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Cytochrome C Oxidase 6B2 Reflects the Mitochondrial Status Through the Oxidative Phosphorylation

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Abstract

Background: Asthenozoospermia (astheno) is a common male infertility disorder associated with low sperm motility. The progressive movement of sperm is an important factor in the fertilization rate, and it requires a high level of adenosine triphosphate (ATP).

Objectives: This experimental study aimed to identify the role of cytochrome c oxidase 6B2 (COX6B2) as an important functional subunit of Cytochrome-c Oxidase in sperm motility.

Methods: According to the World Health Organization (WHO) criteria, Semen samples were collected from 14 asthenozoospermia and 16 normospermia individuals that were referring to the Infertility Research and Treatment Center of Khuzestan, Iran in October 2016- May 2017. The sperm from two groups was isolated via the Percoll density gradient centrifugation to prepare healthy, motile sperm for COX6B2 immunofluorescent staining and real-time polymerase chain reaction (PCR). In addition, apoptosis assessment was carried out simultaneously to compare apoptosis and the COX6B2 expression level. To analyze the data, descriptive statistics including frequency and mean and analytical statistics including Fisher's exact test and two independent samples were used.

Results: COX6B2 was detected in midpiece of the sperm by immunofluorescence assays. In addition, the percentage of COX6B2 positive sperm in the astheno samples was almost half that of the normal group (49.0 ± 15.8 to 28.7 ± 14.1, $P = 0.641$). Real-time PCR definitely reconfirmed the immunofluorescent staining result. A decrease in apoptosis was shown in as the no samples compared with the normal group (19.1 ± 0.4 to 9 ± 0.2, $P = 0.04$).

Conclusions: The expression of COX6B2 in the sperm midpiece represents the OXPHOS pathway and functionality of mitochondria in sperm. This study introduced COX6B2 staining as a potential functional test for the recognition of competent mitochondria in sperm and it could be assigned as a biomarker in male-factor patients.

Keywords: Asthenozoospermia, Cytochrome-c Oxidase 6B2, Immunofluorescent, Oxidative Phosphorylation, Staining

1. Background

About half of infertility in couples relates to the male factor, and asthenozoospermia (astheno) is a common defect in male fertility, with a prevalence of more than 18% (1). It is diagnosed by low motility. The progressive movement of sperm requires a high level of energy; therefore, a disability in energy production could impair sperm function (2).

There are two important pathways for adenosine triphosphate (ATP) production in the cells, including the glycolysis and oxidative phosphorylation (OXPHOS) pathways. The glycolysis pathway occurs in the head of the sperm and the principal portion of the flagellum, while the OXPHOS pathway occurs in the midpiece, where the mitochondria are located. The glycolysis and OXPHOS pathways are both necessary for sperm motility and fertilization (3).

The OXPHOS pathway contains an electron transport system (ETS) that includes four multimeric protein complexes (I - IV), two mobile electron carriers (ubiquinone and cytochrome c) and the F1F0-ATPase/ATP synthase complex. To produce ATP, the protons must be transmitted to the intermembrane space through complexes I, III, and

IV to form a pH gradient (ΔH^+); in addition, the electron must be gradually transported from complex I to complex IV/cytochrome c oxidase (COX) to provide an electrical gradient ($\Delta\Psi_m$). This electrochemical gradient is required for ATP synthesis. COX/complex IV are the final electron acceptors of the ETS complex. This is composed of two monomers, each one comprising 14 subunits (I, II, III, IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, VIII, NDUFA4). The three largest subunits (I - III) are the catalytic core of the enzyme that require two copper ions center. The center site is encoded by mitochondrial RNA (mtRNA), and the remaining 11 subunits are nuclear-encoded (nRNA), reviewed by Sinkler, et al. (4). The function of COXIV depends on dimerization, copper chaperones, and all other nuclear-encoded regulatory proteins.

Cytochrome c oxidase 6B (COX6B) is a nuclear-encoded protein with two isoforms, COX6B1 and COX6B2. The latter is highly expressed in the testis; thus, it is called "testis specific cytochrome oxidase 6B2" (5). COX6B creates a bridge between two monomers of COXIV and dimerizes it as an integrated functional unit. There is no known mutation for *COX6B2*; however, removal of COX6B2 by positively charged gel filtration increases the complex COXIV activity. Previous research has shown that COX6B2 functions as an inhibitory control on the COXIV activity, in addition to the binding activity of the complex COXIV monomer (6).

