Research Article

Urine total glutathione levels as a potential marker of increased oxidative stress in autism

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Abstract

Objective: Glutathione supplement has been prescribed as a therapy in autism often without proper glutathione quantification in the body. Though, increased speech and increased imaginary play is reported, increased screaming and loosening of stools have been reported as side effects. In this scenario, for an effective management of glutathione administration, we suggest a simple and reliable means of glutathione quantification before its administration. This study pertains to the quantification of total glutathione in autistic children using noninvasively taken urine as sample. **Materials & Methods:** Urine samples were collected from ten autistic and nine neurotypical children of similar age and sex. Total glutathione was quantified using enzymatic recycling method. **Results**: Mean levels of total glutathione in autistic children were significantly lower than the neurotypical children (p-value <0.001). **Conclusion:** We conclude that total glutathione is lower in autistic children and noninvasively collected urine can be used for glutathione quantification before administering glutathione as a therapeutic measure.

Key words: Autism, Enzymatic recycling, Glutathione assay.

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Introduction

Autism is now a key public health issue. The burgeoning number of autism cases (1 in 88) [1] is quite alarming. Yet to find the exact etiology, both genetic and environmental factors have been attributed for autism etiopathogenesis, apart from immunological alterations and oxidative stress [2, 3, and 4]. Oxidative stress is considered as an imbalance between the production of reactive oxygen species and anti oxidant defenses [5]. Association of glutathione depletion has been proposed in many neurodegenerative disorders like Alzheimer's and Parkinson's diseases [6]. Studies also point out that oxidative stress by glutathione depletion causes severe hypertension in animal model [7] which cannot be otherwise in humans. Anecdotal reports say that glutathione supplement administration does benefit autistic patients. As a result, there are physicians who prescribe glutathione supplement even without checking the levels in the patient's body. A blind administration of this supplement can sometimes harm the person concerned. In certain subtypes of autism, the candidate could be having optimal levels of glutathione in the body and the administration of the supplement can do no good.

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Having comprehended the paramount importance of glutathione as an antioxidant and as an agent of minimizing the oxidative stress, its prospective role in exacerbation of autistic symptoms is worth studying. Random studies across the labs have reported that autistic candidates have lower levels of glutathione in their body which need further extensive validation using different subgroups. Most times, such studies prove to be laborious and costly. Invasively taken samples such as blood or tissues, though effectively

record the glutathione levels, the added trauma of the patients, parents and their care takers involved in the procedure make it a non approachable method. In this scenario, we propose a pilot study in assessing the total glutathione in children using noninvasively collected urine as an efficient source for total glutathione quantification. Ten autistic candidates and equal number of sex matched and age matched neurotypical children were enrolled for the study.

The total glutathione was estimated using enzymatic recycling method. Total glutathione was successfully quantified in urine. The total glutathione was found to be significantly lower in autistic (GA) group (17.48 ±12.35) than neurotypical (GN) group (87.30 ± 39.75), ($p < 0.001$) suggesting the role oxidative stress in exacerbation of autism.

The Total Glutathione Assay utilizes a carefully optimized enzymatic recycling method for the quantification of glutathione. Glutathione Reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). The sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB) that absorbs at 405 or 414 nm. The rate of TNB production is directly proportional to the concentration of glutathione in the sample. The measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of glutathione in the sample.

Materials & Methods

Subjects and Urine sample collection- Ten autistic and nine neurotypical age and sex matched children were involved in the study. The test candidates were diagnosed with autism by the experts in the field based on CARS scores. Children with a CARS score above 30 were identified and incorporated into the study with informed consent. Urine was collected in sterile containers and immediately four volumes of ice-cold 5% (w/v) Metaphosporic acid were added. After mixing it thoroughly, transferred into ice was transported to the lab and was frozen in -80^0 C for further use.

