Attention deficit-hyperactivity disorder suffers from mitochondrial dysfunction

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1. Introduction

Inattention, impulsiveness and hyperactivity depict phenotypes of attention-deficit hyperactivity disorder (ADHD), a heritable and heterogeneous childhood disorder with a prevalence of 6–7% in children and adolescents [1]. Mitochondrial dysfunction is a suggested vulnerability factor in the pathogenesis of various neuropsychiatric disorders [2–5]. Bioenergetic crisis during brain development, mitochondrial DNA (mtDNA) mutation or deletion may cause neurodevelopmental disorders. Autism spectrum disorder probands with mitochondrial defects exhibit symptoms of ADHD [6–8], suggesting possibility of bioenergetics defects in ADHD too [9]. Other than a report on significant stimulant effect of the ADHD drug, methylphenidate on neuronal firing and mitochondrial transport chain (ETC) enzyme activities in rats [10], no report is available on direct involvement of mitochondrial defects in ADHD. We investigated involvement, if any of mitochondrial bioenergetics in the pathophysiology of ADHD, by creating control and ADHD cybrids from an Indian population.

Cybrids, the mitochondrial transgenic cells created by fusion of mitochondria-less neuronal ρ0-cells prepared from SH-SY5Y neuroblastoma cell line with blood platelets from patients, mimic pathological conditions of disease phenotypes of post-mortem brains as shown for Parkinson' disease [11] and other diseases [12]. We created cybrids using blood platelets from ADHD probands and ethnically-matched controls, and investigated their mitochondrial status and functions. One-time creation of cybrids has advantage over repeated blood sampling from young volunteers, since cybrids could be cryopreserved, differentiated into neurons and used continually for investigations.
2. Materials and Methods

2.1. Materials

The human neuroblastoma cell line SH-SY5Y was obtained from National Centre for Cell Sciences, Pune, India. Low glucose Dulbecco’s modified Eagle’s medium, minimal essential medium modified for suspension culture (S-MEM), fetal bovine serum, Gentamicin, tetracylmethyldihemolamine, methyl ester (TMRM), were procured from GIBCO, Invitrogen Corporation (CA, USA). Lymphocyte separation medium (LSM), polyethylene glycol 1000, manitol, were purchased from MP Biomedicals (France). Picogreen®, MitoSOX™ were purchased from Molecular probes (OR, USA). Potassium chloride, KH₂PO₄, NaCl and Na₂HPO₄·2H₂O, magnesium sulfate, lead nitrate, sucrose, were procured from Sisco Research laboratories (India). Amphotericin B, pyruvate, uridine, ethidium bromide, TRI reagent, 6-aminoacaproic acid, Bis-Tris HCl, N-Dodecyl β-D-Maltoside, bis acrylamide, ATP, EGTA were purchased from Sigma (MO, USA). Coomassie Brilliant Blue G-250 and R-250 dyes were purchased from BioRad (CA, USA) and acrylamide was purchased from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Cybrid preparation

ADHD probands (two aged 13; one aged 10 years) and ethnically-matched controls (a 5 years-old female, three males—5 years, 6 years 8 months and 9 years old respectively) were recruited by mental health professionals of Manovikas Kendra following psychological evaluations through Conners’ Parents and Teachers Rating Scale [13] and Wechsler’s Intelligence Scale for Children [14] for assessing inattention/hyperactivity and Intelligence Quotient (IQ) levels, respectively. Patients with other neuropsychiatric disorders, pervasive developmental disorders, mental retardation (IQ >70) including Fragile-X syndrome were excluded. Informed written consent for participation was obtained from the parents. Institutional Human ethics committee approved the study protocol.

2.2.2. Confirmation of mtDNA transfer

ρ₀-Cells prepared from SH-SY5Y cell line were fused with platelets isolated from 3 ADHD male probands and two healthy controls to produce cybrids [15]. Genomic-DNA isolated from SH-SY5Y, ρ₀-Cells and cybrids created were subjected to long template PCR using internal primers that amplify a 5.8 kb fragment present in both nuclear and mitochondrial genomes and the ampiclon was nested with external primers to amplify a 5.9 kb mtDNA-sequence [16]. Picogreen® and Mitotracker Green® (Invitrogen Corporation, CA) staining were used for examining absence and presence of mtDNA in these cells and to locate mitochondria in live cells, respectively. Images of cells stained with Picogreen® or Mitotracker Green® were captured using confocal microscope.

