS for two times and concentrated by centrifugation respectively. Sperm pellets were homogenized by sonication in 100 μL of RIPA lysis buffer (BIOTEKE, Beijing, China), containing 1% protease inhibitor Cocktail (Calbiochem, CA, USA). After lysis for 1 h at 4°C, the samples were centrifuged at 12,000 r/min for 5 min. Sperm protein concentrations were determined by BCA protein assay (BIOTEKE, Beijing, China). The supernatants were collected and stored immediately at −80°C until use.

Western blot analysis

Sperm protein samples were diluted in 4× loading buffer (Solarbio, Beijing, China) containing dithiothreitol (DTT) and bromophenol blue. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) and were boiled for 5 min before being transferred to SDS-polyacrylamide gel (20 μg per lane). Then the gels were transferred to nitrocellulose membranes. After being blocked with TBST (125 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.1% Tween 20) containing 5% BSA (Merk, Darmstadt, German), the membranes were incubated with monoclonal mouse anti-MFN2 (1:400, Abcam, MA, USA) and polyclonal rabbit anti-GAPDH (1:10,000, Abcam, MA, USA) primary antibodies at 4°C overnight. After being washed with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:1000, Santa Cruz, TX, USA) or anti-rabbit (1:5000, Santa Cruz, TX, USA) secondary antibodies. The reaction was developed with the ECL kit (Pierce Chemical, Rockford, USA) according to the manufacturer's directions. The band density was quantified using AlphaEaseFC software.

Detection of human sperm mitochondrial membrane potential

JC-1 is most widely used for (MMP) mitochondrial membrane potential detection, which shows potential-dependent accumulation in mitochondria.[17] In sperm with high MMP, JC-1 gathers in mitochondrial matrix to form J-aggregates and shows red fluorescence, while JC-1 maintains monomeric form and shows green fluorescence in sperm with low MMP. Semen samples were centrifuged at 700 *g* for 4 min to remove the seminal plasma, and a pellet of 6×10^5 sperm was labeled with JC-1 reagent (JC-1 MMP Detection Kit, Beyotime, Shanghai, China) for 20 min at 37°C. After washing, human sperm was analyzed for MMP on a flow cytometer (BD Bioscience, MD, USA) or observed by fluorescence microscope (IX71, Olympus, Tokyo, Japan).

Detection of human sperm apoptosis

To investigate the sperm apoptosis, an in situ cell death detection Kit (Roche, Mannheim, Germany) was used for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining according to the manufacturer's instructions. Briefly, smears were dried and fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. After washing with PBS for three times, the slides were incubated with 0.3% H_2O_2 in methanol for 1 h to quench endogenous peroxidase activity and permeabilized using 0.1% Triton X-100 for 5 min at 4°C. After that, the samples were incubated with 50 μL TUNEL reaction mixture in a humidified chamber at 37°C for 1 h and washed in PBS. Then, we visualized the POD retained in the immune complex using a DAB (3, 3-diaminobezidine tetrahydrochloride) detection kit (DAKO, Glostrup, Denmark). At last, samples were dehydrated in ethanol, cleared in xylene (Sigma, St. Louis, USA), and mounted. The slides were assessed under an optical microscope (Olympus, Tokyo, Japan). For each sample, at least 200 sperm nuclei were counted. A negative control (sample was treated with 50 μL of labeled solution without terminal transferase instead of TUNEL reaction mixture) was also performed.

Statistical analysis

Data are presented as mean ± SD. Statistical analyses were performed using either a *t*-test or a one-way analysis of variance (ANOVA) when appropriate (significance was set at $P<0.05$). Statistical analysis was carried out using SPSS 17.0 software.

Results

The expression and location of MFN2 in human sperm

Immunofluorescence was used to examine the localization of MFN2 in normal human sperm. The results showed that the expression of MFN2 was specifically localized in the midpiece of the sperm tail and that the 5–7 μm midpiece between the neck and main part was positive but all other parts of human sperm tail were negative for MFN2 (Figure 1). So the localization pattern of MFN2 was consistent with that of mitochondria in human sperm.

