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Research Article

ANALYSIS OF SALIVARY COMPONENTS TO EVALUATE THE PATHOGENESIS OF AUTISM IN CHILDREN

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ABSTRACT

Objective: Autism is a neurodevelopmental disorder affecting the cognitive and social skills with severe implications on the affected individual's ability to lead productive and independent life. The present study is focused on evaluating the alteration in the levels of salivary components including antioxidants, in children with different grades of severity of autism.

Materials and Methods: Unstimulated whole saliva sample was collected from normal, and autistic children grouped as medium functioning autism (MFA) and low functioning autism (LFA) based on childhood autism rating scale score (n-20 in each group). Concentration of protein, cholesterol, thiocyanate (SCN^-), mucin, uric acid, lipid peroxides (LPO), reduced glutathione (GSH), α-amylase and antioxidant enzymes activity were determined in saliva.

Results: LFA group showed elevated levels (p=0.000) of protein, SCN^-, mucin, uric acid, α-amylase and LPO when compared to MFA group and normal children. Antioxidant enzymes, cholesterol and GSH levels were significantly decreased (p=0.000) in LFA than in MFA and normal children. Significant elevation in the levels of SCN^- (p=0.001) and mucin (p=0.004) was observed in LFA than in MFA. The electrophoretic pattern revealed that protein corresponding to 52-63 kD are significantly elevated, and 63-76 kD are decreased in autistic children. Western blot of salivary glutathione-S-transferase-2 (GST-2) showed decreased activity in LFA than in MFA and normal children.

Conclusion: The results showed that alteration in salivary components, including antioxidant enzymes, especially GST was proportional to the severity of autism, which can act as biological marker for diagnosing autism and also saliva can be considered as a non-invasive specimen to study the pathogenesis of autism like other biological specimen.

Keywords: Antioxidants, Autism, Glutathione-S-transferase-2, Low functioning autism, Medium functioning autism, Protein marker, Unstimulated saliva

INTRODUCTION

Autism is a behaviorally defined neurodevelopmental disorder in children predominantly in males between the age group of 2 and 10. In India, the awareness of autism is being created in many forms to make the parents understand the existence of autism. Data released from Centers for Disease Control in April 2012 placed the prevalence of autism at approximately 1 in 88 children. Truly, no data are available in India to provide an India specific prevalence of autism. The clinical picture of autism is modified by age and intellect but always encompasses the triad of impairments in speech and language, social cognition and imaginary thought [1]. This behaviorally defined disorder resulting in a number of diverse factors that may affect the way the brain functions causing delay or impairment in information processing by brain. Though many causative factors have been shown to be associated in the pathogenesis including environmental pollutants [2,3] vaccines containing thimerosal [4], maternal infection during pregnancy, and prenatal stress [5] hence is completely proved with consistent reports. No specific markers have been identified so far, which could be used for early intervention and for understanding the mechanism underlying the pathophysiology of autism. Until now, there is no specific laboratory test to diagnose autism.

Human saliva is an attractive body fluid for diagnosis of disease and prognosis because testing of saliva is simple, safe, low-cost and non-invasive. Oral fluid or whole saliva (WS) is an important complex chemical milieu of teeth and oral soft tissues, which maintains the homeostasis of the oral cavity. Various functions include lubrication, buffering, antimicrobial protection, and the maintenance of mucosal integrity. An impressive amount of about 1000-2000 ml/day are produced by parotid, submandibular and sublingual glands and consist mainly of water, essential electrolytes, glycoproteins, antimicrobial enzymes and numerous other important constituents like glucose, amylase and thiocyanate (SCN^-). The most abundant protein in saliva is amylase, accounting for approximately 20% of salivary proteins. Erosion and abrasion of the tooth surface is protected by salivary proline-rich proteins and mucins are important for pellicle formation. The histatin family of proteins has anti-fungal activity. Immunoglobulins and other non-immune factors maintain the antimicrobial effects. In addition, WS may be analyzed for the diagnosis of human systemic diseases since it can be readily collected and contains identifiable positive correlation with the serum constituents [6].

