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Oxidative stress and *ATPase6* mutation is associated with primary ovarian insufficiency

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Abstract
Purpose Primary ovarian insufficiency (POI) is a heterogeneous, multifactorial disorder. Though genetic anomalies, infections, autoimmune disorder and hormonal imbalance are few of the causes of POI, in the majority of patients (50–60%) no etiology has been identified. Mitochondrial bioenergetics and biogenesis play an important role in oocyte and embryo development, whereas mtDNA integrity and content are essential for the normal development of oocytes. ATPase6 helps to maintain the mt genome integrity, and mutations in *ATPase6* are associated with overproduction of reactive oxygen species (ROS) in a variety of diseases; however, its role in POI has not been evaluated. Therefore, we planned to evaluate the potential role of *ATPase6* gene mutations and associated oxidative stress in idiopathic cases of POI.
Methods This pilot study included: 20 cases of POI with FSH level of >40 mIU/ml; 4 cases of occult ovarian insufficiency (occult OI) with irregular menses and mean FSH levels of 16.4 mIU/ml; and 20 age-matched healthy female controls (FSH 2–5 mIU/ml). ROS levels in blood plasma were measured by luminol-dependent chemiluminescence assay and the ROS values were expressed as relative light unit per minute (RLU/min). mtDNA *ATPase6* gene was amplified and sequenced from the blood lymphocyte DNA.
Results Of all, 50% patients showed nucleotide changes in the *ATPase6* gene, as compared to 10% in controls, and the majority of these mutations were non-synonymous. *ATPase6* mt.8684 C>T and mt.9094 C>T were found to be significantly ($P < 0.005$) higher in cases as compared to controls. ROS levels were found to be significantly ($P < 0.005$) higher in POI and occult OI patients compared to controls and nucleotide changes were found to positively correlate with ROS levels. Moreover, ROS production was found to positively correlate ($r = 0.7038$, $P < 0.001$) with FSH levels of the patients (POI and OI) compared to controls.
Conclusions This pilot study clearly demonstrates for the first time *ATPase6* gene nucleotide alterations and elevated ROS levels in idiopathic cases of POI. Therefore, it may be possible that OS associated with *ATPase6* gene mutation may be causal in idiopathic cases of premature OI. However, larger studies with inclusion of more cases of both POI and occult OI are required to strongly establish the correlation between oxidative stress and mitochondrial

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nucleotide alterations in the pathogenesis of POI. Such cases with OS-induced POI may benefit immensely by early diagnosis and prompt antioxidant administration.

Keywords Premature ovarian failure (POF) · Reactive oxygen species (ROS) · mtDNA mutation · Oxidative stress · ATPase6 · Primary ovarian insufficiency (POI)