2.2.3. Mitochondrial membrane potential and oxidative stress

Mitochondrial membrane potential was evaluated by staining cells with tetracylmethylrhodamine, methyl ester (TMRM). Equal number (1 × 10⁶ for fluorimetry and 2 × 10⁵ for flowcytometry) of cells were plated in 6 well plates for TMRM staining. Next day cells were trypsinized and the pellet was resuspended in fresh medium containing 50 nM TMRM, incubated at 37 °C for 30 min in the dark in a CO₂ incubator. The medium containing the stain was then removed and the cells were washed twice with D-PBS. Red fluorescence was measured using a spectrofluorimeter (PerkinElmer, USA). The excitation and emission wavelengths for TMRM staining were 530 nm and 570 nm respectively. Membrane potential was also measured with the same dye by using flowcytometry in cybrids. Cells were processed in the same way and the population of TMRM stained cells was analysed by flowcytometry (BD LSRSortessa fluorescence activated cell sorting (FACS), Software-FACS Diva 6.2.)

Cells were stained with MitoSOX™ to assess mitochondrial superoxide generation in these cybrids. Cybrids were collected by trypsinization and stained with 2.5 μM of MitoSOX™ for 30 min at 37 °C in the dark in a CO2 incubator. The stain containing medium was then removed and the cells were washed twice with D-PBS. Red fluorescence was measured using a spectrofluorimeter (PerkinElmer, USA, excitation: 510 nm, emission: 580 nm). MitoSOX™ stained cells were also analysed by flowcytometry (BD LSRSortessa fluorescence activated cell sorting (FACS), Software-FACS Diva 6.2.) [19].

2.2.4. Respiration

Whole cell respiration and digitonin permeabilized cells’ mitochondrial respiration were measured in cybrids using Oxigraph respirometer (Hansatech Instruments Ltd, England). Cells were trypsinized and added to the counter chamber of the Oxigraph respirometer containing D-PBS and the rate of oxygen consumption was measured for 8 min. For mitochondrial respiration the cells were permeablized with digitonin (0.01%), in mitochondria isolation buffer (MIB, 225 mM Manitol, 75 mM Sucrose, 5 mM MOPS, 1 mM EGTA, dissolved in water, pH 7.4). The cells were washed two times to remove traces of digitonin and oxygen consumption was measured for 10 min. The representation of the amount of oxygen consumed was given as nmol of oxygen/min/number of cells use. [17].

2.2.5. Transcriptome analysis of ATP6/8 subunits

For real time PCR, RNA was isolated using TRI reagent according to the manufacturer’s protocol. Five μg of the total RNA was reverse transcribed using MuLV reverse transcriptase (conditions: 70 °C for 5 min, 37 °C for 5 min, then 42°C for 60 min, and 70 °C 10 min, final hold at 4 °C). Relative quantification was performed using real-time PCR (Thermal Cycler Dice Real Time System TP800, Takara, Japan) with 100 ng of cDNA and SYBR Premix. The thermal cycling involved initial denaturation of 95 °C for 5 min followed by 40 cycles of 95 °C for 20 s, 58 °C for 30 s, 72 °C for 20 s. 18S RNA was used as endogenous control. The primers were designed using Primer 3 software (TAPase 6 - Forward 5’-GCCCTAGCCCACTTCTTACC-3’, Reverse 5’-TTAAGGCGACACTAAGCCTTTAC-3’; ATPase 8 - Forward 5’-CACCTACTCCTCCCTCACAAA-3’, Reverse 5’-CTAGGATTGTGGGGGCAAT-3’, 18sRNA – Forward 5’-CATG GCCCTTCTATTGTTGTT-3’, Reverse 5’ CCGAACATCTAAGGGCATC-3’), data were analyzed using the 2-ΔΔCT method [18].