Preferences for soft and sweetened food make autistic children susceptible to caries [7]. Due to the lack of protective mechanism, children with autism may pose a threat to the oral health. A routine dental care is very difficult in autistic children due to a wide spectrum of medical, psychological and behavioral symptoms exhibited by them and hence there is a limitation in maintaining good oral hygiene [8].

Mental retardation is evident in approximately 70% of individuals with autism and many psychiatric disorders including autism are associated with increased oxidative stress (OS). Protection against reactive oxygen species (ROS) is provided by the antioxidants that are released locally at sites of inflammation by polymorphonuclear leukocytes and other cells. Free radicals or highly ROS are formed by exogenous chemicals or endogenous metabolic processes in the human body. These are

capable of oxidizing bio-molecules viz nucleic acids, proteins, lipids and DNA and can initiate different degenerative diseases like neurological disorders, cancer, emphysema, cirrhosis, atherosclerosis, arthritis, etc. Antioxidants are the compounds that terminate the attack of free radicals and thus reduce the risk of these disorders [9]. OS is generally determined by the measurement of total antioxidant capacity and also by estimating the products of peroxidation [10].

The aim of the present study is to evaluate whether the salivary components level are altered based on the severity of autism. The study also investigated whether the OS measured in saliva is proportional to different stages of autism.

MATERIALS AND METHODS

Reagents and chemicals

Tris, glycine, acrylamide, methylene bis-acrylamide, sodium dodecyl sulfate (SDS), ammonium per sulphate, N, N, N', N' tetramethylethylenediamine, a full range molecular weight protein marker, phast gel blue R-350 tablets (coomassie stain) were purchased from GE healthcare Bio-Sciences Limited, Kowloon, Hong Kong. Monoclonal anti-glutathione-S-transferase (GST) clone GST-2 in mouse (1:1000 in tris buffered saline [TBS] containing 1% bovine serum albumin [BSA]) - primary antibody, and other chemicals malondialdehyde (MDA), thiobarbituric acid (TBA), dinitrosalicylic acid, trichloro acetic acid, cholesterol, BSA, uric acid, glacial acetic acid (aldehyde free), sulphuric acid (Analar), sodium tungstate, alcian blue, SCN^-, ethylene diamine tetra acetic acid, phenazine methosulphate, nitroblue tetrazollium, nicotinamide adenine dinucleotide, glutathione (GSH) reduced, dithionitrobenzoic acid were purchased from Sigma Aldrich Chemicals Private Limited, Bangalore, India. Rabbit anti-mouse immunoglobulin G coupled with peroxidase (1:1000 in TBS)-secondary antibody was purchased from Genei, Bangalore, India. All the other solvents and chemicals used were of analytical grade.

Subject selection

The Correspondents of the special schools Maruti Seva, Bala Vihar, AIKYA and three other training centres at Chennai, Tamil Nadu, India, were approached with the help of Social Welfare Board, Government of Tamil Nadu and obtained prior permission to collect saliva samples from autistic children currently attending these schools.

Autism was assessed using a checklist of autism in toddlers. After getting consent from the parents, autistic children of both sexes (n=20) were selected with the help of a pediatrician and were grouped according to the method adopted from childhood autism rating scale as medium functioning autism (MFA) and low functioning autism (LFA). Children in the age group of 4-12 years were selected, and the boys and girls ratio was 4:1. Saliva samples were also collected from age and sex matched healthy children (n=20) and analyzed for comparison. The study protocol has been approved by the Institutional Ethics Committee, Madras Medical College and Hospital, Chennai, Tamil Nadu, India and EC No. 39062012.

Collection of saliva sample

Oral and periodontal examination was done for each individual and any child with symptoms and signs of any active oral inflammation and advanced periodontitis was excluded. Unstimulated WS sample was collected using sterile disposable cups and centrifuged at 1000 rpm for 10 minutes to remove unwanted debris. The clinical history of the children involved in the study is presented in Table 1.