Introduction

Primary ovarian insufficiency (POI) is a heterogeneous disorder defined as premature menopause in woman under the age of 40 years. It is multifactorial in origin and occurs in 1 in 10,000 women under the age of 20 years, 1 in 1,000 under 30 years and 1 in 100 at 40 years characterized by primary or secondary amenorrhea, which may be familial or sporadic [1]. The known causes of POI include genetic abnormalities, infection, autoimmune disorders, iatrogenic, chemotherapy and metabolic disorders. Genetic anomalies usually result in decreased gene dosage or impaired meiosis resulting in accelerated germ cell apoptosis [2]. This may lead to decreased follicle production or increased follicle artesia resulting in premature loss of germ cells [3]. Though the above causes are well defined in POI, approximately 50–60% of the cases are idiopathic [1]. Mitochondrial bioenergetics and biogenesis play an important role in oocyte maturation and development of embryo. Studies have reported that women with ovarian insufficiency have significantly lower mtDNA content in the oocytes [4, 5]. Bentov et al. [6] reported that aging and age-related pathogenesis are associated with loss of mitochondrial function, mainly due to accumulation of mtDNA mutations and deletions. Oocytes grow in a relatively hypoxic environment in the ovarian cortex and, due to their quiet metabolism, are exposed to low reactive oxygen species (ROS) levels. However, only during meiosis I and meiosis II the energy demand increases to extrude the first and second polar body and this energy is supplied solely by mitochondria. The oocyte has the largest number of mtDNA copies than any cell in the body, even more than cells with higher energy requirements (muscles and neurons) [6]. Thus, it is possible that there is increased ROS production, and mtDNA is the first target of ROS. In mitochondria, ATP-synthase plays a crucial role in the production of ATP by converting ADP with the help of proton obtained during the electron transportation in the respiratory chain. Single nucleotide polymorphisms in *ATPase6* gene could decrease the efficiency of mtDNA replication as ATPase6 synthase is one of the most important candidates that maintain mitochondrial integrity [7]. It has been reported that mutations in *ATPase6* gene are associated with the overproduction of ROS in various disorders [8]. ROS are a group of free radicals produced by the cells as the by-product of oxidative phosphorylation. Optimal levels of ROS are required for various physiological functions, whereas supra-physiological ROS levels adversely affect various organ systems, especially germ cells [9]. Therefore, ROS production that exceeds the antioxidant defense capacity may severely damage the cells and induce lipid peroxidation and lead to irreversible mtDNA and nuclear DNA damage [10, 11]. Bentov et al. reported that maintenance and repair of the mt genome relies on enzymes that maintain the nuclear genome, making its repair less efficient, and thus mtDNA tends to accumulate mutations. Mutations also accumulate in mtDNA because of it being a naked molecule not protected by histones or protamines and its close proximity to the inner mitochondrial membrane. In women, physiological ROS levels help in folliculogenesis, steroidogenesis and oocyte maturation [12]; however, excess ROS levels establish oxidative stress and may damage follicles by peroxidizing lipids and oxidizing proteins and DNA. Several studies have demonstrated the role of OS in male infertility and some female infertility conditions such as endometriosis and polycystic ovaries. However, the role of OS and mt gene (*ATPase6)* nucleotide changes in the pathogenesis of POI has not been studied. Therefore, this pilot study was aimed to assess if mt nucleotide alteration in *ATPase6* gene and raised ROS levels are causal in POI. Establishing this correlation would help in better management of such cases using antioxidant therapy and could prevent or delay onset of POI.

Materials and methods

Patients

A total of 44 subjects including 20 idiopathic cases of POI, 4 idiopathic cases of occult OI and 20 age-matched healthy female controls were enrolled in the pilot study after informed consent and institute ethical clearance. Peripheral blood (10 ml) was collected for cytogenetic, ROS analysis and DNA isolation. The subject's height, weight, age, medical history and pedigree were recorded in a predesigned proforma to exclude known causes of POI. Only cases with normal chromosomal complement and no recent history of infection, fever or drug intake were enrolled in this study.

ATPase gene mutation analysis

Blood lymphocyte DNA was isolated by the phenol chloroform method. mtDNA *ATPase6* gene was amplified by

using the two sets of primers (1. forward: ACGAG TACACCGACTACGGC; reverse: TGGGTGGTTGGTGT AAATGA, and 2. forward: TTTCCCCCTCTATTGA TCCC; reverse: GTGGCCTTGGTATGTGCTTT) [10]. PCR conditions were as follows: briefly, initial denaturation at 95°C for 10 min, 35 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 2 min and final extension at 72°C for 7 min. Sequencing of PCR products was carried out using 100.0 ng (2.0 µl) of PCR product and 4 pmol (1.0 µl) of primer (forward), 4.0 µl of BigDye Terminator ready reaction kit and 3.0 µl of double-distilled water to adjust the volume to 10.0 µl. Cycle sequencing conditions: 30 cycles at 96°C for 10 s, 50°C for 5 s and 62°C for 4 min. Samples were dissolved in 10 µl of 50% Hi-Di formamide and analyzed in automated DNA analyzer (Applied Biosystems). The obtained sequences were analyzed using chromas software and compared with the reference sequence to find out the nucleotide changes using Genebee multiple alignment (http://www.genebee.msu.su).

ROS measurement

Fresh blood (1 ml) was centrifuged at 300×*g* for 7 min and the plasma was removed and transferred into a microcentrifuge tube. To 400 µl of the plasma, 10 µl of 5 M luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma) was added to the mixture and served as a probe. Levels of ROS were assessed by measuring the luminol-dependant chemiluminescence with the luminometer (Sirius, Berthold) in the integrated mode for 15 min. The results were expressed as relative light unit per minute (RLU/min) per 400 µl of blood plasma.

