Altitude can alter the mtDNA copy number and nDNA integrity in sperm

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Abstract

Objective Mitochondria are factories for energy production and genetic alterations in mtDNA will directly impact OXPHOS function. The copy number of mtDNA (i.e., the number of mtDNA per spermatozoon) is one of the major mitochondrial genetic features. Besides mtDNA copy number, the change of either mtDNA or nDNA integrity is another important factor causing asthenospermia, or poor sperm motility in infertile men. In this study, we investigated the mtDNA copy number and the integrities of mtDNA and nDNA respectively in semen samples from different donors at 5,300 m altitudes.

Methods Total DNA was extracted from semen samples from donors in two different altitudes. Quantitative PCR

was performed to evaluate the mtDNA copy number. PCR amplification was used to examine the integrity of sperm mtDNA. Flow cytometry was carried out to investigate sperm nDNA integrity. All data were analyzed to show the statistical significance.

Results Sperm mtDNA copy number for those living at high altitude (5,300 m) for one month was significantly higher ($P<0.05$) than for those at the lower altitude (1,400 m) or in donors who had been living at the 5,300 m altitude for 1 year. In addition, sperm mtDNA copy numbers were remarkably decreased ($P<0.05$) in those who had lived at the greater altitude for 1 year compared to those who had lived there for one month. The ratio of nDNA integrity among the 10,000 sperms at high altitude for one month was significantly lower ($P<0.05$) than that at the lower altitude (1,400 m) or at 5,300 m for 1 year, and the ratio of nDNA integrity sperms at high altitude for 1 year was increased, and higher than that for at the lower altitude ($P<0.05$).

Conclusion High altitude can alter the mtDNA copy number and nDNA integrity in the sperms.

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Introduction

Mitochondria are factories for the production of energy, whose oxidative phosphorylation system (OXPHOS) drives sperm motility [1, 2]. Most of the enzymatic complexes in the OXPHOS are partially encoded by mitochondrial DNA (mtDNA). Therefore, the genetic alteration of mtDNA will directly impact on the function of the OXPHOS, and may ultimately cause fertility problems [3]. The copy number of mtDNA (i.e., the number of mtDNA per spermatozoon) is

one of the major mitochondrial genetic features, and plays a critical role in mtDNA-mediated pathological pathways [4–7]. For example, in infertile men with abnormal sperm quality, mtDNA content was found to be significantly increased, and could serve as a marker for spermatogenic dysfunction [8].

Besides mtDNA copy number, the change of mtDNA integrity is another important factor causing asthenospermia, or poor sperm motility, in infertile men. Patients with low sperm motility were observed to have remarkably compromised mtDNA integrity [9]. Various large-scale deletions in mtDNA have been identified in spermatozoa from infertile men with poor semen quality [3, 9–12]. It has also been established that the integrity of mtDNA is closely related to nuclear DNA (nDNA) integrity in sperm. The disruption of nDNA in sperm can induce aberrance and deletions in the mtDNA [13]. As a result, mtDNA quantity and integrity sustained by nDNA integrity may serve as useful diagnostic markers of male infertility.

High altitude is a complex environment with hypoxia, cold, strong UV and windy and among them, hypoxia is the main factor impacting on health. It has been widely reported that hypoxia can reduce the fertility of rats [14], rhesus monkeys [15], and men [16, 17]. Among the causes of hypoxia, high altitude mountaineering and temporary accommodation at high altitudes are two common factors, which may influence male reproductive function. For example, men who regularly engage in high altitude mountaineering were observed to have a lower sperm count and decreased motility [16, 17], suggesting that the quantity and quality of sperm may be heavily compromised by high altitude. Mitochondria are the major organelles responsible for normal energy supply, and they react to various physiological and pathological conditions, including hypoxia. However, no scientific connections have been established between genetic alteration of mitochondria and male infertility induced by high altitude, thus hindering the understanding of human reproductive biology in specific geographical surroundings.

In this study, we investigated the mtDNA copy number and the integrities of mtDNA and nDNA respectively in semen samples from different donors at different altitudes.