Study protocol

Protein content in the saliva sample was estimated by the method of Bradford [11] by using BSA as standard. Method of Zak's [12] was used for cholesterol content determination in saliva, and its concentration was expressed as mg/dl saliva. Uric acid was estimated by the method of Caraway [13] and expressed as mg/dl saliva. Mucin content in saliva was determined spectrophotometrically at 605 nm [14,15], and expressed as mg alcian blue equivalent/mg protein. Estimation of salivary SCN^- was done by the method of Bowler [16], and the color intensity was read at 460 nm and expressed as mg of SCN^-/dl saliva. The activity of salivary amylase one of the important enzymes in saliva was determined by the method of Bernfeld [17] and expressed as μM maltose formed/minutes/mg protein. MDA concentration (nM of MDA/mg protein) in the saliva sample was examined by Draper and Hadley method [18]. The level of reduced GSH (μg/mg protein) [19], activity of enzymatic antioxidants superoxide dismutase (SOD) (units/mg protein) [20], catalase (CAT) (units/mg protein) [21], glutathione peroxidase (GPx) (nM of GSH consumed/minutes/mg protein) [22] and GST (units/g protein) [23] were also determined. Salivary proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (10% separating gel, 5% stacking gel) according to the method of Laemmli [24]. The Western blot analysis for GST-2 was carried out by the method of Towbin [25]. Densitometric analysis of protein and GST-2 were carried out by using Digital Gel Documentation and Analysis software (DGelDAS - advanced) and the values are shown in the form of histogram.

Table 1: Clinical history of autistic and normal children taken for the study

Characteristic	Autistic children	Normal children
Number of children	40 (20 in each group)	20
CARS value (15-60)	LFA=46-60 MFA=31-45	<10
Male/female ratio	27/13	12/8
Age in years	4-12	4-12
Children with gluten sensitivity	LFA=9/20 MFA=5/20	
Children with special talents (dancing, humming, drawing, jumble picture)	LFA=6/20 MFA=8/20	14/20
Number of children on antibiotic treatment	LFA=3/20 MFA=2/20	
Number of children with gastrointestinal problems	11/40	
Number of parents given their cooperation	37/40	20
Number of parents appreciated the study	38/40	20

CARS: Childhood autism rating scale, MFA: Medium functioning autism, LFA: Low functioning autism

Statistical analysis

The results are presented as mean±standard deviations. The groups were compared with one-way ANOVA with *post-hoc* Bonferroni test and the $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Autism is a severely affecting lifelong neurodevelopmental disorder that typically occurs in the first 3 years of life. Biological samples can be collected from the affected children only through non-invasive procedure without affecting/harming the children. In this regard urine, saliva, nail and hair samples are preferred for analysis.

The present study investigated the biochemical alterations in the saliva of autistic children. If any metabolite is detected abnormally in saliva, it may be used as a biomarker for the diagnosis of autism. The clinical history of autistic children presented in Table 1 revealed that most of the children are at the age group of 4-12 years with a male and female ratio of 4:1. Some of the autistic children have shown gastrointestinal complication, and few of them were under antibiotic treatment for infections.

The tests conducted in saliva samples collected from autistic children revealed the following results: Table 2 shows the levels of total protein,

cholesterol and SCN^- in saliva collected from normal and autistic children. The result shows that the total protein level is significantly increased in MFA (p=0.002) and LFA (p=0.000) groups when compared with that of age and sex matched normal children. When the comparison was made between LFA and MFA, there was a significant increase (p=0.000) in LFA group of autistic children.

Salivary proteins are extremely important in the homeostasis of the oral tissues and protection from infectious agents. Currently, more than 1166 proteins have been totally identified, among this 914 from parotid and 917 from sublingual glands [26]. Human saliva contains various proteins with a molecular weight ranging from <20 to 90 kD [27].

