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Systemic Oxidative Stress, as Measured by Urinary Allantoin and F₂-isoprostanes, Is Not Increased in Down Syndrome

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Abstract

Oxidative stress has been implicated in Down syndrome (DS) pathology. This study compares DS individuals and controls on their urinary levels of allantoin and 2,3-dinor-iPF₂α-III; these biomarkers have been previously validated in a clinical model of oxidative stress. Urine samples were collected from 48 individuals with DS and 130 controls. Biomarkers were assayed by ultra-performance liquid chromatography-tandem mass spectrometry, normalized by urinary creatinine concentration. After adjusting for age and gender, mean allantoin levels were lower among DS individuals *versus* controls ($p = 0.04$). The adjusted mean levels of 2,3-dinor-iPF₂α-III were similar in DS individuals and controls ($p = 0.7$). Our results do not support the hypothesis that DS individuals have chronic systemic oxidative stress.

Keywords

Down syndrome; biomarker; urine; tandem mass spectrometry

Introduction

Down Syndrome (DS) results from chromosomal aneuploidy (trisomy 21) and is a common genetic disorder recognizable at birth. Individuals with DS are characterized by specific cardiac and gastrointestinal congenital malformations, increased risk for leukemias, a particular cognitive profile, and early onset of Alzheimer's disease (1). The etiology of the clinical manifestations in DS is not fully understood. There is accumulating evidence that individuals with DS have increased susceptibility to oxidative stress (2). The increased copy number of Cu²⁺/Zn²⁺ superoxide dismutase (SOD-1) due to trisomy 21 (the gene for SOD-1 is localized on chromosome 21) compared to catalase (CAT) and glutathione peroxidase (GPX) may result in a high levels of hydrogen peroxide production by SOD-1, that CAT and GPX cannot neutralize, leading to high levels of reactive oxygen species (ROS) (3). If this

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were true, the antioxidant treatments would then hold the most promise to ameliorate DS-associated morbidities (4). However, the clinical studies in DS individuals have not demonstrated positive effects of antioxidants on cognitive functions (5-7). Without questioning the premise that oxidative stress underlies DS-associated pathologies, we instead hypothesized that the major gap in these studies is the absence of clinical monitoring of the direct effect of antioxidants on the levels of oxidative damage in individuals with DS using oxidative damage biomarkers. Without such monitoring, it will be impossible to conclude whether an antioxidant-specific regimen (dose and combination of antioxidant compounds) could decrease oxidative stress. Furthermore, it would not be clear whether antioxidant treatment is an effective and mechanistically relevant approach to alleviating DS-associated morbidities. With this in mind, we chose two biomarkers of oxidative stress that have been previously validated in a clinical (8) and/or animal models (9) as being sensitive indicators of systemic oxidative stress (allantoin and 2,3-dinor-iPF_{2α}-III). We compared the levels of these biomarkers in individuals with DS *versus* individuals without DS. This study was envisioned as the first step to incorporating these biomarkers into clinical studies which use antioxidant interventions for treatment of DS-associated morbidities.

Subjects and Methods

Study Participants

Forty-eight individuals with DS were recruited from the Duke Down Syndrome Programs at their regular clinic visits. All DS individuals provided signed informed consent. When applicable, legal guardians were consented, with assent given by the participants. For the controls, anonymous urine samples were collected. Flyers inviting volunteers to participate were placed at different Duke locations. Volunteers were asked for an anonymous urine sample. The flyers indicated where kits for urine collection were placed and where in a refrigerator these samples should be left. Volunteers were asked only to indicate their gender and age on the sample label. Every morning the refrigerator was checked for new samples. By definition, no consent form could be obtained for an anonymous sample. All procedures were approved by Duke Institutional Review Board.