The sperm with proper morphology, counts, and motility according to conventional semen analysis is not homogeneous populations. Rather, they show distinct biochemical and physiological properties, and only a small percentage of sperm have the potential to participate in the fertilization process (7, 8). Selecting competent sperm is the main concern of modern andrology. The mitochondrial assessment appears to be a mirror of sperm quality, as a significant correlation has been observed between sperm mitochondrial membrane potential (MMP) and the motility, viability, capacitation, acrosome, and chromatin integrity of sperm (9, 10). The development of new functional criteria for sperm selection with healthy mitochondria could improve clinical pregnancy outcomes (11, 12). Mitochondrial status and MMP can be measured by different Fluorescent probes, such as mitotracker, JC-1, TMRM etc. (13-15), but there are some drawbacks that prompt us to define the new mitochondrial tests for selecting functional sperm and fertilization.

2. Objectives

The present study was aimed to evaluate COX6B2 as a mitochondrial functional test in mild astheno patients that it could improve the assisted reproductive technology (ART) results and it might be a predictive biomarker for male fertilization capacity. We also measured the OXPHOS activity through the COX6B2 assessment to determine whether the mitochondrial status is expressed through the COX6B2 fold change and protein staining in astheno patients compared with normal subjects. We found that even in the case of mild astheno patients the OXPHSO pathways in mitochondria was impaired that it threatens the sperm health.

3. Methods

3.1. Sample Collection

Semen samples were collected from 20 - 44 years old individuals from patients that referring to the Infertility Research and Treatment Center of Khuzestan that is a governmental institute and referral center in western area in Iran in October 2016- May 2017.

In this study, according to Hashemitabar et al. (16) that mean score 4.82 $\pm$ 2.44 in astheno and 27.89 $\pm$ 4.45 in normal group were estimated and considering the 95% confidence level and the test power of 90%, the final sample size was estimated according to the Equation 1. Eleven of person in each groups were estimated. However, to increase the accuracy of the study, the study was done on 16 individuals in normal and 14 patients.

$$n = \frac{\left(s_1^2 + s_2^2\right)\left(z_{1-\frac{\alpha}{2}} + z_{1-\beta}\right)^2}{\left(\bar{x}_1 - \bar{x}_2\right)^2} \quad (1)$$

A total of 800 patients were enrolled, among them who were the criteria for entering the study, according to the sample size and systematic random sampling; 14 were selected as the patient group and 16 as the normal group.

The samples were collected by masturbation after a 3- to 5-day ejaculatory abstinence interval, and the liquefaction time was 30 - 60 min. Following the World Health Organization (WHO) guidelines for sperm parameters (17), the volume, count, motility, and morphology were evaluated. The sperm parameters were manually checked by two expert observers. Normozoospermia (defined as sperm concentration $\geq 15 \times 10^6$ /mL, total motility grades A + B + C $\geq$ 40%, progressive motility A + B $\geq$ 32%, normal sperm morphology $\geq$ 4%) and astheno semen samples (sperm concentration $\geq 15 \times 10^6$/mL, total of motility grades A + B + C $\leq$ 40%, progressive motility A + B $\leq$ 32%, normal sperm morphology $\geq$ 4%). Motility was calculated according to the following categories: rapidly progressive (type A), slowly progressive (type B), not progressive (type C), and immotile (type D). All participants consented to

their sperm samples being assessed in the research. Smokers, alcoholics, drug consumers, and patients with an infectious disease history, cancer, diabetes, high blood pressure, depression, X-ray exposure, and cryptorchidism were excluded. In addition, men with obesity, varicocele, and a history of fever (previous 90 days) were excluded.

This experimental study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of IR.AJUMS.REC.1394.375 (Iran, Ahvaz Jundishapur University of Medical Sciences, 2015)

3.2. Sample Preparation

The discontinuous Percoll density gradient (45-001-754; GE Healthcare, Sweden) was employed to remove debris, leucocytes, and immature germ cells from motile cells in semen. Ham's F10 was used to dilute the Percoll and wash the samples. Two milliliters of liquefied semen were carefully loaded on 40% and 80% Percoll, each consisting of a 10-mL layer (18) and centrifuged at 1,000 × g for 10 min at room temperature (RT; 25°C). The motile sperm was isolated from the pellet, washed twice with Ham's F10, and counted with a Neubauer chamber.

3.3. Immunofluorescence Assay

The prepared smears from the sperm samples were fixed with 4% paraformaldehyde for 20 min, then washed twice with phosphate-buffered saline (PBS). Following this, the samples were incubated in glycine for 15 min and washed three times with PBS using 1.5% Triton X-100 (9002-93-1, Sigma-Aldrich) for 10 min to permeabilize the cells. Sperm was incubated in 10% rabbit serum for 1 h. Anti-COX6B2 antibody (ab134960) was applied as the primary antibody in a concentration of 1/100, at 4°C overnight. It was washed three times, and then a secondary antibody, goat anti-rabbit immunoglobulin G (IgG) H&L TRITC conjugated (ab50598) was used (19). DAPI staining was applied as the counter and nuclear staining. Images were visualized via two single-band-pass filters. Two observers manually counted the immunostaining samples using an Olympus BX51 phase contrast fluorescent microscope. To determine the agreement between the two observers, the kappa coefficient was calculated (K = 0.9).