The protein biomarkers can give valuable information about the diagnosis and the prevalence of autism. The present investigation shows that there is an abnormal increase in proteins with a molecular weight ranging from 52 to 63 kD. SDS-PAGE analysis (Fig. 1a and b) clearly shows that there is the elevation in the level of certain proteins that may act as the diagnostic markers for autism. The electrophoretogram also shows that the proteins with 63-76 kD are decreased in autism samples. The exact protein which was altered in saliva of autistic children has to be studied clearly by completely analyzing the salivary protein. The results consistently show that there are both abnormal and subnormal proteins present in saliva collected from autistic children. However, the exact protein or the peptide can be detected by using advanced techniques such as proteome analysis.

The lipids present in saliva are mostly glandular origin, and some are believed to diffuse directly from serum. Cholesterol in excess can be harmful but is crucial for the proper development and maintenance of the brain. Mental dysfunction can be caused due to lower the level of cholesterol during the early period of growth. Cholesterol is also essential for normal development of embryos and fetuses, and in central nervous system for both synaptic fusion and myelin membrane growth [28]. Always there is a correlation between serum and salivary cholesterol, and the abnormal pathological condition is reflected in the level of both saliva and serum cholesterol. We found that there is a significant decrease (p=0.000) in the level of salivary cholesterol in LFA and MFA group when compared to the normal group. When LFA was compared with MFA, a significant decrease (p=0.000) was observed in LFA group of children (Table 2).

Overall there was low cholesterol level observed in the autistic group when compared to the normal group. Cholesterol is delivered from mother to fetus in the form of cholesterol sulfate which is amphiphilic in nature and would cross the placental barrier much more readily than cholesterol. Low level of cholesterol in autism might be due to an insufficient supply of cholesterol sulfate to the fetus from the mother during the gestation period although elevated level of cholesterol is seen in the mother [28]. It is also proved that Smith-Lemli-Opitz Syndrome, a genetic condition of impaired cholesterol biosynthesis is associated with autism [29].

The level of SCN^- in both the group of autistic children (Table 2) showed a significant increase (p=0.000) when compared to that of normal healthy children. When the comparison was made between LFA and MFA, a significant increase (p=0.001) was observed in LFA group. SCN^- is an important physiological anion involved in innate defense of mucosal surfaces. It is a metabolite of cyanide and the product of detoxification of compounds containing cyanide through a reaction catalyzed by the enzyme rhodanese [30,31]. Everyone has SCN^- in their saliva, which means that we are exposed to cyanide sources throughout our daily lives. There are indispensable parts of host defense system that act as a substrate for lactoperoxidase that oxidizes airway surface lipid SCN^- thereby generating antimicrobial agent hypo-SCN^- [32,33].

It has been reported that SCN^- level is a marker of nicotine toxicity in cigarette smokers [34]. The elevated SCN^- level in autism may be attributed to low rhodanese activity as reported by Waring and Klovrza [35] who stated that the raised ratio of thiosulfate and SCN^- may be due to reduced rhodanese activity. The level of alteration is proportional to the severity of autism.

Uric acid, one of the nitrogenous compounds of urine, is found in serum, blood and saliva. Uric acid is the main final product of purine metabolism and its determination in urine is a powerful indicator of metabolic diseases [36]. The salivary uric acid is reported to be elevated in patients with metabolic syndrome and correlated with several cardiometabolic risk factors including blood pressure, triglyceride levels, high-density lipoprotein and fasting blood glucose.

Table 2: Levels of protein, cholesterol and SCN^- in saliva of normal, LFA and MFA group of autistic children

Subjects	Protein (mg/ml)	Cholesterol (mg/dl)	Thiocyanate (mg/dl)
Normal (n=20)	1.85±0.24	7.54±0.84	2.10±0.32
MFA (n=20)	2.19±0.33[ł]	5.83±0.60*	2.57±0.33*
LFA (n=20)	2.60±0.24*,*	3.55±0.50*,*	2.96±0.33*,[#]