Materials and methods

Semen collection, DNA preparation and data analysis

Donors of similar age (18–20 years old) and health were asked to collect semen by masturbation after 7 days abstinence. Thirteen of these donors had lived for 1 year at an altitude of 1,400 m; 10 donors had lived at an altitude of 5,300 m for one month; 15 had lived at an altitude of

5,300 m for one year. Since men in high altitude may suffer various physical and emotional stresses including increased intense physical exertion, heat and cold. To avoid experimental bias induced by those stresses, the experimental subjects were chosen with similar physical and emotional conditions and with as few as stresses. All participants had the similar diet and no vitamins or other anti-oxidants were taken. Semen samples were stored in -80°C prior to experiments. Written consent was obtained from all subjects in agreement with the guidelines from the Declaration of Helsinki, and it was approved by the IRB of Third Military Medical University. All the samples were standardized for sperm concentration (10,000 sperms/ μL) and then DNA in sperm samples was extracted with a genome kit (Omega, USA).

Analysis of sperm mtDNA copy number

We used quantitative real-time PCR (qPCR) to determine the amount of mtDNA relative to nDNA by measuring the ratio of the encoding mitochondrial gene 16S rRNA, and a nuclear gene, GAPDH [8]. Total DNA was extracted from each semen sample by using a DNA isolation kit (Sangon, China) according to the manufacturer's instructions. DNA concentration was measured by a NanoDrop ND-1000 spectrophotometer. To determine the ratio of 16S rRNA/GAPDH, qPCR was performed with Opticon1 (MJ Research, USA) and SYBR kit (Takara, Japan). The primers used for 16s RNA and GAPDH amplification were synthesized by Sangon Biotech (Sangon, China) and sequences were as follows: 16s rRNA sense: 5'-ACTTTGCAAGGAGAGCCAAA-3', and anti-sense: 5'-TGGACAACCAGCTATCACCA-3'. GAPDH sense: 5'-GGATGATGTTCTGGAAGAGCC-3', and anti-sense: 5'-AACAGCCTCAAGATCATCAGC-3'. PCR reactions were carried out under standard conditions, with 50 cycles using 10 ng of total DNA template in a 25 μL volume containing 12.5 SYB RGreen Supermix (including MgCl_2 , DNA polymerase, dNTP and SYBR; Toyobo, Japan), 10 pmol of each primer. Each of 50 cycles consisted of a denaturation step of 15 s at 95°C , and a hybridization and extension step of 15 s at 60°C . A melting curve analysis verified the accuracy of amplification by taking one second readings at 0.2°C intervals from 50°C to 95°C .

Integrity evaluation of sperm mtDNA

It has been well documented that the integrity of sperm mtDNA is disrupted in many infertile men [8, 9]. To investigate whether the disruption also exists in sperm mtDNA from those living at high altitude, we examined the integrity of a specific mtDNA fragment (8.7 kb) using PCR amplification. We applied long PCR amplification to 8.7 kb out of the 16-kb mitochondrial genome in all samples. This

8.7-kb fragment contains several genes encoding subunits of respiratory complexes as well as the common deletion types (4.9 kb and 7.4 kb) [8]. Half of the mitochondrial genome (8.7 kb) was amplified with high fidelity long-Taq polymerase (Takara, Japan). The forward primers were 5'-AAGGATCCTCTAGAGCCCACTGTAAAG-3', and the reverse primer was 5'-TTGGATCCAGTGCATACCGC CAAAAG-3'. The long chain PCR reactions were carried out with 50 ng of template mtDNA in a 50 μ L volume containing 1.5 mM $MgCl_2$, 10 pmol of each primer, 0.1 mmol/L of each dNTP and 2.5 U DNA polymerase (Takara, Japan). The parameters for PCR included one cycle of 95°C for 2 min followed by 35 cycles with a denaturation step of 15 s at 95°C, a hybridization step of 60 s at 62°C, and an extension step of 9 min at 68°C. PCR products were examined by electrophoresis with 0.8% agarose gel and visualized by ethidium bromide staining.

Integrity evaluation of sperm nDNA

Since the compromise of nDNA integrity may result in lose of mtDNA integrity [18], nDNA integrity was measured by sperm chromosomal structure assay with AO staining followed by FCM analysis. When AO binds to nucleic acids, it emits a green fluorescence for double-stranded DNA or red fluorescence for single-stranded DNA. According to this, the lower right quadrant with green fluorescence indicated intact sperm nDNA; upper right quadrant with red fluorescence indicated sperm single-strand DNA. The evaluation of the integrity of sperm nDNA was performed as described previously [8]. Briefly, 50 μ L of semen and acridine orange (AO) liquid (6 μ g/mL) were mixed, and incubated for 10 min in the dark at 37°C. After that, 2 mL of PBS (0.04 mol/L, pH 7.2) was added into the mixture followed by centrifugation to remove unbound dye. An appropriate volume of PBS was then added to modulate sperm density to reach a concentration of 1×10^6 /mL. Finally, flow cytometry (FCM) was performed to measure the red and green fluorescence signal of samples. In the FCM images, the FL1 (530/30 nm) green fluorescence represented double-stranded DNA, while the FL3 (630/22 nm) red fluorescence represented single-stranded DNA. The percentage of sperm with intact nDNA was calculated by testing 1×10^4 sperm in each group.