2.2.6. Complex V activity

For Blue Native - Polyacrylamide gel electrophoresis (BN-PAGE), mitochondria (P2 fraction) were isolated in mitochondria isolation buffer (MIB, 225 mM Manitol, 75 mM Sucrose, 5 mM MOPS, 1mM EGTA, dissolved in water, pH 7.4), after quantification stored in -80°C. Next day pellet was dissolved in sample buffer (1 M 6-aminoacaproic acid and 50 mM Bis-Tris HCl, dissolved in water, pH 7.0), along with freshly prepared 10% (w/v) N-Dodecyl β-D-Maltoside, kept on ice for 10 min to dissolve the membrane proteins in the solution. Centrifuge the samples at 20,000xg for 30 min at 4°C. Collect the supernatant and add the gel loading buffer (5% w/v Coomassie Brilliant Blue G-250 dissolved in 1 M 6-aminoacaproic acid).

Gradient gel (5-13%) was prepared by using 30% acrylamide solution (by dissolving 29.22 g of acrylamide, 0.78 g of bis acrylamide in 100 ml of distilled water) in gel buffer (150 mM Bis-Tris, 1.5 mM aminocaproic acid, pH 7.0). The sample protein (60 μg) was run at, 100 V for 4 hrs at 4°C on this gradient gel using 1X Cathode Buffer (10X Cathode Buffer- 50 mM Tricine, 15 mM bis-Tris HCl, pH 7.0, 0.02% Coomassie G-250) and 1X Anode buffer (10X Anode buffer- 500 mM Bis-Tris HCl, pH 7.0). Two gels were run using same protein, once run was complete one gel was kept for coomassie staining (for protein quantification, 0.25 % coomassie brilliant blue R250 in 40% methanol, 7% acetic acid) for one hour and stained overnight in 10% acetic acid and 10%
methanol. Another gel was kept for complex V reaction buffer (35 mM tris, 270 mM glycine, 14 mM magnesium sulfate, 0.2 % lead nitrate, 8 mM ATP, pH 7.8) for overnight at room temperature. Next day gels were photographed, reaction gels shows white bands [19].

2.2.7. Serotonin analysis

For HPLC analysis 5 × 10^5 cells were plated and differentiated for six days. On the seventh day, the medium was replaced with fresh medium. After 16 h the cells were washed, sonicated in 50 μl of ice-cold 0.4 M perchloric acid, centrifuged at 12,500 rpm for 5 min and 10 μl of the supernatant was injected into the HPLC-ECD system. A standard solution containing 4 pmol of all the biogenic amines (Norepinephrine, DOPAC, Dopamine, 5-HIAA, HVA and 5-HT) were assayed prior to and at the end of the sample injections to confirm the retention time of the analytes in the column. The flow rate was 0.7 ml/min and the electrochemical detection was performed at 0.74 V. The sensitivity of the HPLC-ECD was set at 20 nA [21].

Cybrids cultured on poly-L-lysine coated cover-slips at 10^5 cells/ml/35 mm dish were immunostained for monoamine oxidase-A (MAO-A) and MAO-B proteins, images were captured employing confocal microscope, and fluorescence Intensity was measured using Image J software (NIH, USA) (Data are not shown)

3. Results

3.1. Inheritance of mitochondria

The inheritance of mitochondria was tested in ADHD samples employing long-template PCR method. The primer sets were designed for PCR so that the internal primers amplified a 5.8 kb sequence present in both the nuclear (chromosome 1) and mitochondrial genomes and its amplicon was nested in that of the external primers, which amplified a 5.9 kb mtDNA-specific sequence (Fig. 1A), in case of SH-SY5Y, control and ADHD cybrids PCR product of external and internal primers is obtained, while in ρ0 cells lacking mitochondria, only nuclear sequence amplified by internal primers was observed. PicoGreen dye binds to minor groove of double stranded DNA and gives green fluorescence, so we can see the punctate mitochondria in the cytoplasm of SH-SY5Y cells. The punctate fluorescence is absent in ρ0 cells, but show only nuclear fluorescence because they do not contain mtDNA (Fig. 1B). The punctates observed in control, ADHD cybrids confirm their inheritance of mitochondria from platelets of healthy individuals and patients.