Results are expressed as mean±standard deviation. Statistical significance was calculated by comparing normal versus MFA, normal versus LFA and MFA versus LFA. Variables were examined using one-way ANOVA and symbols represent significant differences from *post-hoc* Bonferroni test. *p=0.000 (Control vs. MFA and LFA, MFA vs. LFA), [ł]p=0.002 (Control vs. MFA), [#]p=0.001 (MFA vs. LFA). MFA: Medium functioning autism, LFA: Low functioning autism, SCN^-: Thiocyanate

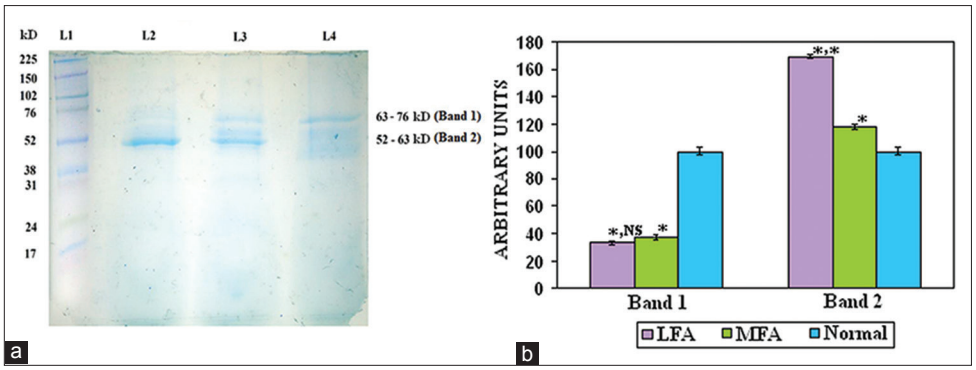

Fig. 1: (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of proteins in saliva of low functioning autism, medium functioning autism and age-sex matched normal children. L1-Molecular weight of protein marker, L2-Low functioning autism, L3-Medium functioning autism, L4-Nomal (b) Densitometry analysis of proteins (Bands 1 and 2) in the saliva of low functioning autism (LFA), medium functioning autism (MFA) and age-sex matched normal children. Values are expressed as mean±standard deviation for n=3. Statistically significant variations are expressed as *p=0.000 (Control vs. MFA and LFA, MFA vs. LFA), NS-Non-significant (MFA vs. LFA)

Table 3: Concentration of mucin, uric acid and activity of amylase in saliva of LFA and MFA group of autistic children compared with age and sex matched normal children

Subjects	Mucin (mg alcian blue/ mg protein)	Uric acid (mg/dl)	Amylase (µM maltose/minutes/ mg protein)
Normal (n=20)	2.79±0.29	1.99±0.46	0.066±0.013
MFA (n=20)	3.86±0.46*	2.75±0.51$^{\#}$	0.082±0.019$^{\$}$
LFA (n=20)	4.30±0.47*,$^{@}$	4.13±0.77*,*	0.127±0.017*,*

Results are expressed as mean±standard deviation. Statistical significance was calculated by comparing normal versus MFA, normal versus LFA and MFA versus LFA. Variables were examined using one-way ANOVA and symbols represent significant differences from *post-hoc* Bonferroni test. *p=0.000 (Control vs. MFA and LFA, MFA vs. LFA), $^{\#}$p=0.001, $^{\$}$p=0.011 (Control vs. MFA), $^{@}$p=0.004 (MFA vs. LFA). MFA: Medium functioning autism, LFA: Low functioning autism

Table 4: Activity levels of SOD, CAT and GST in saliva from normal, LFA and MFA group of autistic children

Subjects	SOD (units/ mg protein)	Catalase (units/mg protein)	GST (units/ g protein)
Normal (n=20)	4.70±0.50	107.81±12.36	0.86±0.11
MFA (n=20)	3.84±0.66*	88.93±10.42*	0.64±0.10*
LFA (n=20)	2.54±0.55*,*	71.87±15.87*,*	0.47±0.12*,*