3.4. qPCR Analysis of COX6B2

Total RNA was extracted from sperm by an EZ-10 spin column total RNA minipreps super kit (BS583). The total RNA was quantified by Nanodrop (Thermo Scientific NanoDrop 2000C) and then stored at -80°C until use. To produce *COX6B2* cDNA, 0.5 μg of total RNA from each sample was reverse transcribed with a TaKaRa PrimeScript™ RT Reagent Kit (RR037Q). The cDNA reaction mixture contained the following: 5 × PrimeScript™ buffer, PrimeScriptTM RT Enzyme Mix I, Oligo dT Primer, random 6-mers, total RNA, and RNase free water. cDNA reaction mixture was incubated at 37°C for 15 min to activate the reverse transcription. It was then transferred to 85°C for 5 s to inactivate the reverse transcriptase. The final volume of cDNA was stored at -20°C until further use.

The relative mRNA expression of *COX6B2* in ejaculated sperm was analyzed using an ABI (StepOnePlus Real-Time PCR System). The *ACTB* and *COX6B2*, accession number, and primer sequences are summarized in Table 1.

qPCR was performed using SYBR® Premix Ex Taq™ II (RR820Q; TaKaRa) under the following cycle conditions: 95°C for 10 s, denaturation at 95°C for 5 s, annealing at 56.5°C for 20 s, and a final extension at 72°C for 30 s for 45 cycles. Standard curve analysis was performed for the *COX6B2* and *ACTB* using serial dilutions of complementary DNA (cDNA) prepared from motile sperm to confirm the amplification efficiency, which was 97%.

3.5. Apoptosis Assessment

The FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) was applied to determine the percentage of apoptotic sperm in the astheno and normal groups (20). Briefly, following the kit instructions, sperm were washed twice with PBS and resuspended in 1X Annexin V Binding Buffer at a concentration of 1×10^6 sperm/mL. Following this, 100 μL of the solution (1×10^5 cells) was transferred to a 5-mL culture tube; 5 μL of Propidium iodide (PI) and 5 μL of FITC Annexin V were added and vortexed gently, then incubated in a dark place for 15 min at RT. Finally, 400 μL of 1X Binding Buffer was added to each tube and analyzed using a flow cytometer (FACSCalibur; BD, USA).

We have not any missing value in our study, and before the experiments all of instruments and equipment were calibrate and checked.

3.6. Statistical Analysis

Descriptive statistics, including the frequency, mean, and standard deviation, were used in this study. The Shapiro-Wilk test was used to examine the normality of the

Table 1. Primers Properties

	COX6B2	*ACTB*
Accession No.	NM-144613.4	NM-001101.3
Forward 5' - 3'	GCAGCCCTGCGAGTACTATT	TGGACTTCGAGCAAGAGATG
Reverse 5' - 3'	CCCGTTCTTGATCTGCTCGT	GAAGGAAGGCTGGAAGAGTG
Product size	88 bp	137 bp

main quantitative variables. The results of the test showed that the variables were normal (P> 0.05). Thus, to compare the mean of the main outcomes in the normal and astheno groups, the *t*-test and Fisher's exact test were used. A P value < 0.05 was considered significant. The IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., Armork, N.Y., USA) was used to analyze the data.

4. Results

4.1. Semen Parameters

The normozoospermic and asthenozoospermic semen information is summarized in Table 2 and Figure 1. The most important results were as follows: age, 30.40 ± 4.52 and 31.8 ± 5.78 years; volume, 4.80 ± 2.11 and 2.90 ± 1.29 mL; sperm concentration, 55.96 ± 24.61 × 10^6/mL and 51.39 ± 17.74 × 10^6/mL; total motility, 47.0% and 37.8%; and progressive motility, 38% and 30.6%.

Normal sperm morphology value (shape and size) in the normal group and the astheno group were 5 ± 0.42 and 4.21 ± 0.11 that the results of chi-square test showed that the percentage of healthy sperms did not significantly differ between the normal and patient groups (P = 0.121).

The motile sperm was isolated from the sediment plate of Percoll gradient and examined under a light microscope. The progressive motility was increased to more than 95% for both groups. The Papa Nicola staining showed no morphological difference between the two groups (data not shown).

4.2. COX6B2 Staining by Immunofluorescence

After preparation of the sperm for immunofluorescence assay, at least 200 sperm were randomly counted. To visualize the sperm contour, they were counterstained with DAPI, and the COX6B2 location was visualized by immunofluorescence microscopy. Although the sperm morphology was normal in both groups (data not shown), COX6B2 was accumulated in the midpieces of the sperm in both groups; in contrast, in the sperm with non-activated OXPHOS pathway, the midpieces were not stained (Figure 2H and I), or the heads were stained instead of the midpieces (Figure 2E and F).