Oxidative Stress Biomarkers

The urine samples were stored at no warmer than -70°C , prior to analysis. Allantoin and 2,3-dinor-iPF_{2α}-III (an F₂-isoprostane) levels in urine were determined by using UPLC-MS/MS (ultra performance liquid chromatography-tandem mass spectrometry), as previously described (10, 11). Analyses were performed using an Acquity UPLC system and TQD triple quadrupole mass spectrometer, equipped with an ESI source (Waters Corp., Milford, MA). For allantoin analysis, equal amounts of urine and a DL-allantoin-5-¹³C;1-¹⁵N internal standard solution were mixed and diluted into the matrix [0.5% formic acid in acetonitrile: DI-H₂O (95:5, v/v)]. The 5 l sample mix was injected onto an Acquity UPLC™ BEH HILIC, 1.7 m, 2.1× 100 mm column (Waters Corp., Milford, MA) and chromatographic separation was achieved by isocratic elution. Allantoin was detected in positive ion mode, using selective reaction monitoring (SRM). For 2,3-dinor-iPF_{2α}-III analysis, urine and a iPF_{2α}-III-d₄ internal (Cayman Chemicals, Ann Arbor, MI) standard mixture was mixed and purified by solid phase extraction (SPE) using Oasis HLB 30 mg cartridges (Waters Corp., Milford, MA). Purified samples were injected onto an Acquity UPLC™ BEH C18 1.7 m 2.1× 50 mm column (Waters Corp., Milford, MA) and separated using gradient elution. 2,3-dinor-iPF_{2α}-III was detected in negative ion mode, using SRM. Urinary levels of both biomarkers were determined relative to creatinine, as previously described (12, 13).

Statistical Analysis

Statistical analysis was performed with SAS, version 9.2 (Cary, NC). Spearman correlation coefficients, stratified by age-groups, were used to describe the relationships between biomarkers and age. Mean levels of the biomarkers, adjusted for age and gender, were obtained from the generalized linear models function. The linear model for each biomarker was stratified by a child/adult category, and within each strata, the levels of biomarkers were further adjusted for age as a continuous variable.

Results

The mean age in the DS group (14 years, range: 2-52, $n = 48$) was lower than the overall control group age (mean: 34 years, range: 4-78, $n = 130$) (Table 1). Therefore, we performed an additional stratified analysis within the child and adult strata.

Overall, the mean allantoin level, adjusted for age and gender, was lower for the DS group than for controls ($p < 0.04$) (Table 2). However, this difference was limited to children and was not found among adults. The age- and gender-adjusted mean levels of 2,3-dinor-iPF_{2α}-III among all individuals, regardless of DS-status or child/adult stratification were similar (Table 3). Taking into consideration that age is an important determinant of DS pathology and oxidative status, we conducted a sensitivity analysis by excluding controls older than DS individuals in this study population, i.e. older than 52. Exclusion of older controls did not influence the results: p -values for the comparisons of the biomarkers' mean levels between DS individuals and controls (ages 18-52) were 0.7 and 0.2 for allantoin and 2,3-dinor-iPF_{2α}-III, respectively. As shown in Tables 2 and 3, both biomarkers inversely correlated with age among DS individuals and controls. In case of allantoin, this correlation was marginally significant among children and was not present among adults. Correlations between 2,3-dinor-iPF_{2α}-III and age were inverse among all considered groups, with a varying degree of statistical significance: not significant among children with DS, significant among children controls, and marginally significant among adults with or without DS. The correlations with age were very similar for both biomarkers among adults after exclusion of controls above age of 52: Spearman correlation coefficients were -0.07 ($p = 0.5$) and -0.25 ($p = 0.07$) for allantoin and 2,3-dinor-iPF_{2α}-III, respectively.

Discussion

Our study used two urinary biomarkers of systemic oxidative status, allantoin and 2,3-dinor-iPF_{2α}-III, which we have previously validated in a clinical model of oxidative stress (8). Investigation of these biomarkers in the urine from individuals with DS has not been reported before. Contrary to our expectations, neither allantoin nor 2,3-dinor-iPF_{2α}-III levels were elevated among the individuals with DS. In contrast, we determined tendencies of increased levels of oxidative stress biomarkers in certain control sub-groups, for example, higher levels of allantoin in the pediatric control group (Table 2).