Results are expressed as mean±standard deviation. Statistical significance was calculated by comparing normal versus MFA, normal versus LFA and MFA versus LFA. Variables were examined using one-way ANOVA and symbols represent significant differences from *post-hoc* Bonferroni test. *p=0.000 (Control vs. MFA and LFA, MFA vs. LFA). MFA: Medium functioning autism, LFA: Low functioning autism, SOD: Superoxide dismutase, GST: Glutathione-S-transferase, CAT: Catalase

The level of uric acid in saliva shows a highly significant elevation (p=0.000) in LFA group of autistic children when compared to that of normal children and MFA (Table 3). When the comparison is made between MFA and normal children the uric acid level showed a significant elevation (p=0.001). Uric acid has been considered as a marker of OS and may have a potential therapeutic role as an antioxidant [37]. On the other hand, like other strong reducing substances such as ascorbate, uric acid can also act as a pro-oxidant, particularly at elevated level [38]. Elevated level of uric acid might contribute to OS in autistic children.

There is a significant increase in the level of mucin in LFA and MFA group of children (p=0.000) when compared to normal healthy children. As shown in Table 3 significant increase (p=0.004) in salivary mucin content was observed in LFA when compared with MFA. Mucins are high molecular weight glycoproteins with a protein backbone and high carbohydrate content. Secretary salivary mucins are synthesized and released by the submandibular and sublingual gland and small glands of the oral mucosa. Currently, two main groups of salivary mucins are identified as MG1 (>1000 kD) and MG2 (200-300 kD) [39].

Salivary mucins are well-recognized as an important factor in the preservation of oral cavity health. These large glycoproteins play major role in the formation of protective coat covering the tooth enamel and oral mucosa, which act as a dynamic functional barrier capable of modulating the harmful effects of oral environment, and are of significance to the process occurring with the epithelial perimeter of mucosal defense. It is well known that low molecular weight form of mucin rich in sialic acid, aggregates bacteria. It has also been shown that salivary mucins actively participate in the modulation of oral mucosal calcium channel activity through the inhibition of protein tyrosine phosphorylation [40]. It is surprising to know that the salivary mucin level is elevated in autistic children. In this regard, it is essential to study the nature of mucin and its carbohydrate content which are important in the defense activity of the mucins.

The α-amylase activity was found to be significantly increased in MFA (p=0.011) and LFA (p=0.000) group of autistic children when compared to that of normal children. LFA, when compared with MFA, showed a significant increase (p=0.000) in the activity of salivary α-amylase (Table 3). Human salivary α-amylase is predominantly produced from the parotid and submandibular acinar cells [41].

Salivary α-amylase activity is an increasingly investigated biomarker for the activation of the autonomic nervous system. Hence, the high activity of α-amylase in autistic children shows that there is hyperstimulation of autonomic nerves, which may be associated with hyperactive nature of autistic children. The information collected from the parents of study subjects revealed that all the autistic children are hyperactive in nature. From this study, it is clear that the salivary α-amylase might be a new biomarker for autism in the future.

Salivary α-amylase has been used as a marker of the adrenergic component of the stress response [42]. Measurement of α-amylase activity in the saliva of children has been used as an indicator of the aggressive response involving sympathetic nervous system [41,43,44]. However, the isoenzymic forms of amylase need to be investigated in autism.

Antioxidants are present in all body fluids and tissues which protect the cells from endogenously-formed free radicals, collectively known as ROS. OS represents the imbalance between the production of highly ROS, including reactive nitrogen species and the antioxidant defense system [45]. OS can result in DNA damage, including oxidation of lipids, proteins and nucleic acids. Saliva is rich in antioxidants which includes enzymatic activities such as SOD, CAT, GPx, GST and non-enzymatic antioxidants including vitamins A, E, C, melatonin and reduced GSH [46,47].