Statistical analysis

All data were expressed as mean $\pm$ SD, and the independent sample ANOVA was evaluated by Statistical Package for the Social Sciences (SPSS) 12.0 software. The value of $P < 0.05$ was considered statistically significant; $P < 0.01$ was considered statistically very significant. All the statistical tests were two-sided probability.

Results

Initially, we compared the sperm mtDNA copy numbers from donors living at different altitude. We found that there were significantly more sperm mtDNA copy numbers in donors living at higher altitude (5,300 m) at one month or 1 year than those at lower altitude (1,400 m). In addition, sperm mtDNA copy numbers were remarkably decreased in those who had lived at the higher altitude for only 1 year compared to those who had lived for one month. Integrity analysis showed that there was no long mtDNA deletion in sperm among all the donors at higher altitude (5,300 m). Furthermore, the sperm nDNA integrity in donors at the higher altitude at 1 month or 1 year was significantly lower than those at the lower altitude.

Copy number of sperm mtDNA increases at high altitude

In order to compare the sperm mtDNA content from donors in different altitudes, total sperm DNA was extracted and then used as template DNA to specifically amplify 16S rRNA and GAPDH with quantitative PCR. The ratio of 16S rRNA/GAPDH reflects the relative sperm mtDNA copy number [8]. As shown by the linearity of the standard curve (Supplemental figure 1A), the mtDNA PCR efficiency was constant over the standard sample dilution. Analysis of melting curve also showed that specific amplification occurred (Supplemental figure 1B). According to the quantification results of the qPCR, we next calculated the average ratio of 16S rRNA/GAPDH from all the 38 samples. As shown in Fig. 1, whether at high altitude (5,300 m) for one month (49.11 ± 8.09) or 1 year (18.60 ± 3.14), the ratio of 16S rRNA/GAPDH at high altitude is significantly higher ($P < 0.05$) than that at low altitude

Fig. 1 Changes in mtDNA copy number of sperm at different altitudes. Sperm mtDNA copy number at high altitude (5,300 m) for one month was significantly higher than at high altitude (5,300 m) for 1 year, or in the plain (1,400 m)

Fig. 2 Long PCR product amplification of mtDNA at different altitudes. There was no 8.7 kb deletion in any of the samples. Lane 1–3, from plain samples; lane 4–5, from hypoxia 30 days samples; lane 7–9, from hypoxia 1 year samples

(1,400 m) (7.42 ± 1.87), suggesting that living temporarily at high altitude may induce mitochondrial-related male infertility issues. In addition, for donors living at the same higher altitude (5,300 m), the ratio after one month is also much higher ($P < 0.05$) than for 1 year.

Integrity of sperm mtDNA is not disrupted at higher altitude

As half of the whole mtDNA genome (16.6 kb), this 8.7 kb fragment contains essential mitochondrial genes encoding subunits of respiratory complexes and plays critical roles in mitochondrially induced male infertility [8]. If the integrity of sperm mtDNA from donors at high altitude was compromised, then the DNA fragmentations or deletions in this fragment would be detected by PCR amplification. Clearly, among all the 38 samples including in this experiments, there was no 8.7 kb fragment deleted (Fig. 2, lane 1).

Fig. 3 FCM graph of the nDNA integrity analysis at different altitudes. The lower right quadrant with green fluorescence indicated intact sperm nDNA; upper left quadrant with red fluorescence indicated sperm single-stranded DNA

Fig. 4 The ratio of nDNA integrity in 10,000 sperm at different altitudes. Ratio of nDNA integrity of sperm at high altitude (5,300 m) for one month was significantly lower than at high altitude (5,300 m) for 1 year, or at the plain altitude (1,400 m)

Detection integrity of sperm nDNA

In sperm nDNA samples from donors living at high altitude for one month, we observed the whole genome with both double-stranded nDNA and single-stranded nDNA (Fig. 3), suggesting that the sperm nDNA integrity was compromised at high altitude. To comprehensively evaluate the sperm nDNA integrity, we further calculated the ratio of nDNA integrity among 10,000 sperm taken from different donors at the two altitudes. The smaller ratio indicates a worsening of nDNA integrity. At the higher altitude, the ratios of sperm nDNA integrity after one month (0.4438 ± 0.1098) and 1 year (0.5766 ± 0.082) were both lower ($P < 0.05$) than at the lower, 1,400 m altitude (0.6679 ± 0.0587 ; Fig. 4).