Computational assessment of missense mutations

Two homology-based programs PolyPhen (polymorphism phenotyping; Division of Genetics, Department of Medicine, Brigham and Women's Hospital/Harvard Medical School, Boston, MA) and sorting intolerant from tolerant (SIFT; The J. Craig Venter Institute Rockville, MD and La Jolla, CA) were used in this study to predict the functional impact of missense mutation. PolyPhen scores of >2.0 indicate that the polymorphism probably damages protein function. Scores of 1.5–2.0 are possibly damaging, and scores of <1.5 are likely to be benign. However, SIFT is based on the premise that protein evolution is correlated with protein function. Positions important for function should be conserved in an alignment of the protein family, whereas unimportant positions should appear diverse in an alignment. Positions with normalized probabilities <0.05 are predicted to be deleterious and those ≥0.05 are predicted to be tolerated.

Statistics

Fisher's exact test was used to find out the significance in *ATPse6* nucleotide changes between cases and controls. Kruskal–Wallis test was used to find out the significant difference in ROS levels between patients and controls. The values were expressed as median (minimum, maximum) range. Correlation between ROS levels and nucleotide changes was analyzed by Spearman correlation coefficient test. $P < 0.05$ was considered as significant unless otherwise stated. The statistical analysis was performed using Stata 9.0 version software.

Results

Out of 24 cases (20 POI and 4 OI) screened for mitochondrial *ATPase6* gene, 50% were found to have one or more nucleotide changes and the majority of these changes were non-synonymous. *ATPase6* mt.8684 C>T and 9094 C>T were found to be significantly ($P < 0.005$) higher in cases compared to controls (Table 1). All these mutations were either G>A or C>T except for one case, which showed a silent polymorphism A>G at the nucleotide position 8679. The majority of nucleotide alterations in the *ATPase6* gene in these cases were non-synonymous as compared to controls. The overall median ROS range was found to be significantly ($P < 0.005$) higher in patients compared to controls (Table 2). A significant positive correlation ($r = 0.6636$) was found between nucleotide changes and ROS levels in these cases (Fig. 1). Among the patients, 50% (10/20) had very high ROS levels, 20% (4/20) had moderately elevated levels and 30% (6/20) were found to have normal values comparable to controls at 340 RLU/min (120, 5,094) (Fig. 2). Cases with occult OI had moderate elevation of ROS levels and two cases harbored one nucleotide alteration (8684 C>T, 9094 C>T) each. A positive correlation ($r = 0.7038$, $P < 0.001$) between ROS levels and FSH values was found in these cases (Fig. 3).

ATPase6 mt.8684 C>T resulted in amino acid change from threonine to isoleucine (T>I). The Polyphen analysis of this non-synonymous change showed PSIC score of 0.219, which is benign, and an SIFT score of 1 showed that the resulting protein structure is not deleterious. However, it resulted in replacement of a threonine, a hydrophilic amino acid, to isoleucine, a nonpolar hydrophobic amino acid. Similarly, the other two missense mutation of ATPase6:9094 C>T resulted in amino acid change from leucine to phenylalanine (L>F), both being nonpolar hydrophobic amino acids showed PSIC score of 0.002 (benign) and SIFT score of 0.73 (tolerant) and 9064. A>T resulted in amino acid change from alanine to threonine

Table 1 Comparison of ROS and FSH levels of POI, occult OI patients and control subjects

Subjects	ROS (RLU/min)	Age (years)	FSH (mIU/ml)
POI ($n = 20$)	50,480 (120, 132,966)*	25.55 ± 3.51	109.60 (64.21, 162.31)
Occult OI ($n = 4$)	15,046 (1,225, 21,356)	23.25 ± 2.5	38.40 (25.20, 55.60)
Controls ($n = 20$)	340 (120, 5,094)	25.95 ± 3.25	2.86 (1.80, 6.23)
Significance (P)	<0.005	0.3249	<0.0001

Values in parentheses are medians (minimum, maximum)

* $P < 0.005$ is considered as significant by Kruskal–Wallis test

Table 2 *ATPase6* nucleotide changes in POI, occult OI patients and controls

Subjects	8679 A>G	*8684 C>T	8865 G>A	*9064 G>A	*9094 C>T	9123 G>A
Amino acid change	No change	T>I	No change	A>T	L>F	No change
POI ($n = 20$)	1	7[a]	1	1	5[a]	5
Occult OI ($n = 4$)	0	1	0	0	1	0
Control ($n = 20$)	0	0	1	0	0	1
Odds ratio (95% confidence interval)	3.154 (0.1210–82.23)	22.77 (1.19–432.90)	1.0 (0.058–17.19)	3.154 (0.1210–82.23)	14.548 (0.746–283.58)	6.33 (0.633–60.196)