The activity levels of salivary antioxidant enzymes are presented in Table 4. SOD catalyses the dismutation of superoxide anions (O_2^-) into hydrogen peroxide and molecular oxygen. SOD level in saliva was found to be significantly low (p=0.000) in both MFA and LFA group of autistic children when compared to normal children and when LFA and MFA comparison was done, it showed a significant decrease (p=0.000) in LFA. This clearly explains the inverse correlation between antioxidants level and the severity of autism. Decreased level of salivary SOD was reported in oral leukoplakia and oral cancer patients. Plasma level of SOD was reported to be low in autistic patients [48].

CAT is a prime enzyme involved in detoxifying H_2O_2 by hydrolyzing hydrogen peroxide into water and O_2 and is widely used as an acceptable OS marker. Decreased activity of CAT has been shown in the plasma of autistic patients [49]. In the present study, the activity of CAT decreased significantly (p=0.000) in the saliva of autistic children of LFA and MFA groups when compared with that of normal healthy children. While comparing the MFA and LFA group, the activity of CAT also showed a significant decrease (p=0.000) in LFA group.

The level of lipid peroxidation (LPO) products thiobarbituric acid reacting substances (TBARS) is shown in Table 5. The level of TBARS is significantly increased in LFA (p=0.000) when compared to that of MFA and normal children. LPO can be defined as the oxidative deterioration of lipids containing carbon-carbon double bonds [50]. MDA is an end product of the peroxidation of polyunsaturated fatty acids and related esters which is used as a marker of LPO.

When MFA, LFA was compared with the age and sex-matched normal subjects the salivary GSH was found to be significantly low (p=0.000) in LFA group of autistic than the other groups which show their vulnerability to severe OS (Table 5). Children are more vulnerable than adults to OS because of the low availability of GSH from conception through infancy [51,52], and the young brain has an immature antioxidant system that may be unable to get protected against the harmful effects of ROS [53]. It has been shown that the

plasma concentration of GSH is lower, and the ratio of oxidized GSH to reduced GSH is higher in autistic children when compared to healthy controls [54]. In general, salivary GSH level has been reported to be decreased in periodontal diseases [55] and diabetic patients [56]. The depletion of GSH may be due to its active consumption for the neutralization of free radicals.

GPx is a predominant defense enzyme participating in the detoxification of hydrogen peroxide. GPx is an enzyme containing selenium as cofactor and catalyses the breakdown of H_2O_2 and organic hydroperoxides. When this enzyme is dispensable, it causes OS in autistic children [57]. Table 5 shows the activity of GPx in normal and autistic children. The activity of GPx was found to be significantly decreased (p=0.000) in MFA and LFA group children when compared to the normal individuals, whereas a significant decrease (p=0.014) was observed in LFA when the comparison was made between MFA and LFA. The results consistently show that the depleted level of these defensive enzymes might have contributed for OS in autistic children especially in LFA group of autistic children.

GST is a family of multifunctional enzymes that plays an important role in the detoxification of xenobiotics including carcinogens. Activity of GST in saliva was found to be significantly decreased (p=0.000) in both the groups of autistic children when compared to normal children (Table 4). GST plays a critical role in cellular protection against OS and toxic foreign chemicals. GST also detoxifies a variety of electrophilic compounds including oxidized lipid, DNA and catechol products generated by ROS [58]. Lower activity of GST in plasma has been reported in autistic children [59].

Table 5: Levels of LPO, GSH and activity of GPx in saliva of normal, LFA and MFA group of autistic children

Subjects	Lipid peroxides (nM of MDA/mg protein)	Reduced GSH (µg/mg protein)	GPx (nM of GSH consumed/ minutes/mg protein)
Normal (n=20)	0.45±0.14	62.54±13.47	6.21±1.10
MFA (n=20)	0.65±0.14[£]	48.15±9.53*	4.15±0.98*
LFA (n=20)	0.99±0.34*,*	32.04±9.91*,*	3.19±0.99*,[a]