Total Glutathione assay- Total glutathione assay was performed using Glutathione (total), detection kit by Enzo Life sciences following the manufacturer's protocol. Glutathione standard curve was set up. 50 µl of 1 X Assay Buffer was added to all the wells in rows A through E, columns 1,2, and 3 of a microtiter plate. 50 µL of the 4 µM oxidized glutathione (GSSG) was to wells A1, A2, and A3 using a multichannel pipette. 50 µL from wells A1, A2 and A3 was transferred to wells B1, B2, and B3 and in a similar fashion to row C and D discarding the last 50 µL so as to have the oxidized glutathione content (GSSG) 100pmoles/well, 50 pmoles/well, 25 pmoles/well, and 12.5 pmoles/ well respectively. 50 µL of urine sample was added to the wells in columns 4 to 12. Using a multichannel pipette, 150 µL of freshly prepared reaction Mix was added to each well. The absorbance in the wells at 405 nm was recorded using a plate reader at 1 minute intervals over a time of 10 minute period.

Determination of Total Glutathione Concentration- Average of the triplicate absorbance readings for each standard, experimental sample, and blank at each time point was taken. The average of each standard, experimental sample, and background absorbance (A45nm) versus incubation time was plotted and the slope was determined from the linear portion of each curve. Net slopes of the GSSG standards versus pmoles of Glutathione were plotted. The net slopes of the experimental samples with those of the standard curve were compared in order to determine the pmoles of GSSG for each experimental sample.

Statistical Analysis- Statistical analysis was done using IBM SPSS Statistics 20 Windows (SPSS Inc., Chicago, USA). For all the continuous variables, the results are given in Mean ± SD and for categorical variables as percentage. To compare the means of continuous variables between two groups, Student's independent samples t test was performed. Fisher's Exact test was used for finding the association between two categorical variables (study group and sex). A p-value <0.05 was considered statistically significant. All tests of statistical significance were two tailed.

Results

The following tables enumerate the total glutathione in picograms in neurotypical and autistic children. A substantial difference can be observed between the two groups with higher levels of total glutathione in neurotypical children suggesting a better overall health in terms of immunity, detoxification, proper synthesis of proteins, DNA synthesis etc. The mean value obtained from the normal participants is much higher than the autistic candidates.

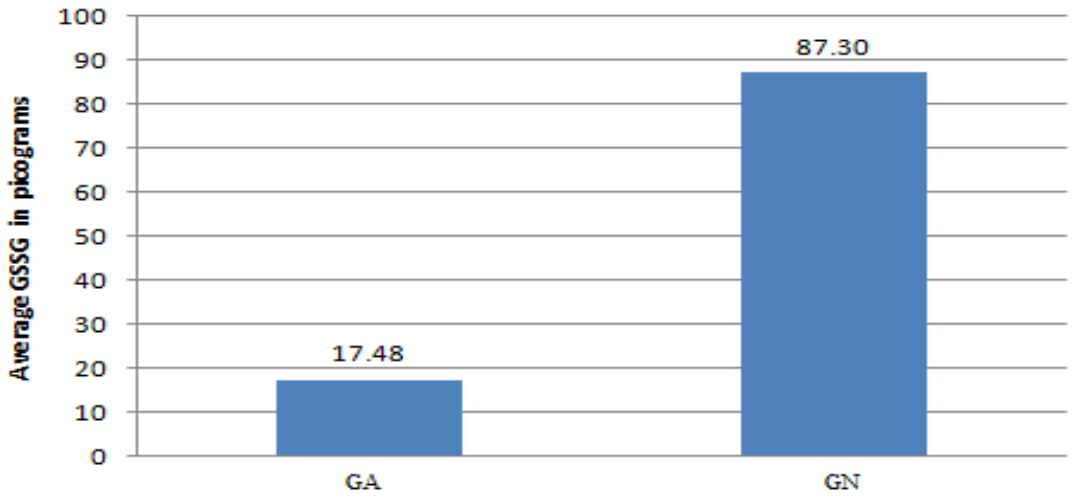

Figure-1: Average Total Glutathione in Picograms (GA: Autistic, GN: Normal)

Table-1: Total glutathione in Normal neurotypical children.

