Mitochondrial dysfunction in Autism spectrum disorders (ASD) children from central India: Clinical, biochemical, neuroimaging and genetic screening

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Abstract

Autism spectrum disorder (ASD) encompasses neurodevelopmental disorders that are defined by behavioural observations, in particular dysfunctions in social interaction and communication skills, as well as repetitive behaviours. Several studies have revealed immune and neuronal dysregulation in autistic subjects. Many children with ASD have associated underlying medical comorbidities, like epilepsy, sleep disruptions, mitochondrial dysfunction (MD) and gastrointestinal (GI) abnormalities. Few studies have hypothesized that individuals with ASD may have an abnormality in carbohydrate metabolism should be tested for disorder of impaired Mitochondrial function. Many studies has provided evidence (19-43%) that individuals with ASD have concomitant MD and proposed a "Mitochondrial autism subgroup". Mitochondria exist in nearly every cell that generates adenosine triphosphate. In MD, the mitochondria cannot convert food and oxygen into life-sustaining energy. Earlier considered as uncommon disease, diagnosis of MD can be challenging. It is identification is based on several objectives, clinical, histological, biochemical, molecular, neuroimaging and enzymatic findings. Screening for underlying MD is important in ASD, because the children clinically look and act in both. Proposed study is an attempt screen ASD children and finding out the role of co-occurrences of MD for the better understanding and management of the disease.

Objective

1. To collate features of mitochondrial dysfunction in the general population of children of Bhopal with Autism spectrum disorders (ASD) by clinical, biochemical, neuroimaging and genetic screening.
2. To compare the characteristics of the MD children among the ASD group with those of the general population (without ASD) (Evaluation of digestive enzymes is patients with ASD GI-abnormalities).

Materials &Methods

Clinical Criteria for case selection: Clinical history of the patient were taken by the Paediatrician, mainly on Family history, Regression, Seizures, Fatigue/ lethargy, Ataxia, motor delay, GI abnormalities and cardiomyopathy. ASD children of age between 3-18 years are registered after taking written approval from their parents and Institutes Human Ethical Committee (IHEC) AIIMS Bhopal.

Patient collection: Autistic Patients registered at AIIMS paediatrics department were contacted. Inclen diagnostic tool was used for diagnosis of autistic spectrum disorder.

Project study was explained to parents. Ten autistic parents children have accepted to get their child registered in the study. Out of these ten registered ASD patients Paediatrician has selected only three children after taking there clinical history on the basis of selection criteria; Regression Seizures, Fatigue/Lethargy, Ataxia, Motor Delay, GI abnormalities, Cardiomyopathy. Institutional Human Ethical committee has reviewed and approved the study.

Biochemical Parameters: 1 ml blood sample was taken. Serum Lactate, Pyruvate, Carnitine (Free and Total) Creatine Kinase, AST, ALT, Amylase, Glucose, Quantitative plasma amino acids was tested on automatic auto-analysers at the department of Biochemistry, AIIMS Bhopal. Sample was stored at -20 Deg C if required for any conformation testing. For GI abnormalities evaluation of digestive enzymes test was done in view of impaired carbohydrate metabolism.

Neuro Imaging: It would be done wherever to assess the phosphocreatine level in the prefrontal cortex for autistic children and to estimate the lactate peak in brain parenchyma in patients suffering from mitochondrial disorders (11, 13). Three parameters will be taken into consideration for the MR spectroscopy which are – NAA, Choline and creatinine. These were then compared to normal children and the levels compared to find a decrease or increase in the value. A series of sagittal, coronal and axial T1-weighted anatomic scans serving as MRS localized (TR/TE = 250/3.8 ms, flip angle = 70 degrees, 5 mm slide thickness, 1.5 mm gap, 512*512 matrix) will be acquired for spectroscopic voxel placement. The MRS voxel will be placed in a region of the bilateral ACC and PCC.

Genetic Tests, Using PCR for Mitochondria DNA

Mitochondria are found in all eukaryotic cells. The mitochondrial genome is 16,569 bp in length and contains 37 genes. Recently, forensic scientists, anthropologists, and evolutionary biologists have looked at mutations within the DNA of the mitochondrion to explore differences between peoples and populations.

This step typically dislodges hundreds of cells from the cheek epithelium. An aliquot of the mouthwash solution is centrifuged to collect the dislodged cells, which are then re-suspended in a small volume of saline. Take the expel saline in the 1.5 ml Eppendorf tube and label it. Transfer 1000 µL to 1500 µL (1 mL to 1.5 mL) of the saline/cell suspension into the labelled microfuge tube. In a micro-centrifuge, spin your saline cell suspension for 1 minute to pellet the cells at 10,000 RPM. Take the supernatant to store till PCR at -20 Deg C is done.

PCR amplification of the mitochondrial D-loop region using the primers for this protocol is ued to produce 440 bp product seen on agarose gel electrophoresis. PCR products were sent for sequencing for the analysis by using NCBI BLAST tools of Mt DNA genome SNPs.