Results are expressed as mean±standard deviation. Statistical significance was calculated by comparing normal versus MFA, normal versus LFA and MFA versus LFA. Variables were examined using one-way ANOVA and symbols represent significant differences from *post-hoc* Bonferroni test. *p=0.000 (Control vs. MFA and LFA, MFA vs. LFA), [£]p=0.023 (Control vs. MFA), [a]p=0.014 (MFA vs. LFA). MFA: Medium functioning autism, LFA: Low functioning autism, GSH: Reduced glutathione, MDA: Malondialdehyde, GPx: Glutathione peroxidase, GSH: Glutathione, LPO: Lipid peroxides

Western blotting analysis showed decreased activity of salivary GST-2 in autistic children when compared to normal children (Fig. 2a and b). GST's are phase II metabolic isozymes, which known to play a key role in the detoxification and reduction of ROS. GST's are able to conjugate GSH to the toxic reactive compounds which are generated during the oxidation of membranes [58]. GST's are classified under three superfamilies, cytosolic, mitochondrial and microsomal. Cytosolic GST's are divided into 13 classes based upon their structure: Alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, sigma, tau, phi, and omega. Mitochondrial GSTs are in class kappa. The microsomal superfamily consists of GST subgroups designated I-IV. Human cytosolic GST's belong to the alpha, zeta, theta, mu, pi, sigma, and omega classes, while six isozymes belonging to classes I, II, and IV of the microsomal superfamily are known to exist. The present investigation shows that GST-2 is significantly decreased (p=0.000) in LFA when compared to normal children, and this might be due to lack of the substrate GSH and hence may be accounted for the reduced detoxifying capacity of GST in autistic children. The activity of the enzyme may possibly be affected, if there is any genetic polymorphism in GST. Some supportive genetic evidence-related to GST M1, GST P1 in autistic individuals also suggest increased the risk of OS in autistic children [60,61].

CONCLUSION

The current study involving saliva, a non-invasive biological specimen shows that there is an alteration in the level of proteins with molecular weight ranging between 52 and 76 kD. The results also suggest that there is significant alteration in the level of salivary components and antioxidants, especially GST with a decreased activity in LFA group of autistic children, which reveals that OS is involved in the pathogenesis of autism. Altered GST activity in saliva of autistic children may be due to a polymorphism in the GST genes, which has to be studied much more clearly so that it could act as biological protein marker in diagnosing autism in the future. However, the exact protein which is differentially expressed in saliva can also be identified by proteomic analysis.

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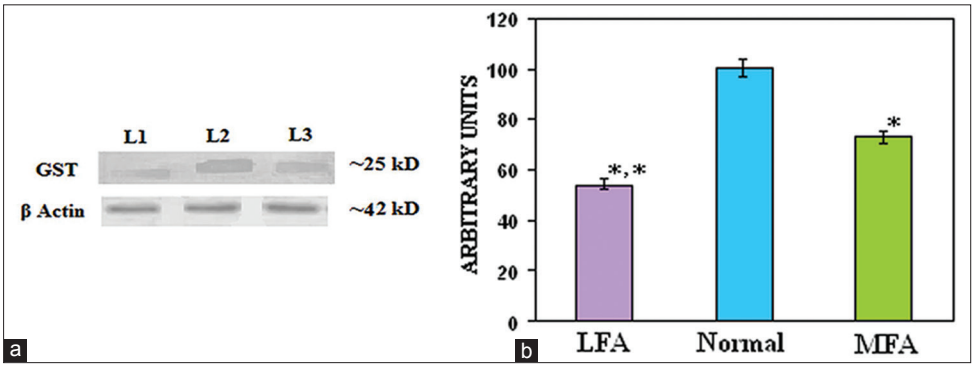

Fig. 2: (a) Western blotting of salivary glutathione S transferase in low functioning autism (LFA), medium functioning autism (MFA) and age-sex matched normal children. L1-LFA, L2- Normal, L3-MFA (b) Densitometry analysis of salivary glutathione S transferase in low functioning autism (LFA), medium functioning autism (MFA) and age-sex matched normal children. Values are expressed as mean±standard deviation for n=3. Statistically significant variations are expressed as *p=0.000 (Control vs. MFA and LFA, MFA vs. LFA)

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