MFN2 expression level analysis in human sperm with asthenozoospermia

A total of 10 patients with clinical asthenozoospermia and 10 healthy fertile men were included in this study. All the participants had no diagnosed diseases related to mitochondrial quality and no history of smoking and alcohol intake. Mean ages were 30.2 years (range: 27–35) for asthenozoospermia patients and 29.6 years (range: 26–32) for healthy fertile men, with no significant difference between the two groups. The parameters of their semen samples are listed in Table 1. The average sperm motility of the asthenozoospermia patients was 22.22%, which was significantly lower than that of healthy fertile men ($P<0.0001$). And there is no difference in sperm concentration between the two groups. As determined by Western blot, the expression of MFN2 (protein level) in sperm of patients with asthenozoospermia was lower than that in healthy fertile men (Figure 2).

Sperm MMP analysis in human sperm with different motility

The results of flow cytometry and JC-1 immunofluorescence showed that the sperm of asthenozoospermia group had a lower MMP ($P < 0.05$), indicating high percentage of potential apoptosis (Figure 3(a) to (d)). Therefore, we used TUNEL assay to detect sperm apoptosis. However, our result showed that there were no significant differences in the rate of sperm apoptosis in asthenozoospermia and normozoospermia group (Figure 3(e) and (f)).

Relationship of MFN2 expression level to cryoprotective potentials

According to the CSRs, normal semen samples were divided into two groups: freeze-resistant group with cryo-survival rate over 40% and freeze-intolerant group with cryo-survival rate lower than 40%. Western blot analysis showed the presence of MFN2 in both groups which was in accordance with immunofluorescence staining results

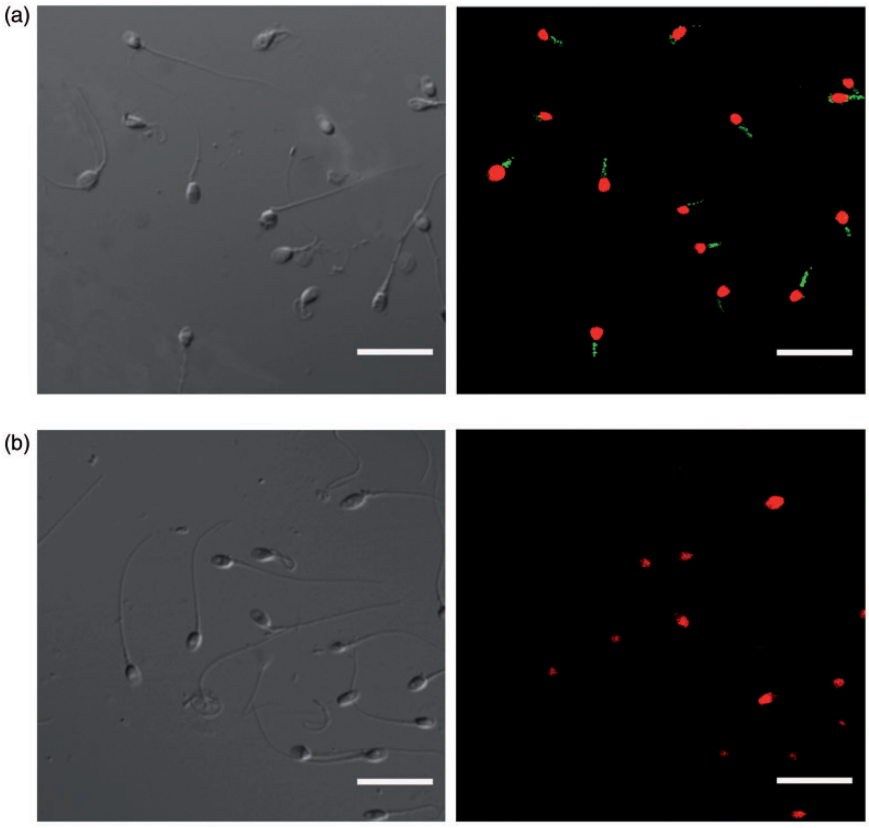

Figure 1. The expression and location of MFN2 in human sperm. (a) Representative images from confocal scanning microscope show MFN2 localizes in the midpiece of human sperm (green). The head of sperm is indicated by PI staining (red) of the nuclei. The bright field is shown in adjacent left panel. Scale bar: 50 um. (b) Negative control for MFN2 immunofluorescent staining. (A color version of this figure is available in the online journal.)