Normal	Age	Sex	Total Gluathione in Picogram
GN1	8	M	87.592
GN2	12	M	45
GN3	9	F	138.907
GN4	9	M	125.855
GN5	11	M	119.828
GN6	9	M	121.973
GN7	7	M	61.276
GN8	7	F	77.723
GN9	11	F	79.697

Table-2: Total glutathione in Autistic children.

Autistic	Age	Sex	Total Gluathione in Picogram
GA1	8	M	9.302
GA2	12	M	27.065
GA3	8	F	20.486
GA4	9	M	29.697
GA5	11	M	4.697
GA6	10	M	2.065
GA7	7	M	6.013
GA8	5	F	36.276
GA 9	11	F	21.802
GA10	8	M	15.223

Table-3: p- values.

	Group	Number	Mean	Std. Deviation	p-value
GSSG in Picogram	GA	9	17.489222	12.3568350	<0.001
	GN	10	87.307400	39.7510307	

Discussion

Prevalence of autism has significantly increased in recent years. It shows an increase from 4.2 per 1,000 in 1996 to 15.5 per 1,000 in 2010 by 119.4% in U.S children from 2000 to 2010[8]. Considered as the fastest-growing developmental disability, 1 in 88 children are found to be autistic in U.S.

The scenario is not far different with proportional propensity being reflected in other parts of the world. Metabolic abnormalities are implicated [9] in several neurodevelopmental disorders including autism. Oxidative damage by free radicals has been a concern in various diseases such as cancer, atherosclerosis, inflammation, and certain other neurodegenerative disorders [10, 11].

Glutathione is known as body's chief detoxifier. Elevated Glutathione levels have generally been associated with overall well being among the elderly [12]. The present study also establishes the role of glutathione in this regard.

A recent study carried out using conventionally raised (CONV-R) and germ-free (GF) mice by Mardinoglu et al., suggests that the gut microbiota modulates host amino acid and glutathione metabolism in mice[13].

It is a known fact that gut is inhabited by trillions of bacteria that have profound effect on host physiology [14]. Valicenti-McDermott [15], Horvath [16] and Taylor[17] are among a few scientists who have detailed the role of Gut/Intestine issues in the exacerbation of autism. Analysis has also shown to have higher rates of diarrhea, constipation and abdominal pain in autistic children [18] compared to normal children.

Anecdotal reports and other evidences suggest potential links between dietary, metabolic, infective, and gastrointestinal factors in exacerbation of autistic behavior. Corroborating this hypothesis, it is also observed that propionic acid (PPA) and its related short-chain fatty acids (SCFAs) are able to have extensive effects on gut, brain, behavior etc.

SCFA's are produced as fermentation products from Autism Spectrum Disorder associated bacteria like Clostridia, Bacteriodetes, Desulfovibrio etc. PPA and SCFAs when administered intraventricularly in rats were found to induce abnormal motor movements, repetitive interests, electrographic changes, cognitive deficits, and impaired social interactions [19]. Thus, the low glutathione level evident from our assay becomes more significant as it can be attributed by the presence of aberrant microorganisms implicated in autism.

Low glutathione levels are correlated with aberrant dopamine manufacture which can lead to impaired cognitive functions [20]. As cognitive dysfunction is observed in autism, glutathione administration and thereby fixing the dopamine manufacture could lead to improved cognitive functions in autism to a certain extent. Clinical trials of glutathione supplementation in autism spectrum disorders have shown to improve transsulfuration metabolites [21]. But there are anecdotal reports that glutathione supplement can cause abdominal pain and loose bowel movements.

Therefore quantification of total glutathione can be a diagnostic tool for ASD's in order to fix problems related with low glutathione levels implicated in autism using simple and effective assays as we have implemented which can be routinely undertaken through enzymatic recycling of urine. The children could then be recommended different doses depending on the levels of glutathione than blindly suggesting tailor made quantities of total glutathione.

Conclusion

Autistic children are tend to have lesser total glutathione in their body and enzymatic recycling estimation of total glutathione in urine, noninvasively collected, is an easy and efficient method for glutathione quantification.

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