3.1.2. ADHD cybrids show reduced mitochondrial mass and membrane potential

Mitotracker Green enters mitochondrial matrix where it covalently reacts with free thiol groups of cysteine residues of mitochondrial proteins and produce green fluorescence which allows visualization of the mitochondria. SH-SY5Y cells are showing elongated mitochondria and ρ0 cells have fragmented mitochondria (Fig. 2A). Bar diagrams represents the fluorescence intensity in the control and ADHD cybrids measured using Image J software which shows significant reduction in ADHD cybrids compared to controls (Fig. 2B). Mitochondrial membrane potential was examined in cybrid neurons with TMRM staining followed by spectrofluorimetric (Fig. 2C) quantification as well as flow cytometric (Fig. 2E and F) procedure. Significantly low TMRM fluorescence observed in ADHD cybrid-neurons implied higher mitochondrial depolarisation in these cells. Mitochondrial membrane potential was also analysed in differentiated cybrid neurons [20] by fluorimetric and FACS analysis and we observed similar results (data are not shown) as in undifferentiated neurons.

3.1.3. Higher mitochondrial oxidative stress in ADHD cybrids

Likewise MitoSOX™ staining was carried out to test the mitochondrial oxidative stress generated in these cybrids neurons employing spectrofluorimetric (Fig. 2D) and flow cytometric (Fig. 2G).
and H) analyses. Interestingly ADHD cybrids displayed higher levels of superoxide radicals, as evidenced from the significantly increased MitoSOX™ fluorescence in the cells.

3.1.4. Reduced oxygen consumption due to complex V deficiency in diseased cybrids

Oxygen consumption was monitored in controls and ADHD non-permeabilized (Fig. 3A and B) and permeabilized cells (Fig. 3C and D) employing a sensitive Oxygraph in order to understand the respiratory capability of the cybrids. Significantly reduced rate of respiration found in ADHD cybrids, as well as in the mitochondria of these disease-cybrids, pointed to a significant loss of mitochondrial functions in both these diseases. Lower respiration rate could be resulting from the considerable loss in the ATPase 6/8 transcript levels in ADHD cybrids revealed in quantitative PCR analysis as seen in the present study (Fig. 3E and F).

3.1.5. Higher level of 5HT in ADHD cybrid neurons and reduced activity of complex V

Serotonin level, measured by HPLC-ECD in differentiated neurons, was found to be higher in ADHD cybrids as compared to controls (Fig. 3G). BN-PAGE in-gel activity studies demonstrated a significant deficiency in mitochondrial complex V activity in ADHD cybrids, when compared to the control cybrids (Fig. 3H to J).

4. Discussion

Most important feature of the present study is the successful creation of control and ADHD cybrids from an east-Indian population. This is confirmed by successful incorporation of mtDNA into ρ0-cells, which did not have mtDNA before fusion with the patient or control platelets. This is the first ever report on production of mitochondrial cell-hybrids (cybrids) for the neurodevelopmental disorder, ADHD. Another important attribute of this study is the finding of significant mitochondrial aberrations and dysfunctions, as evidenced by reduced staining intensity of Mitotracker Green®, TMRM and increase in MitoSOX™ fluorescence, and significant reduction in oxygen consumption concurrent with low levels of ATPase 6/8 transcripts in ADHD cybrids in comparison to control cybrid-neurons. These observations support a significant bioenergetic crisis in ADHD, which was proposed [9], but never demonstrated. The third important observation made in the present study is the significant increase in the levels of serotonin in the differentiated ADHD cybrid-neurons as compared to control cybrid-neurons, which is a topic of interest and intense debate.