Table 1. Semen parameters of the men from different groups (normozoospermia or asthenozoospermia).

Group	Volume (mL)	Sperm concentration ($\times 10^6$/mL)	Total motility (%)	Progressive motility (%)	Immotility (%)
Normozoospermia (n=10)					
Min	2.2	17.743	44.34	39.00	22.58
Max	5.4	182.68	77.42	67.00	55.66
Mean	4.13	99.01	64.44	53.00	35.56
SD	1.05	61.85	11.26	9.75	11.26
Asthenozoospermia (n=10)					
Min	2.2	26.44	5.68	3.41	32.72
Max	4.5	154.32	34.60	25.00	94.32
Mean	3.31	84.71	22.22[a]	15.40[b]	74.51[c]
SD	0.77	49.04	9.25	6.75	16.80

Note: [a]$P<0.0001$, [b]$P<0.0001$, [c]$P<0.0001$, compared with normozoospermia group.

(Figure 4(a)). Quantitative analysis of MFN2 expression level was determined by gray intensity analysis of the Western blot bands. The expression level of MFN2 in freeze-resistant group was significantly higher than that of freeze-intolerant group ($P < 0.05$) (Figure 4(b)).

Discussion

MFN2 not only plays an important role in mitochondrial fusion to maintain mitochondrial morphology, it has also been demonstrated to be associated with the regulation of mitochondrial function like cell respiration and intracellular signal transduction in mammalian cells.[18,19] However, few studies about the expression and function of MFN2 in human sperm have been reported recently. In this study, we detected the expression and location of MFN2 in human sperm and confirmed that MFN2 was specifically localized in the 5–7 μm midpiece between the neck and main part of human sperm, which was in line with the distribution of mitochondria, suggesting that MFN2 may be related to sperm motility.

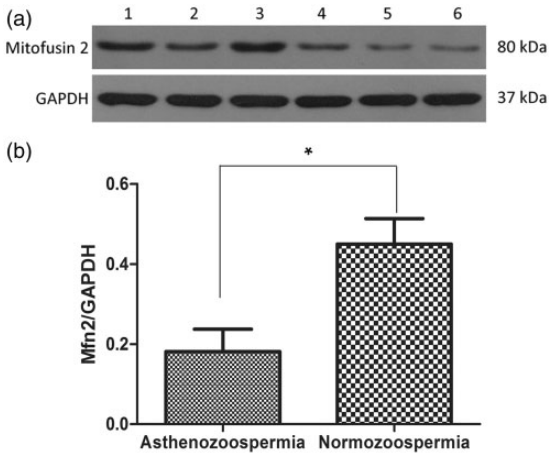

Figure 2. MFN2 expression levels in groups with different sperm motility.(a) Western blot analysis of MFN2 expression level in normozoospermia group (1–3) and asthenozoospermia group (4–6). (b) Quantitative analysis of MFN2 expression differences by gray intensity analysis with AlphaEaseFC software. GAPDH was utilized as an internal control to estimate the total protein content of samples. Figures show representative results from 10 independent replicates. Error bars represent the standard error of the mean. *$P < 0.05$.

To explore the relationship of MFN2 to sperm motility, we investigated the expression level of MFN2 in human sperm with different motility. The results of Western blot showed that the expression level of MFN2 in sperm of patients with asthenozoospermia was lower than that of healthy fertile men. Furthermore, we found that the sperm of asthenozoospermia had obviously decreased MMP when compared with sperm of normozoospermia. However, the results of TUNEL assay did not show any significant difference in the sperm apoptosis between both groups. Normally, mitochondrial morphology is maintained by a dynamic balance of fusion and fission. Mitochondrial fusion plays a crucial role in the maintenance of a tubular mitochondrial network. Disruption of fusion results in mitochondrial dysfunction, like increasing mitochondrial fission and rapid fragmentation of mitochondria.[19–21] Increasing evidence has identified that cells lacking MFN2 have low levels of mitochondrial fusion with a series of severe cellular defects, including poor cell growth, decreased cellular respiration, and MMP.[19,22] Based on the distribution and function of MFN2 in