* Non-synonymous changes

[a] $P < 0.05$ is considered as significant by Fisher's exact test compared to controls

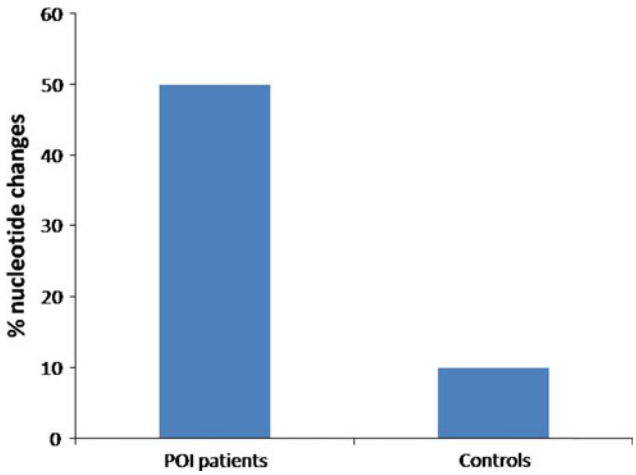

Fig. 1 Percentage of patients and controls with *ATPase6* nucleotide change

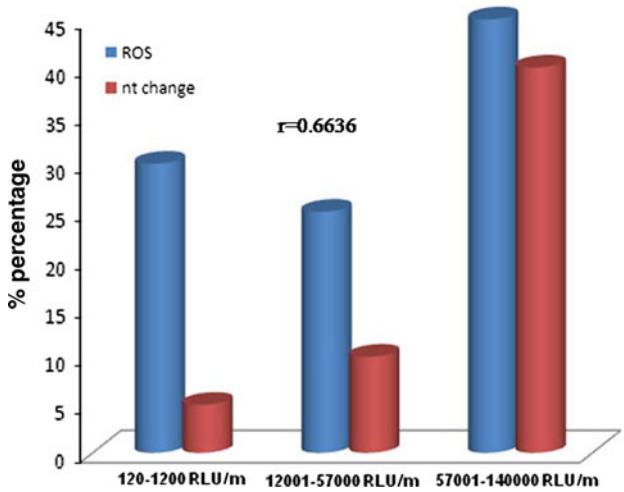

Fig. 2 Percentage of patients with ROS range and nucleotide changes

(A>T), showing a PSIC score of 0.021 (Benign) and SIFT score of 0.37 (tolerant). Alanine is a nonpolar hydrophilic amino acid and threonine is a polar hydrophilic amino acid.

Discussions

Mitochondrial energy production plays an important role in oogenesis, follicle maturation, ovulation and embryogenesis. This energy demand is met solely by mitochondria and thus the oocyte has the largest number of mitochondria and mt DNA copies of any cell [4]. The electrons transported through the OXPHOS system are used to convert ADP into ATP by ATPas6 synthase. Studies have reported that mutations in *ATPase6* gene correlate with the overproduction of ROS in various disorders [8–10] and the *ATPase6* gene maintains mt genome stability and integrity [7]. This preliminary pilot study showed that non-synonymous changes in *ATPase6* gene and elevated ROS levels were observed in the POI patients and thus it is possible that OS