The percentage of midpiece COX6B2$^+$ in the astheno group was 28.7 ± 14.1, while in the normal group, it was 49.0 ± 15.8, P = 0.641 (Figure 3). Thus, the amount of positive midpiece staining in the spermatozoa of normal samples was almost twice that in the astheno group.

4.3. The mRNA Level of COX6B2 by qPCR

The relative difference of the *COX6B2* mRNA abundance between the normal and astheno samples was determined. At first, ΔCT was calculated between *COX6B2* and *BACTIN* in each groups (2) and then ΔΔCT was calculated by Equations 2 and 3:

$$\Delta CT = CT\,(COX6B2) - CT\,(ACTB) \quad (2)$$

$$\Delta\Delta CT = \Delta CT\,(asthenozoospermia) - \Delta CT\ \ (normozoospermia) \quad (3)$$

Then, fold change calculated by Equation 4:

$$Fold\ change = 2 - \Delta\Delta CT \quad (4)$$

The data displayed a significant difference of the *COX6B2* mRNA levels between the two groups. The relative mRNA abundance of *COX6B2* in the astheno group was considerably lower than that in the normal group (256-fold; Figure 4).

4.4. Annexin V Assessment

The percentages of PI and FITC Annexin V-positive sperm were sorted by a flow cytometer and analyzed using FLOWJO software. The percentage of apoptotic sperm in the astheno and normal groups were 9 ± 0.2 and19.1 ± 0.4 respectively P = 0.04.

5. Discussion

Progressive motility is an important aspect of sperm biology and fertilizing the oocyte. However, the molecular mechanisms behind sperm motility are not fully understood (21, 22). During intracytoplasmic sperm injection (ICSI), other requirements for sperm, such as capacitation, acrosome reaction, sperm-oocyte fusion, and penetration are bypassed, and more investigations are needed to clarify these parameters' validity. The new functional sperm tests, such as TUNEL assays, DNA fragmentation assays, and reactive oxygen species (ROS) and MMP assessments, are implemented in modern andrology to evaluate the sperm's fertilizing capacity.

Mitochondria provide part of the sperm energy requirements, and their dysfunction may cause idiopathic astheno (23). Mitochondrial dysfunction manifests a significant decrease in MMP and increases in oxidative stress in infertile men (24). High production of ROS has an indirect relationship with MMP in sperm mitochondria, and it increases the sperm's susceptibility to apoptosis (25, 26).

Previous studies employing 2D gel electrophoresis demonstrated that the amount of COX6B2 decreased in the tail (16) and severe astheno patients compared with the

Table 2. Sample Properties in Astheno and Normal Group[a]

	Normal, N = 16	Astheno, N = 14	P Value[b]
Mean age of patients, y	30.41 ± 4.52	31.78 ± 5.78	0.464
Volume, mL	4.78 ± 2.11	2.9 ± 1.29	0.012
Sperm count, × 10^6 sperm/mL	55.96 ± 24.61	51.39 ± 17.74	> 0.999
Motility, %	47.06 ± 6.26	37.85 ± 1.02	< 0.001
Motility A, rapid progressive %	7.68 ± 3.65	3.67 ± 1.87	0.002
Motility B, slow or sluggish %	31.14 ± 10.9	26.92 ± 3.76	0.138
Motility C, nonprogressive %	6.23 ± 1.08	7.53 ± 2.35	0.063
A + B	38.09 ± 12.29	30.60 ± 2.78	< 0.001

[a]Values are expressed as mean ± SD.
[b]P value from *t*-test.