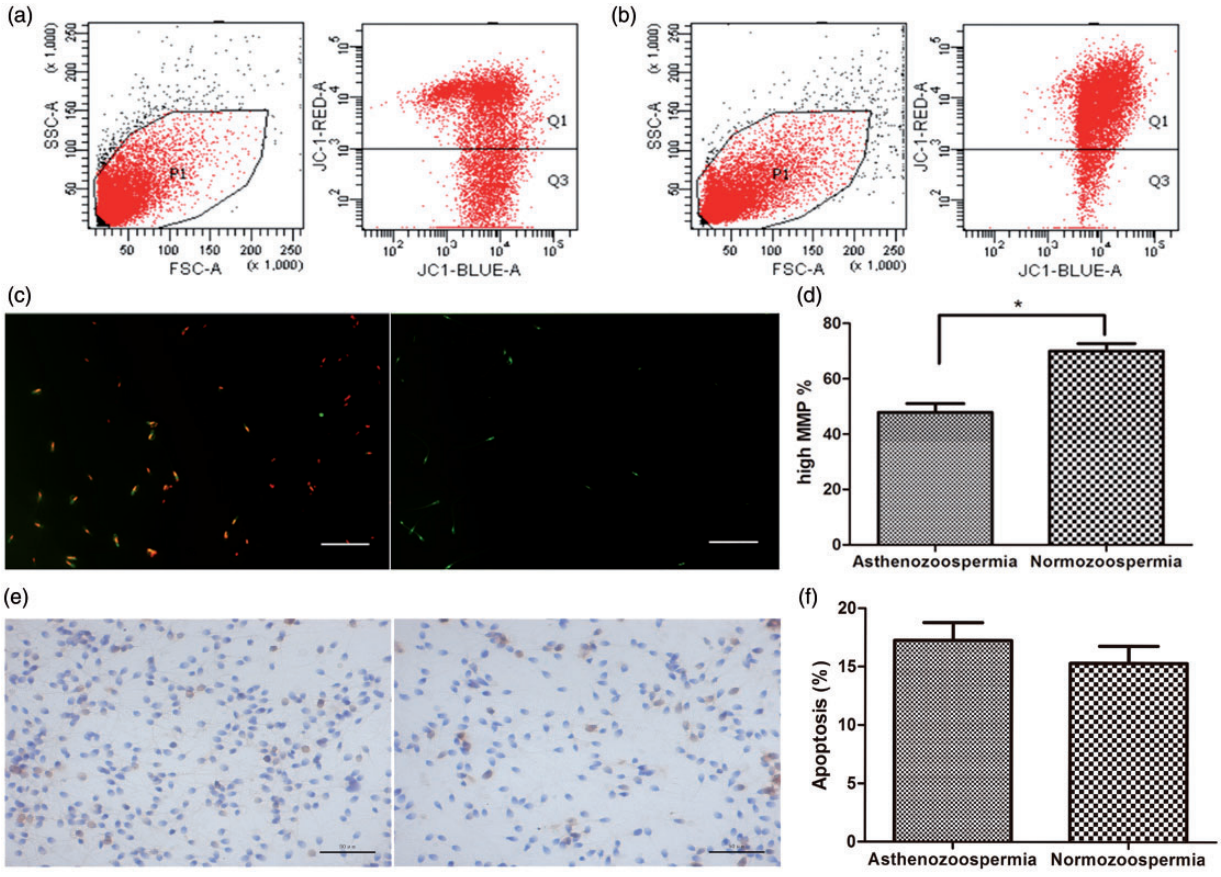

Figure 3. Sperm mitochondrial membrane potential (MMP) analysis for men with different sperm motility. (a) Representative flow cytometry plots for asthenozoospermia group; (b) Representative flow cytometry plots for normozoospermia group; (c) Representative images from fluorescence microscope show different sperm MMP in normozoospermia group (left) and asthenozoospermia group (right). Sperm with high MMP show red fluorescence, and sperm with low MMP show green fluorescence. Scale bar: 50 um. (d) Quantitative analysis of sperm MMP in normozoospermia group and asthenozoospermia group, indicated by percentage of sperm with red fluorescence. Figures show representative results from 10 independent replicates. Error bars represent the standard error of the mean.*$P < 0.05$. (e) TUNEL staining in normozoospermia group (left) and asthenozoospermia group (right). Brown stained sperm shows positive for apoptosis, and light colored sperm indicates negative for apoptosis. (f) Quantitative analysis of sperm apoptosis in normozoospermia group and asthenozoospermia group, indicated by percentage of brown stained sperm. Figures show representative results from 10 independent replicates. Error bars represent the standard error of the mean.*$P > 0.05$. (A color version of this figure is available in the online journal.)