Mitotracker green dye accumulates in the mitochondrial matrix where it covalently reacts with free thiol groups of cysteine residues and produce green fluorescence and allows to visualize the mostly inter-netted, tubular fluorescing mitochondria in the cytoplasm of control cybrids, but punctate (due to fragmented mitochondria) fluorescence in diseased cybrids. Quantification of mitotracker green staining by ImageJ software gives the total mass of the mitochondria present in the cytosol [23] which was found less in ADHD cybrids as compared to the control cybrids. This data speculates that due to fragmentation of mitochondria the total mass in the cytoplasm decreases. Significantly low TMRM fluorescence observed in ADHD cybrid-neurons implied higher mitochondrial depolarisation in these cells. No report is available in literature which deals with mitochondrial membrane potential and ADHD, and therefore the present report is...
the first in literature. ADHD cybrids displayed higher levels of superoxide radicals, as evidenced from the significantly increased MitoSOX™ fluorescence in the cells. These amply provide proof that in ADHD there exist a serious oxidative stress condition, and that could be detrimental to the health of the neurons. In one study (48 children and adolescents (34 male, 14 female) with ADHD who had no neurological, systemic, or comorbid psychiatric disorders, and 24 sex- and age-matched healthy controls (17 male and seven female), it was observed that ADHD patients have oxidative imbalance [24]. In another study one group investigated total antioxidative status and total oxidative status of plasma and antioxidant enzyme. Oxidative stress index values and the plasma TOS levels of the patients with ADHD were statistically higher than those of the control group [25]. Sezen et al. [26] also investigated and found that oxidative stress was higher in the ADHD patients than the control group. Bioenergetic dysregulation playing key roles in many neuropsychiatric disorders is recently reviewed [27]. This may result from defective mitochondrial ETC complex function, which is validated by a significant decrease in state3 respiration in ADHD cybrids. Considerable decrease in the ETC complex V ATPase 6/8 subunits in ADHD cybrids further provides positive evidences in this direction. ATPase 6/8 mutations are associated with many neurodevelopmental disorders including autism [28,29], with which ADHD shows overlapping syndromes [30]. The decreased transcript level of ATP6 and ATP8 subunits may lead to the lower activity of complex V in ADHD cybrids as our BN-PAGE data shows. These evidences firmly suggest that mitochondrial dysfunction exists in ADHD.

The status of mitochondrial energetics in ADHD subjects is not available. However, indirect evidences from pharmacological studies using methylphenidate, which is a drug commonly used to treat ADHD have shown that the drug affects the activity of mitochondrial ETC enzymes and results in increase of the extracellular levels of the catecholamines that stimulate glycolysis and release of lactate from the astrocytes, thereby correcting the energy deficiency, and restoring appropriate firing rates [10]. This has led to the hypothesis that impairment of mitochondrial bioenergetic machinery may play a role in autism and ADHD pathogenesis [9,31].

Serotonergic system, known to modulate impulse control and aggression, is challenged in ADHD patients [32]. At a time when mitochondrially-located MAO-A or MAO-B were unaffected in ADHD cybrids, the differentiated cybrid-neurons from ADHD probands exhibited 2-fold increase in serotonin indicating probably an active role of this biogenic amine in the phenotypes of the disease. If this is proved to be true, these cybrid-neurons can be used as a cellular model for ADHD to investigate neuromolecular mechanisms underlying ADHD pathology.

At a time when no direct evidence of mitochondrial dysfunctions in ADHD is available, we created and used ADHD cybrids to assess mitochondrial functions in this neurodevelopmental disorder. Cybrids created by fusion of ρ0-cells and Parkinson’s disease (PD) patient platelets are shown to replicate mitochondrial pathology seen in post-mortem PD brains [11], and differentiation of these cells into neurons are demonstrated to retain the mitochondrial defects [22]. Therefore, ADHD cybrids and the cybrid-neurons created in the present report could be reliable tools to study the mitochondrial dynamics and bioenergetics, and to understand the molecular mechanisms underlying the pathophysiology of ADHD.

In short, this is the first report that identifies mitochondrial dysfunction as an inherent factor of ADHD pathology, and for probable direct
role of serotonergic system in the disease syndromes. Increased serotonin content in the differentiated cybrid-neurons suggests that the mitochondrial defect in these cells can significantly alter the serotonergic neurotransmitter function, which could be a pathological hallmark of ADHD. This study identifies mitochondrial pathology as one of the risk factors for ADHD, and the cybrid model provides a novel, reliable and valid tool to study the molecular basis of ADHD at cellular level.

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Potential conflicts of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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