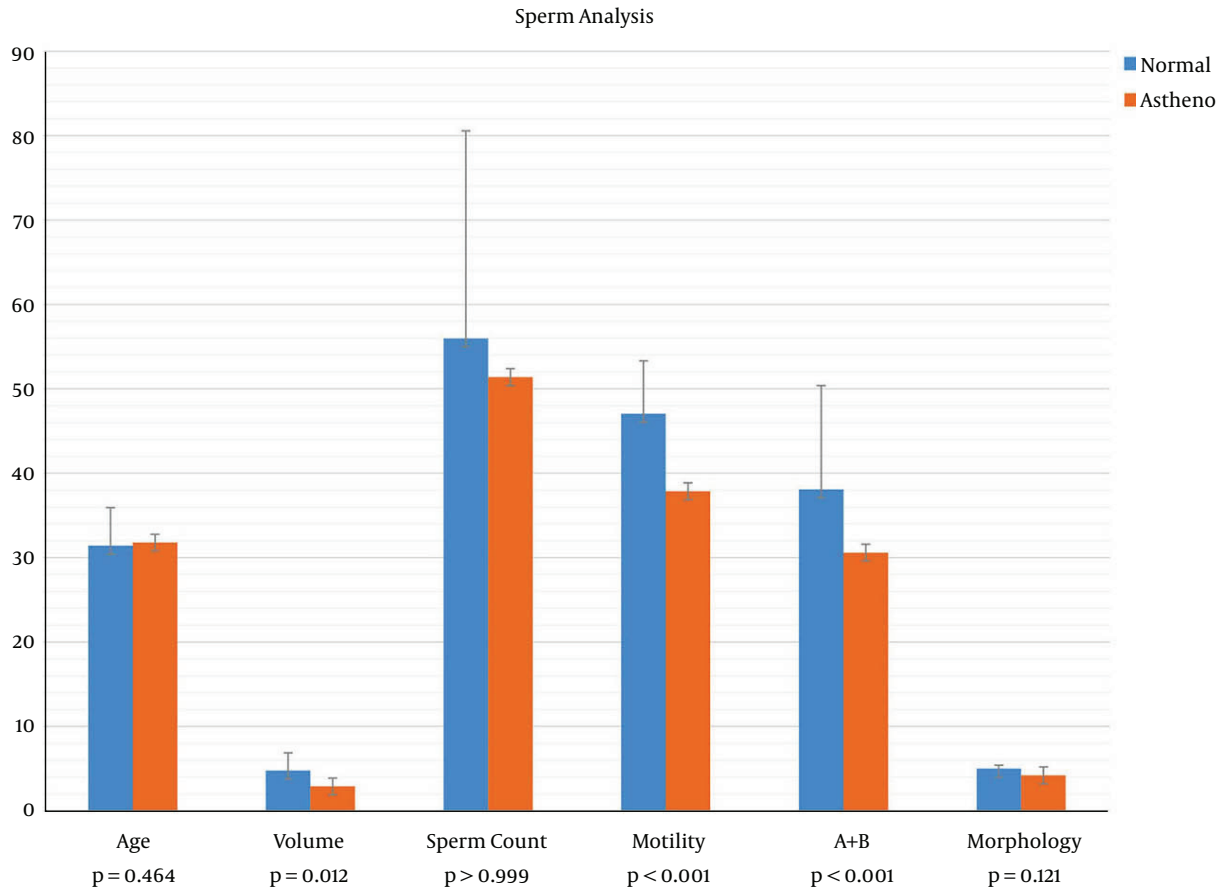

Figure 1. Sperm analysis in astheno and normal group

normal group (27). Comprehensive further analysis, such as *COX6B2* mRNA quantification and immunofluorescence assay, is required to clarify the role of COX6B2 in the mild astheno patients.

The novelty of the current study is to demonstrate that COX6B2 decreases not only in the tail of the sperm of astheno groups comparing to the normal group where there is the mitochondria localization, but also in whole sperm