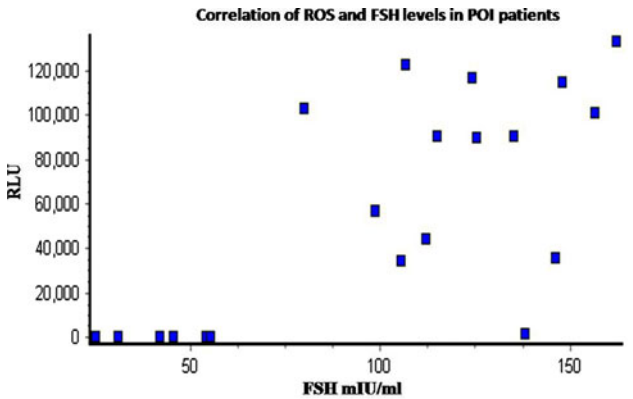

Fig. 3 Correlation of ROS and FSH levels in POI patients

and *ATPase6* mutations may be causal in the premature cessation of ovarian function. In such cases, antioxidant treatment may prove highly beneficial in delaying or preventing ovarian insufficiency. ROS levels damage mitochondrial membrane and cause mt and nuclear DNA damage creating abasic sites, single- and double-stranded breaks, DNA oxidation and mutagenic DNA bases such as 8-hydroxy 2-deoxyguanosine (8OHdG). These mutations may decrease the ATP levels that may lead to cessation of cell growth, development and accelerated atresia/apoptosis of germ cells [13]. Production of ovarian follicles and its maturation play an important role in female reproduction. Oxidative stress is a well-established condition in a variety of diseases including female infertility [14, 15]; however, its role in POI has not been studied. Therefore, this study was planned to screen *ATPase6* gene nucleotide variations and OS in cytogenetically normal idiopathic cases of POI.

Low ROS levels facilitate folliculogenesis, oocyte maturation, ovarian steroidogenesis and fertilization [15, 16]. When the ROS levels overwhelm the antioxidant defense capacity, it leads to oxidative stress, which may damage the adjacent bimolecules leading to peroxidative damage to nucleic acid, lipids and proteins. Therefore, the observed elevated ROS levels in POI cases may have a role in impaired folliculogenesis or accelerated primordial germ cell apoptosis. Oxidative stress induces nucleotide alterations in both mitochondria and nuclear genome [10, 17]. Their first target of damage is mtDNA, which is situated in close proximity to the electron transport chain. As nucleotide alteration in mtDNA accumulate, they result in further increase in ROS production and subsequently the oxidative stress induces nuclear damage as membrane permeability is altered [11, 18, 19]. To confirm this, we screened nucleotide changes in *ATPase6* gene that maintains mt genome integrity. As a majority of nucleotide alterations were non-synonymous resulting in change from polar to nonpolar amino acid, it may adversely affect electron transfer and disrupt electron transport chain by increasing ROS production. Two changes were found to be significantly higher in cases mt.8684 C>T and mt.9094 C>T as compared to controls. Instability and nucleotide alteration of *ATPase6* gene and associated overproduction of ROS have been previously described in primary ovarian carcinomas and male infertility [10, 20]. As the energy levels deplete with decrease in ATP levels, these cells undergo apoptosis, which may explain the premature cessation of ovarian function. A recent study by Bentov et al. [6] clearly demonstrated that supplementation of CoQ10, a natural antioxidant synthesized in all tissues improved oocyte and embryo quality. Almost all tissues in the body synthesize coenzyme Q and it is a major cellular antioxidant, the tissue concentration of which is five- to tenfolds higher than other lipid-soluble antioxidants as vitamin E [21]. They also showed that increased ATP content in a group of embryos cultured with CoQ10 and its supplementation led to better quality of embryos. This may be due to the antioxidant effect of CoQ10, as high ROS levels cause pronuclear block, slow cleavage and impair blastocyte development. Though depleted, ATP levels may be one of the causes of POI; it is possible that increased ROS production leading to OS may also have secondary, additive effect and reduce functional competence of germ cells. The pathological role of ROS has also been demonstrated in various female reproductive disorders such as endometriosis, polycystic ovarian disease, spontaneous abortion, hydatidiform mole and preeclampsia [19]. High ROS levels have been reported in peritoneal fluid and endometrium in these conditions [22], and damage various biomolecules, such as lipids in cell membrane, and mt and nuclear DNA, due to accumulation of highly mutagenic 8-hydroxy-2-deoxyguanosine (8-OHdG) [23], which is a marker of oxidative stress.

Various studies have established OS as the etiopathological factor in several male and female reproductive disorders [11, 12, 15, 24, 25]. Though the majority of the POI patients were found to be idiopathic, this preliminary pilot study showed that approximately 70% of cytogenetically normal women presenting with idiopathic POI had elevated ROS levels and 50% of these cases harbored nucleotide alterations of the *ATPase6* gene. The result of this study highlight *ATPase6* gene mutations and OS may lead to premature cessation of ovarian function. Thus, early detection of oxidative stress and prompt management with antioxidants and mitochondrial nutrients may delay/prevent oxidative stress-induced POI. However, inclusion of a large number of cases with idiopathic POI and occult OI are required to establish a strong correlation between OS and POI.

Conflict of interest statement We declare that we have no conflict of interest.

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