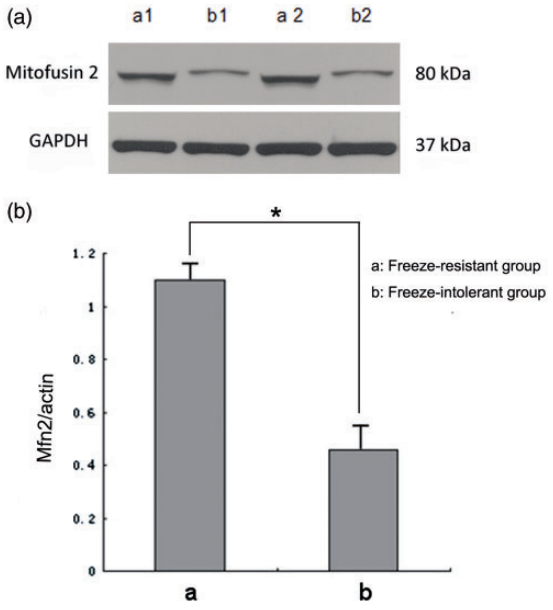

Figure 4. MFN2 expression levels in groups with different cryo-survival rates. (A) Western blot analysis of MFN2 expression level in freeze-resistant group (a) and freeze-intolerant group (b); (B) Quantitative analysis of MFN2 expression differences by gray intensity analysis with AlphaEaseFC software. GAPDH was utilized as an internal control to estimate the total protein content of samples. Figures show representative results from eight independent replicates. Error bars represent the standard error of the mean.*$P<0.05$.

human sperm, we have hypothesized that sperm MFN2 not only serves to promote fusion to keep the tubular mitochondrial structure, but also activates oxidative phosphorylation to trigger mitochondrial energization, as it works in other types of cells to optimize mitochondrial function.

Although cryopreservation is the most efficient method for long-term preservation of human sperm, the freeze-thawing process could influence the sperm quality parameters, sperm functions, and DNA integrity.[23–26] It was widely reported that freezing-thawing could result in decrease in sperm vitality, motility, and percentage of sperm with normal morphology.[27,28] It was also verified that freezing-thawing procedure of sperm can greatly decrease DNA integrity and condensation,[26,29,30] and DNA fragmentation was associated with male infertility and sperm parameters defects.[29,31] The mechanisms underlying the increase of DNA fragmentation in human sperm after cryopreservation are still unclear, but oxidative stress appears to be a possible mechanism.[32,33] It has been previously reported that oxidative stress is related to DNA damage after cryopreservation of human sperm.[34,35] Moreover, reactive oxygen species (ROS) are associated with poor semen quality and defective function of human sperm.[36,37] In fact, cold stress can significantly influence cell metabolism, ROS release, and mitochondrial energy efficiency in mammalian cells.[38,39] All of these changes are related to mitochondrial function, indicating that mitochondrial dysfunction is one of the major causes for cold-induced cell injury. In particularly, MFN2 also plays a key protective role against cold stress, as evidenced by a high rate of cold stress-induced cell death in MFN2 silenced HEK293 cells.[15] We used Western blot to evaluate the differences of MFN2 expression levels between the freeze-resistant and freeze-intolerant human sperm samples. Our result showed that the expression level of MFN2 was closely related to CSRs of human semen samples, revealing that MFN2 has protective effects on mitochondrial function as well as sperm survival during cold stress. Presumably, sperm MFN2 may function to reduce the production of ROS and increase the ATP efficiency to resist the cold-induced injury and to improve the vitality after cryopreservation. However, the mechanism behind this protective potential of MFN2 needs to be elucidated by further investigations.

In conclusion, our study showed that MFN2 expression is related to motility and cryoprotective potentials of human sperm. Therefore, it is suggested that MFN2 could be a new target for the mechanism research of asthenozoospermia. MFN2 may also serve as a protein marker predicting the ability of human sperm to sustain cryopreservation. Nevertheless, we need to recognize that our data are preliminary, so the specific role of MFN2 in human sperm remains to be explored deeply.