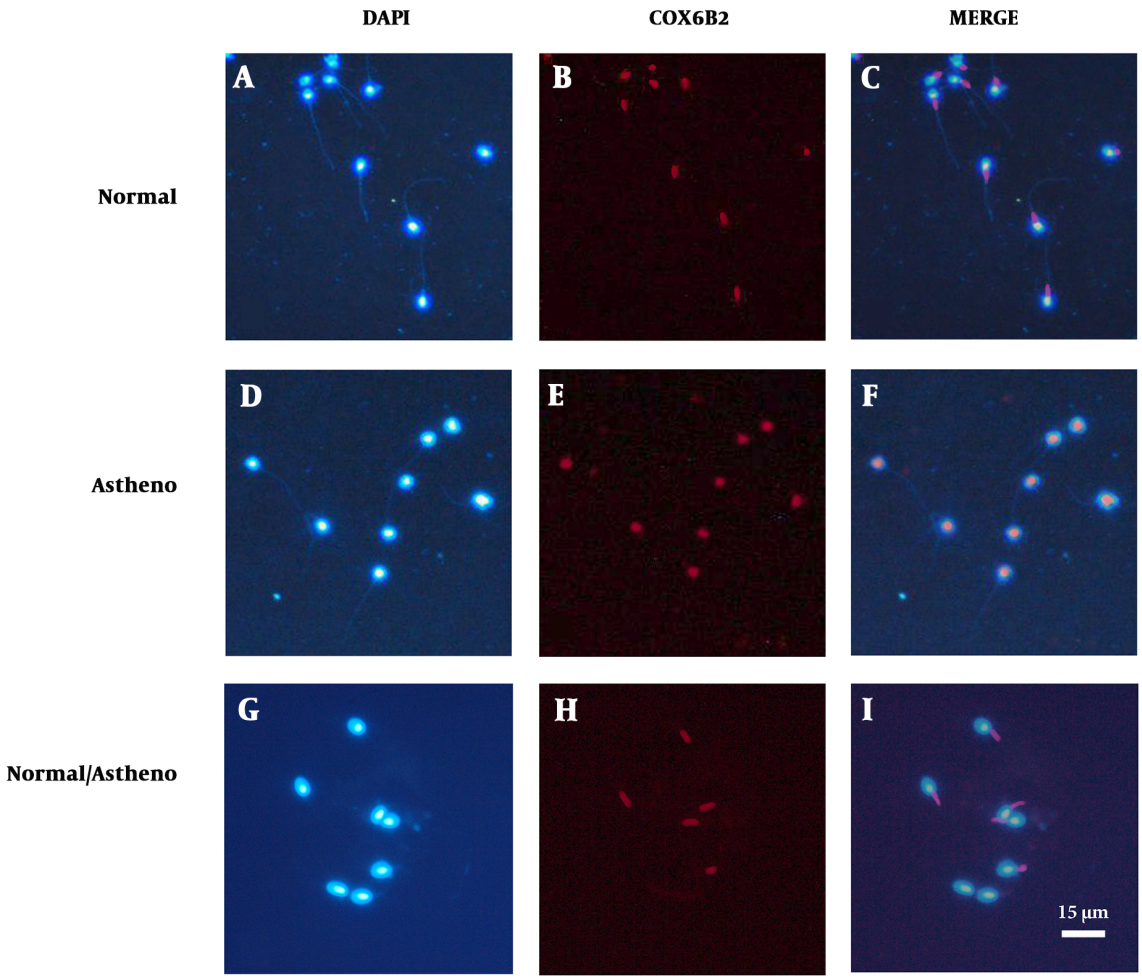

Figure 2. Immunofluorescence localization images of COXVIB2 protein. The midpiece positive staining in human spermatozoa in normal group (A, B, C). COXVIB2 was accumulated in the head of sperm, astheno group (D, E, F). Image of combine sperm midpiece positive and midpiece negative staining, normal or astheno groups (G, H, I). The sperm counterstained with DAPI (A, D, G), the location of COXVIB2 protein was showed with red color in (B, E, H) and the overlap of two previous pictures (C, F, I).

perpetration, the COX6B2 assessment can be represented the mitochondria functionality even in mild astheno patients according to real-time PCR and various proteomic methods.

Immunofluorescence assay revealed the lower percentage of COX6B2 positivity in the sperm midpiece in astheno samples compared with the control. In other words, the sperm with positive midpiece staining represented the active OXPHOS pathway; otherwise, the nuclear-encoded COX6B2 is accumulated in the head and cannot translocate into the mitochondria in the midpieces to setup OXPHOS in the functional sperm.

Nowadays, fluorescent dyes, including mitotracker, JC-1, TMRM are used to evaluate mitochondria (9, 13, 14). Fluorescent dyes do not stain the mitochondria specifically. They produce auto-fluorescence and can interfere with other material, such as antioxidants, decreasing the accuracy of the test, e.g., in the JC1. Generally, NADH and FADH2 created in the Krebs cycle, have similar emission and/or excitation wavelengths as probes, leading to misinterpretation of the results (9). In the case of TMRM, the disadvantage is that it can measure both membrane potential of cell plasma membrane and mitochondria.

In many cases, ART is unsuccessful, associated with no known etiology, and the sperm has no defect in morphology, motility and other parameters. New methods with high efficiency and accuracy are needed to evaluate sperm functionality. Our new mitochondrial assessment can detect mitochondria functional from non-functional among the astheno patients. Therefore, we recommended obtain-

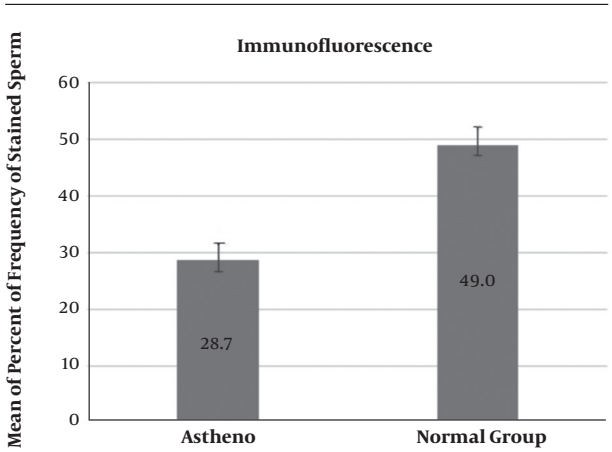

Figure 3. Level of COXVIB2 protein expression by IF in Normal group and Astheo

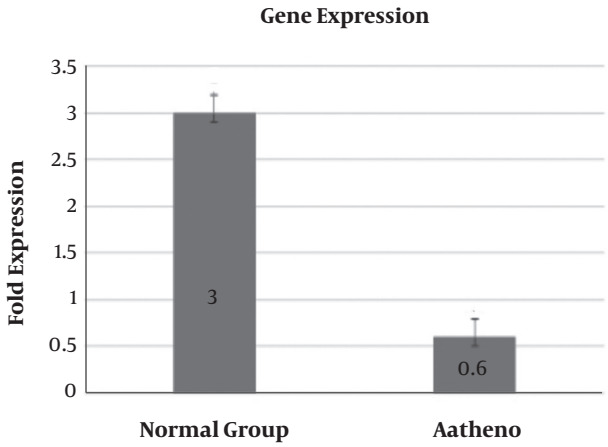

Figure 4. Level of COX6B2 expression in Normal group and Astheno

ing cut-off criteria to achieve a differentially functional sperm detection test by sperm COX6B2 assessment among the further sperm sampling.

