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Attention deficit-hyperactivity disorder suffers from mitochondrial dysfunction

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ABSTRACT

Background: Pathophysiology of attention-deficit hyperactivity disorder (ADHD) is not known, and therefore the present study investigated mitochondrial defects, if any in cybrids created from patients and control population.
Methods: To investigate mitochondrial pathology in ADHD, cybrids cell lines were created from ADHD probands and controls by fusing their platelets with ρ^0-cells prepared from SH-SY5Y neuroblastoma cell line. Cellular respiration, oxidative stress, mitochondrial membrane potential and morphology were evaluated employing oxygraph, mitochondria-specific fluorescence staining and evaluation by FACS, and immunocytochemistry. HPLC-electrochemical detection, quantitative RT-PCR and Blue Native PAGE were employed respectively for assays of serotonin, mitochondrial ATPase 6/8 subunits levels and complex V activity.
Results: Significantly low cellular and mitochondrial respiration, ATPase6/8 transcripts levels, mitochondrial complex V activity and loss of mitochondrial membrane potential and elevated oxidative stress were observed in ADHD cybrids. Expression of monoamine oxidizing mitochondrial enzymes, MAO-A and MAO-B levels remained unaffected. Two-fold increase in serotonin level was noted in differentiated cybrid-neurons.
Conclusions: Since cybrids are shown to replicate mitochondrial defects seen in post-mortem brains, these observed defects in ADHD cybrids strongly suggest mitochondrial pathology in this disorder.
General significance: Mitochondrial defects are detected in ADHD cybrids created from patients' platelets, implying bioenergetics crisis in the mitochondria could be a contributory factor for ADHD pathology and/or phenotypes.

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 ρ^0-cell lines with blood platelets from patients, mimic pathological conditions of disease phenotypes of post-mortem brains as shown for Parkinson's 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.

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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, tetramethylrhodamine, 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, KH_2PO_4, NaCl and $Na_2HPO_4.2H_2O$, magnesium sulfate, lead nitrate, sucrose, were procured from Sisco Research laboratories (India). Amphotericin B, pyruvate, uridine, ethidium bromide, TRI reagent, 6-aminocaproic 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

ρ^0-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, ρ^0-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 amplicon 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 tetramethylrhodamine, methyl ester (TMRM). Equal number (1×10^6 for fluorimetry and 2×10^5 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 CO_2 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 LSRFortessa 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 LSRFortessa 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 Oxygraph respirometer (Hansatech Instruments Ltd, England). Cells were trypsinized and added to the counter chamber of the Oxygraph respirometer containing D-PBS and the rate of oxygen consumption was measured for 8 min. For mitochondrial respiration the cells were permeabilized 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. 18sRNA was used as endogenous control. The primers were designed using Primer 3 software (ATPase 6 - Forward 5'-GCCCTAGCCCACTTCTTACC-3', Reverse 5'-TTAAGGCGACA GCGATTTCT-3'; ATPase 8 - Forward 5'-CACCTACCTCCCTCACCAAA-3', Reverse 5'-CTAGGATTGTGGGGGCAAT-3', 18srRNA – Forward 5'-CATG GCCGTTCTTAGTTGGT-3', Reverse 5' CGGACATCTAAGGGCATCAC-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^{0}C. Next day pellet was dissolved in sample buffer (1 M 6-aminocaproic 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^{0}C. Collect the supernatant and add the gel loading buffer (5% w/v Coomassie Brilliant Blue G-250 dissolved in 1 M 6-aminocaproic 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 ^{0}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 distained overnight in 10% acetic acid and 10%