Authors' contributions: FF, KN and TQM carried out the experiments and wrote the manuscript. JS and XKZ performed data analysis and quality assessment. CLX and TQM contributed equally to conception and design of the study, supervision of the study and revising the paper critically for important intellectual content. All authors read and approved the final version of the manuscript to be published.

ACKNOWLEDGEMENTS

We are grateful to the human sperm bank of Hubei Province, China for kindly providing the semen samples for this study.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from National Science & Technology Pillar Program during the 12th 5-Year Plan Period, China (No. 2012BAI32B03) and National Natural Science Foundation (No. 81370755).

REFERENCES

1. Ramalho-Santos J. A sperm's tail: the importance of getting it right. *Hum Reprod* 2011;**26**:2590–1
2. Luo SM, Ge ZJ, Wang ZW, Jiang ZZ, Wang ZB, Ouyang YC, Hou Y, Schatten H, Sun QY. Unique insights into maternal mitochondrial inheritance in mice. *Proc Natl Acad Sci U S A* 2013;**110**:13038–43
3. Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. *Int J Androl* 2012;**35**:109–24
4. Amaral A, Lourenco B, Marques M, Ramalho-Santos J. Mitochondria functionality and sperm quality. *Reproduction* 2013;**146**:R163–74
5. Du Plessis SS, Agarwal A, Mohanty G, van der Linde M. Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use? *Asian J Androl* 2015;**17**:230–5