Previous studies showed elevated plasma allantoin (14) and urinary 8,12-iso-iPF_{2α}-VI (15) in children with DS. However, studies by Campos *et al.* showed significantly lower urinary iPF_{2α}-III (8-iso-PGF_{2α}) levels in adults (> 40 years) with DS and similar levels were found in DS individuals compared with controls in the younger population (15-40 years) (16, 17). Thus, the results of these studies are essentially inconsistent; and our study further emphasizes this discrepancy. Such inconsistency may be explained by the variability of population-based estimates, frequently observed in epidemiological studies, which can be reconciled within the framework of meta-analysis. At this time, meta-analytical technique is not applicable to the studies of oxidative stress markers in DS individuals, because the studies that use the biomarkers previously validated in at least one model of oxidative stress

(all of which are presented in this discussion) are few. Another likely explanation may be the difference between systemic and local oxidative stress: excessive oxidation may be present in affected tissues but not at the systemic level. However, brain tissue studies examining the levels of oxidative damage markers also showed inconsistent results (18), questioning the existing consensus that individuals with DS are under chronic oxidative stress. Although an *in vitro* study showed increased generation of ROS in Down's syndrome neurons (4), studies in human populations do not uniformly confirm this observation. Absence of definitive evidence for increased systemic oxidative stress in individuals with DS also questions the ability of antioxidant supplementation to improve cognitive function. In fact, the hypothesis that increased SOD-1 activity relative to GPX (SOD-1/GPX ratio) drives the increased production of ROS in DS individuals has not been confirmed. A recent study did not show a correlation between these enzymes' activities and urinary F₂-isoprostanes, and in the same study increased SOD-1/GPX ratio did not correlate with poor cognitive performance (19).

We also documented inverse correlation between both biomarkers and age among DS individuals, which is in agreement with earlier literature (20, 21). This finding has been interpreted as evidence for increased oxidative stress among younger DS individuals. However, similar inverse correlations of the biomarkers with age among controls do not support such an interpretation. In general, pediatric studies show a decrease of urinary F₂-isoprostanes (22) and other oxidative damage biomarkers with age (23). Among adults, findings regarding associations between urinary F₂-isoprostanes and age are inconsistent (24). Whereas for allantoin, to the best of our knowledge, such data have not been published.

In summary, the results of our study emphasize a controversy within the field of Down syndrome. Although the prevailing concept considers oxidative stress the major pathophysiological vector of morbidities associated with DS (18, 25), human studies show conflicting results on whether such oxidative stress exists or on whether such oxidative stress can be reliably measured at the systemic level.

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Table 1

Demographic characteristics of the study participants

	DS subjects (n=48)	Controls for allantoin (n=130)	Controls for 2,3-dinor- iPF2α-III (n=85)
Mean age in years (range)	14 (2-52)	33 (4-78)	35 (4-75)
Females (n)	23	71	49
Males (n)	25	59	36
Children (age < 18 years)	36	36	18
Adults (age 18-78 years)	12	94	67
Adults (age 18-52 years)	12	80	56

Table 2
Urinary allantoin levels (mmol/mol creatinine) in individuals with Down syndrome (DS) and control subjects.

Age groups	Mean (standard error) *		p values	Spearman correlation coefficient for allantoin and age / p-values	
	DS subjects	Controls		DS subjects	Controls
All	9.49 (0.91) n = 48	11.78 (0.53) n = 130	0.04	-0.33/ 0.02	-0.45/ <0.0001
Children (age <18 years)	12.31 (1.05) n = 37	16.41 (1.11) n = 36	0.007	-0.31 / 0.06	-0.28 / 0.09
Adults (age ≥ 18 years)	8.76 (1.54) n = 11	8.86 (0.53) n = 94	1.0	-0.09 / 0.8	-0.12 / 0.3

* Adjusted for age and gender.

Table 3

Urinary 2,3-dinor-iPF_{2a}-III levels (ng/mg creatinine) in individuals with Down syndrome (DS) and control subjects.

Age groups	Mean (standard error)*		p values	Spearman Correlation Coefficient for 2,3-dinor-iPF _{2a} -III and age / p-values	
	DS subjects	Controls		DS subjects	Controls
All	5.21 (0.38) n = 48	5.40 (0.27) n = 85	0.7	-0.28 / 0.05	-0.24 / 0.03
Children (age <18 years)	6.20 (0.44) n = 37	5.63 (0.64) n = 18	0.5	-0.19 / 0.3	-0.59 / 0.01
Adults (age ≥ 18 years)	3.83 (0.65) n = 11	4.86 (0.25) n = 67	0.1	-0.45 / 0.1	-0.21 / 0.08

* Adjusted for age and gender.