Discussion

Mitochondria are the main site of oxidative phosphorylation in the cells. The proper function of mtDNA requires

both structure integrity and copy number. mtDNA copy number is usually considered as an indication of oxidative phosphorylation capacity for total mitochondrial respiratory function [19]. The present study measured relative mtDNA copy number using qPCR, showing a range of 7 to 41 mtDNA copies per spermatozoon. The burden of oxidative stress increases during the time spent at higher altitudes and may even persist for some time upon return to sea level [20]. Excessive levels of ROS could raise mtDNA copy number, because ROS acts as a regulator of mtDNA copy number in the 30 days, acting through the Mhr1-dependent initiation of rolling-circle replication promoted by Ntg1-induced double-stranded break (the oxidized base excision-repair enzyme) in the mtDNA replication origin ori5 [21]. Increased mtDNA copy number per cell is associated with elevated oxidative stress in the aging tissues of rats [22] and humans [23]. Increased sperm mtDNA copy number can be considered as compensation for a decline in mitochondrial function and provide energy for sperm's normal activity during hypoxia, but this compensation still cannot recover the mitochondrial dysfunction and sperm motility which were induced by hypoxia. In this study, we found that the higher copy number of mitochondria under hypoxic conditions was at 30 days at 5,300 m. For those donors living in hypoxic conditions at the higher altitude for 1 year, the mtDNA copy number tapered, although these were still higher than for donors living in the plain. Therefore the change mtDNA copy number could serve as a marker for spermatogenic dysfunction.

Mitochondrial DNA is a 16.6 kb circular molecule with 2 rRNAs, 22 tRNAs and 13 polypeptides, all of which are involved in oxidative phosphorylation. Mitochondrial DNA is located near the inner mitochondrial membrane, where the electron transport chain and the main source of endogenous reactive oxygen species (ROS) production are located. Furthermore, no DNA repair system and some protective DNA-binding proteins, such as histones and protamine, were identified in mitochondrial. In terms of the relationship between mtDNA abnormalities, including deletions and point mutations, and semen quality, earlier studies did not achieve the consistent conclusion [10, 11]. In our studies, however, the 8.7-kb fragment deletion was not found in any of the samples in the present study. The perhaps reasons is the mtDNA replicated speed is very faster, to get high copy number when hypoxia, so the unintegrity mtDNA may be replaced by the normal mtDNA.

Recent researchers have documented that sperm nDNA integrity can be influenced by smoking, cold, radiation and chemicals leading to infertility, habitual abortion or implantation after fertilization and fetal development [24]. Nuclear DNA integrity (i.e., double-stranded chromatin) is an important assessment of male fertility and associated with

the activity ratio of sperm. An increase in the ratio and level of single-stranded chromatin can cause sperm to die or attenuate its activity, minimizing sperm's quality [25]. Many mitochondrial proteins are encoded by nDNA. Therefore the integrity of nDNA can affect mitochondria integrity and functions. For example, an nDNA mutation at the mitochondrial DNA polymerase (POLG) locus is associated with male infertility [18]. Our data illustrated that the sperm nDNA integrity in donors living at 5,300 m for either one month or 1 year was significantly less than the lower altitude controls. Other studies indicated that when stay at the 5,300 m, the spermatozoa is oxidant by the oxidative stress, which causes the incomplete of human sperm chromatin [26]. Further studies are still needed to explore the reasons and mechanisms.

From the discussion above, we may conclude that for donors living at the higher altitude for one month or 1 year, mtDNA copy number always remained greater than those in the plain. Similarly, the number of sperm in which nDNA integrity was compromised in high altitude for one month or 1 year, was always lower than those in low altitude. This may be because donors gradually adapt to the high altitude over time. More studies need to be performed to elucidate the significance of changes in the number of mtDNA copies and nDNA integrity in sperm.

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