6. Meinhardt A, Wilhelm B, Seitz J. Expression of mitochondrial marker proteins during spermatogenesis. *Hum Reprod Update* 1999;**5**:108–19
7. Nogueira V, Rigoulet M, Piquet MA, Devin A, Fontaine E, Leverve XM. Mitochondrial respiratory chain adjustment to cellular energy demand. *J Biol Chem* 2001;**276**:46104–10
8. De Martino C, Floridi A, Marcante ML, Malorni W, Scorza Barcellona P, Bellocci M, Silvestrini B. Morphological, histochemical and biochemical studies on germ cell mitochondria of normal rats. *Cell Tissue Res* 1979;**196**:1–22
9. Yang J, Wang T, Zhang Y, Li R, Wang S, Xu H, Liu J, Ye Z. Altered expression of mitofusin 2 in penile tissues of diabetic rats. *Andrologia* 2014;**46**:522–8
10. Vadnais ML, Lin AM, Gerton GL. Mitochondrial fusion protein MFN2 interacts with the mitostatin-related protein MNS1 required for mouse sperm flagellar structure and function. *Cilia* 2014;**3**:5
11. Ramio-Lluch L, Fernandez-Novell JM, Pena A, Bucci D, Rigau T, Rodriguez-Gil JE. "In vitro" capacitation and subsequent acrosome reaction are related to changes in the expression and location of midpiece actin and mitofusin-2 in boar spermatozoa. *Theriogenology* 2012;**77**:979–88
12. Paoli D, Lombardo F, Lenzi A, Gandini L. Sperm cryopreservation: effects on chromatin structure. In: E Baldi, M Muratori (eds) *Genetic damage in human spermatozoa*. New York: Springer, 2014, pp.137–50
13. Wang SQ, Wang W, Xu Y, Tang M, Fang JZ, Sun HY, Sun YY, Gu MJ, Liu ZL, Zhang ZX, Lin FX, Wu T, Song NH, Wang ZJ, Zhang W, Yin CJ. Proteomic characteristics of human sperm cryopreservation. *Proteomics* 2014;**14**:298–310
14. Flores E, Fernandez-Novell JM, Pena A, Rigau T, Rodriguez-Gil JE. Cryopreservation-induced alterations in boar spermatozoa mitochondrial function are related to changes in the expression and location of midpiece mitofusin-2 and actin network. *Theriogenology* 2010;**74**:354–63
15. Zhang W, Chen Y, Yang Q, Che H, Chen X, Yao T, Zhao F, Liu M, Ke T, Chen J, Luo W. Mitofusin-2 protects against cold stress-induced cell injury in HEK293 cells. *Biochem Biophys Res Commun* 2010;**397**:270–6
16. WHO. *WHO laboratory manual for the examination and processing of human semen*, 5th ed. Geneva: World Health Organization, Department of Reproductive Health and Research, 2010
17. Tang C, Liang J, Qian J, Jin L, Du M, Li M, Li D. Opposing role of JNK-p38 kinase and ERK1/2 in hydrogen peroxide-induced oxidative damage of human trophoblast-like JEG-3 cells. *Int J Clin Exp Pathol* 2014;**7**:959–68
18. Zhao N, Zhang Y, Liu Q, Xiang W. MFN2 affects embryo development via mitochondrial dysfunction and apoptosis. *PLoS One* 2015;**10**:e0125680
19. Chen H, Chomyn A, Chan DC. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J Biol Chem* 2005;**280**:26185–92
20. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and MFN2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol* 2003;**160**:189–200
21. Chen H, Chan DC. Emerging functions of mammalian mitochondrial fusion and fission. *Hum Mol Genet* 2005;**14**:R283–9
22. Pich S, Bach D, Briones P, Liesa M, Camps M, Testar X, Palacin M, Zorzano A. The charcot-marie-tooth type 2A gene product, MFN2, up-regulates fuel oxidation through expression of OXPHOS system. *Hum Mol Genet* 2005;**14**:1405–15
23. Yeste M. Sperm cryopreservation update: cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology* 2016;**85**:47–64
24. Loren AW, Mangu PB, Beck LN, Brennan L, Magdalinski AJ, Partridge AH, Quinn G, Wallace WH, Oktay K. Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. *J Clin Oncol* 2013;**31**:2500–10
25. Comizzoli P, Wildt DE. Mammalian fertility preservation through cryobiology: value of classical comparative studies and the need for new preservation options. *Reprod Fertil Dev* 2013;**26**:91–8
26. Hammadeh ME, Askari AS, Georg T, Rosenbaum P, Schmidt W. Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. *Int J Androl* 1999;**22**:155–62
27. Punyatanasakchai P, Sophonsritsuk A, Weerakiet S, Wansumrit S, Chompurat D. Comparison of cryopreserved human sperm in vapor and liquid phases of liquid nitrogen: effect on motility parameters, morphology, and sperm function. *Fertil Steril* 2008;**90**:1978–82
28. Donnelly ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Hum Reprod* 2001;**16**:1191–9
29. Zribi N, Feki Chakroun N, El Euch H, Gargouri J, Bahloul A, Ammar Keskes L. Effects of cryopreservation on human sperm deoxyribonucleic acid integrity. *Fertil Steril* 2010;**93**:159–66
30. Boitrelle F, Albert M, Theillac C, Ferfouri F, Bergere M, Vialard F, Wainer R, Bailly M, Selva J. Cryopreservation of human spermatozoa decreases the number of motile normal spermatozoa, induces nuclear vacuolization and chromatin decondensation. *J Androl* 2012;**33**:1371–8
31. Ribas-Maynou J, Fernandez-Encinas A, Garcia-Peiro A, Prada E, Abad C, Amengual MJ, Navarro J, Benet J. Human semen cryopreservation: a sperm DNA fragmentation study with alkaline and neutral Comet assay. *Andrology* 2014;**2**:83–7
32. Paoli D, Lombardo F, Lenzi A, Gandini L. Sperm cryopreservation: effects on chromatin structure. *Adv Exp Med Biol* 2014;**791**:137–50
33. Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod* 2009;**24**:2061–70
34. Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J Androl* 1992;**13**:232–41
35. Amidi F, Pazhohan A, Shabani Nashtaei M, Khodarahmian M, Nekoonam S. The role of antioxidants in sperm freezing: a review. *Cell Tissue Bank* 2016;**17**:745–56
36. Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel HMA, Thomas AJ, Jr, Agarwal A. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 2004;**19**:129–38
37. Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertil Steril* 2000;**73**:459–64
38. Rauen U, Polzar B, Stephan H, Mannherz HG, de Groot H. Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. *FASEB J* 1999;**13**:155–68
39. Mollica MP, Lionetti L, Crescenzo R, Tasso R, Barletta A, Liverini G, Iossa S. Cold exposure differently influences mitochondrial energy efficiency in rat liver and skeletal muscle. *FEBS Lett* 2005;**579**:1978–82

(Received April 7, 2018, Accepted June 25, 2018)