When the proper function of OXPHOS and respiratory chain proteins, including ETS protein and their nuclear-encoded regulatory subunits are impaired in the mitochondria, it may occur in the two form, first mitochondrial hyperactivity that is associated with electron leakage, increase in ROS production and increase of $\Delta\psi$m to 180 mV. Secondly, it may occur in the case of mitochondrial hypoactivity with losing the mitochondria membrane integrity and decrease of $\Delta\psi$m to almost zero. However, in the case of a decrease in $\Delta\psi$m and mitochondria hypoactivity, it could not be measured by fluorochrome dye such as mitotracker assay and the mitochondrial situation could not be screened, but our new suggesting method has a potential to be used for examining the OXPHOS status in both hyper or hypo activated sperm mitochondria. The basic question is as follows: What is the main source of sperm energy requirements? Mammalian spermatozoa must be able to utilize both glycolysis and OXPHOS to fertilize an oocyte, as reported previously (28, 29). ATP production through glycolysis is important for the sperm's progressive motility. Pyruvate, the main production of the glycolysis pathway, and the other respiratory chain substrates in the OXPHOS pathway, such as malate, fumarate, citrate, and 2-oxoglutarate, do not increase during the motility of sperm. Therefore, for a long time, andrology scientists concluded that OXPHOS is less efficient in human spermatozoa than glycolysis is (30). Ramalho and college also suggested that the OXPHOS pathway has a pivotal role in sperm's functionality, but it is not essential for sperm motility (28). The OXPHOS pathway exists in a homeostatic balance that could be disturbed through external disease or internal signaling. For example, in the case of insulin resistance, diabetes, obesity, and ischemia/hypoxia, the $\Delta\psi$m is increased to almost 180 mv and converts the mitochondria into a hyperactivated form (31). In the opposite condition, in the case of acute inflammation–increased tumor necrosis factor α (TNFα), the mitochondria are converted into a hypoactive form (32). The $\Delta\psi$m increases in association with a decline in pyruvate consumption, O_2 depletion, fatty acid peroxidation, electron leakage, increase of oxidative stress (e.g., ROS production), apoptosis, that finally, lead to sperm death. While low $\Delta\psi$m is associated with the depletion of ATP, again resulting in sperm death (33) (Figure 5).

There were no mutations reported in *COX6B2* in previous studies, but there have been two reports demonstrating mutations in *COX6B1* (an isoform of *COX6B2*). These mutations are associated with severe infantile encephalomyopathy, hydrocephalus, and cardiomyopathy (34, 35). *COX6B2* in mammals is homologous to COX12 in yeast, which encodes an essential subunit for COXIV assembly. It has been found that deletions in COX12 show a 5% - 15% decrease in COXIV activity compared with the wild type (36). There is evidence that *COX6B* has a regulatory effect on subunit COX2 (mitochondrial encoded) (37). This is an active site where the electrons from ferrocytochrome c enter the complex (38). The electrons are subsequently transferred via heme a to the oxygen reduction center in subunit I.

Downregulation of *COX6B2* in astheno samples was observed in this study and microarray analysis in astheno patients has shown that a decrease in the levels of specific mtRNAs and nRNAs of the *COX6* subunits' transcriptome are associated with low sperm motility (39).

The percentage of apoptosis in normal samples was almost twice that in the astheno group, which is consistent