Biochemical Results

Investigation		Result(s)	Unit(s)	Reference Range*
Plasma glucose	Random	91.0	mg/dL	
Liver function test	SGPT (ALT)	17.0	U/L	< 50 (male); <35 (female)
	SGOT (AST)	34.0	U/L	< 50 (male); <35 (female)
Serum Creatine Kinase (CK)		312.0	U/L	≤ 171 (male) ; ≤ 145 (female)
Serum Amylase		81.0	U/L	28-100
Serum Lactate		16.7	mg/dL	4.5-19.8

Investigation		Result(s)	Unit(s)	Reference Range adults *
Plasma glucose	Random	91.0	mg/dL	
Liver function test	SGPT (ALT)	20.0	U/L	< 50 (male); <35 (female)
	SGOT (AST)	35.0	U/L	< 50 (male); <35 (female)
Serum Amylase		63.0	U/L	28-100
Serum Creatine Kinase (CK)		91.0	U/L	≤ 171 (male) ; ≤ 145 (female)
Serum Lactate		21.2	mg/dL	4.5-19.8

Investigation		Result(s)	Unit(s)	Reference Range*
Plasma glucose	Random	102.0	mg/dL	
Liver function test	SGPT (ALT)	78.0	U/L	< 50 (male); <35 (female)
	SGOT (AST)	40.0	U/L	< 50 (male); <35 (female)
Serum Amylase		58.0	U/L	28-100
Serum Creatine Kinase (CK)		131.0	U/L	≤ 171 (male) ; ≤ 145 (female)
Serum Lactate		16.6	mg/dL	4.5-19.8

Digestive enzymes Disaccharides testing

1. Presence of undigested disaccharide indicated the absences of diasaccharidase enzyme in stool sample of Sample ID STS-2015_002
1. Other tested samples were partially positive for diasaccharidase enzyme .
2. As partially digested disaccharide were present in stool samples of ASD patients.

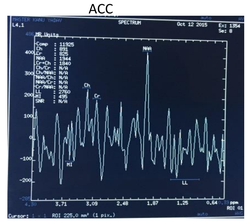
Summary and Conclusion

1. This pilot study was conducted as a part of STS-2015 ICMR project and it has turned out be a very important study to find out mitochondrial dysfunction (MD) present in ASD children of Bhopal. In previously done studies it is published that an estimated 1 out of 110 individuals in America are affected with ASD, with male-to-female ratio of 4.5:1.The etiology of ASD is not known in most cases, but genetic component, possibly involving 15 or more loci, is widely accepted in contribution of ASD. It is becoming apparent that many children with ASD have associated underlying medical comorbidities which may be epilepsy, sleep disruptions, mitochondrial dysfunction and gastrointestinal (GI) abnormalities
2. Mitochondrial dysfunction has been implicated in several psychiatric and neurological disorders, with association with carbohydrate metabolism .In the present study also we could see the necessity of performing MD tests in ASD suspected children. As the biochemical tests performed on them have indicated abnormal values of tests for lactate, Creatine Kinase, Alkaline transaminase (ALT), Aspartate Transaminase (AST), serum amylase and random glucose all of which could indicate towards Mitochondrial Disorders in Autistic children. However, for a better conclusion, study has to be performed on a larger group. These indirect markers can be abnormal for several reasons as suggested in many studies. For example, MD impairs aerobic respiration, leading to a reduction in TCA cycle function resulting in an elevation in pyruvate. Pyruvate is metabolised to lactate and alanine, leading to elevations in these metabolites.
3. MR Spectroscopy revealed increased concentration of NAA. The NAA concentration correlates with the social quotient of the child. Studies have done earlier have shown decreased concentrations of NAA in the Left Amygdala and the bilateral orbito-frontal cortex. These findings suggest presence of neuronal dysfunction in the children with ASD. Our test patient showed increased values but since only one patient could be subjected to MRS, no conclusion could be drawn.
4. In view of MtDNA genetic testing results showing proper amplification of MtDNA and amplified products were sent for sequencing to analyse the nucleotide sequence of single nucleotide Polymorphism (SNP).Analysis of partial genomic sequencing results indicated two point mutation in mtDNA of sample ID STS-2015_002 (Genbank accession no. KU534601).This study has standardised the protocol of MtDNA isolation from cheek cells from ASD children.
5. Stool Sample collected from ASD patients were investigated clinically for carbohydrate metabolism. The observations indicated a gastrointestinal problem if or whenever milk or its products were taken or given in the diet. Testing of collected stool samples from ASD patients shows presence of undigested disaccharides indicated inactivated/ absence of diasaccharidase enzyme in ASD children. A paediatrician (Co-supervisor) in the study has recommended diet without milk or milk product for child for seven days. During that seven days the child had no complain of any stomach-ache, which also suggested that GI disorder in these patients should be investigated as one of the important criteria before prescribing antibiotics for GI disorder symptoms repetitively.

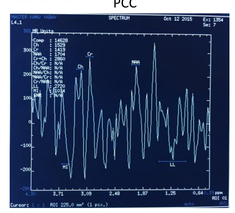
Results

MRS Results

ACC



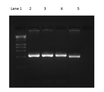
PCC



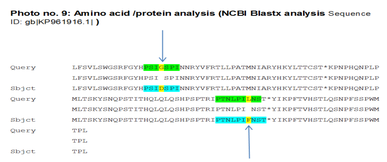
AREAS	METABOLITE	PPM	VALUE
RIGHT AMYGDALA			
	NAA	2	612
	CR	3	495
	CHOLINE	3.5	396
LEFT AMYGDALA			
	NAA	2	288
	CR	3	341
	CHOLINE	3.5	396
ACC			
	NAA	2	44
	CR	3	37
	CHOLINE	3.5	33
PCC			
	NAA	2	1944
	CR	3	825
	CHOLINE	3.5	891

mtDNA testing

mtDNA isolated from cheek cells of ASD pateints PCR amplification showing 320 bp product . Lane 1 Molecular Weight Marker (1kb), Lane 2-4 samples



mtDNA partial Genomic sequencing of sample no. showing Two point mutations.GenBank Accession no. KU534601



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