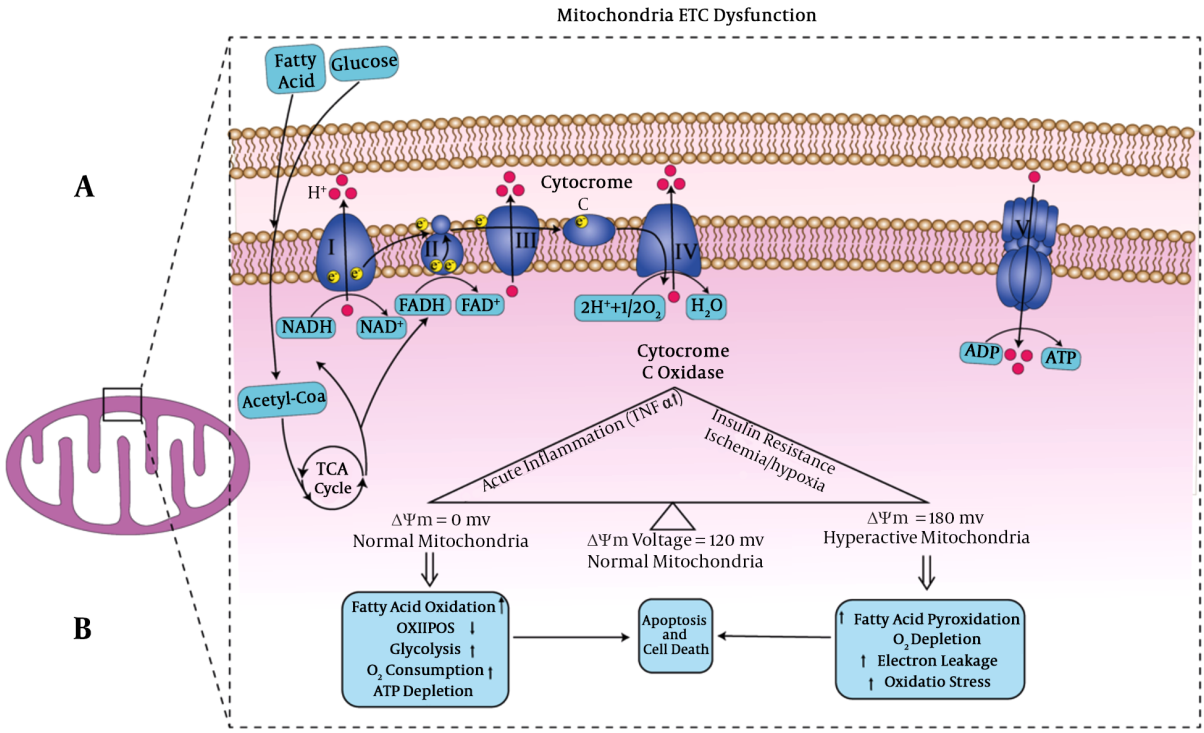

Figure 5. The energy of glucose and fatty acid breaks down through the three carboxylic acid (TCA) cycle and ECT complex. The ETC consists of four protein complex (I - VI), two carriers and F1F0-ATPase/ATP. Cytochrome c oxidase (COXIV) in the center of ETC complex is the last acceptor of the electron. It highly consumes the O_2. It is a metabolic and signaling center that regulates through mtRNA and nuclear-encoded nRNA to sustain the $\Delta\psi$m = 120 mv in normal condition (A). The balance between normal $\Delta\psi$m in ETC complex may be impaired. The hypoactive mitochondria in acute inflammation lead to depletion of ATP and cell death by blocking of OXHPOS and stimulation of glycolysis pathway, the left side (B). Hyperactive mitochondria in insulin resistance condition lead to increasing of $\Delta\psi$m, represent electron leaking and oxidative stress (ROS increase), and cell death, right side (B).

with Erkkila and colleagues' study. They demonstrated that an enhancement in the ATP synthesis in the OXPHOS pathway leads to increased apoptosis in testicular germ cells and showed a direct correlation between ATP production and apoptosis (40). This is true in the case of hyperactive mitochondria and increases ROS production. However, we cannot generalize it to the case of early hypoactive mitochondria and ATP decline.

Therefore, it is assumed that there is a significant relationship between apoptosis and mitochondrial function as a major element in apoptosis induction.

The early hypoactivated sperm mitochondria try to decrease the apoptosis up to the time of ATP depleting. Perhaps one of the most novel findings is to reveal that even in the case of mild/border astheno cases. We have faced to OXPHSO pathways impairment rather than motility. This is the point that has to consider in further application of COX6B2 assessment as a biomarker for sperm healthy in IVF center.

Therefore, although we found a positive influence of OXPHOS on sperm motility, according to our sampling and selecting of mild astheno patients, it seems that COX6B2 has more influence on the sperm function through the decline in ATP production and energy function and energy requirement of sperm rather than on motility. We recommend simple staining to identify sperm with functional mitochondria from nonfunctional sperm utilizing ATP only through the glycolysis pathway. COX6B2 is potentially a suitable biomarker for predicting the functionality of sperm mitochondria.

Footnotes

Authors' Contribution: Mahmoud Hashemitabar designed the project and writing the manuscript. Elham Heidari carried out the experiment, writing the manuscript, and analyzed the data. Maryam Dastoorpoor analyzed the statistics section. Ata A. Ghadiri writing the manuscript. Mahmoud Orazizadeh interpreted the data and revised the manuscript. Susan Sabbagh carried out the experi-

ment and writing the manuscript. Mahsa Afrough collected the samples. All authors read and approved the final manuscript.

Conflict of Interests: The authors declare no conflict of interests.

Ethical Considerations: This study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of IR.AJUMS.REC.1394.375.

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