Plasma Glutathione Concentrations in Non-infected Infants Born From HIV-infected Mothers: Developmental Profile

CAROLINE J. CHANTRY MD*; JORGE L. RODRIGUEZ MT†; IRMA FEBO MD; CLEMENTE DIAZ MD; JOSE F. RODRIGUEZ-ORENGO Ph.Dđ

ABSTRACT. Glutathione (GSH) is the primary antioxidant in humans. Oxidative cellular injury is postulated to be centrally involved in diverse processes including aging, cancer, cardiovascular disease, and Human Immunodeficiency Virus (HIV) disease progression. Normal plasma GSH concentrations have been well characterized in healthy children and adults, but not during infant development. The objectives of this study were to: a) measure plasma GSH concentrations in non-infected infants born from HIV-infected mothers, to b) assess the developmental variations with age and gender, and c) evaluate for possible associations with growth, anemia, and other maternal and infant variables. One hundred and seventy (170) plasma samples from 44 HIV-uninfected infants (birth to 18 mos.) born to HIV-infected mothers from the Women and Infant Transmission Study (Puerto Rico site) were analyzed. The total plasma GSH geometric mean concentration for all samples analyzed was 1.94 (1.06) μmoles/L. A developmental effect of age was seen with lower concentrations in younger infants (0-2 months) than in older infants 4-18 months. There was no significant effect of gender, anemia, zidovudine exposure, maternal age, maternal CD4 cell percent, or infant growth, although a trend towards increasing GSH concentration was seen with increasing weight for height z-score. These findings have multiple clinical ramifications including prediction of capacity to detoxify oxidants at different ages, and partial explanation for the increased viral loads seen in HIV-infected infants. Key words: Glutathione, HIV, Children, Pregnant women

Glutathione (GSH), a thiol containing tripeptide, is the primary intracellular antioxidant in humans. It functions directly as an antioxidant to remove peroxides produced during oxidative metabolism as well as exogenous toxins. Additionally, it maintains other cellular antioxidants in reduced form and enzymes involved in reduction in an active state. Accordingly, GSH and its related enzymes are among the principal protective mechanisms against cellular injury. Oxidative cellular injury is postulated to be centrally involved in diverse processes including aging, cancer, cardiovascular disease, pulmonary diseases such as adult respiratory distress syndrome, Alzheimer's disease, rheumatoid arthritis, and others (1-6).

Antioxidant systems are also integral to optimal immune function, including T-cell activation and proliferation. GSH deficiency has been associated with disease progression and survival in HIV-infected adults (7, 8). HIV-infected children also demonstrate relative GSH deficiency (9, 10), the severity of which correlates with CD4 cell counts and inversely with viral load (9).

Because of its broad implications to health, there have been multiple studies characterizing the concentrations of GSH in humans in both plasma and tissues and analyzing association with age, sex, diet, and various disease states. It has been demonstrated that there is a wide range of plasma GSH concentrations in healthy adults (11). Despite the importance of this compound and its detailed characterization in adults, there are no

* Department of Pediatrics, University of California at Davis, Sacramento California.
† Project GAMMA (Pediatrics AIDS Program) and Puerto Rico WITS Project, Department of Pediatrics, University of Puerto Rico School of Medicine, San Juan PR.
d Department of Biochemistry, University of Puerto Rico School of Medicine, San Juan PR.

This work was supported in part by the following Public Health Service Grants 2U01AI32996, 1P20RR11126, RR02501, AI34858, and R01AI39191 from the National Institute of Health.

Address correspondence to: Dr. Jose F. Rodriguez-Oreno, Department of Biochemistry, University of Puerto Rico School of Medicine, PO Box 365967 San Juan PR 00936-5967. Phone & Fax: (787) 754-4929.
E-mail: j_rodriguez@creaxp.upr.edu

267
published studies demonstrating the developmental pattern of GSH in infants and young children, or exploring possible association with age, sex, growth or other parameters in children. Animal work has been done examining the relationship between growth and GSH in several species with inconsistent results; some studies have shown association with body weight, but other studies have not (12). One study in chickens examined a developmental pattern with lowest whole blood concentrations at day 1 and rapid increase over the first week, declining thereafter (12). This supports the concept that age may be important in evaluating GSH concentrations and relative deficiencies at both ends of the lifespan.

The objectives of this study were to: (i) measure plasma GSH concentrations in a cohort of newborns, infants, and small children perinatally exposed to HIV whom were not infected; (ii) assess the developmental variations of plasma GSH with age and gender; and (iii) evaluate for possible associations with growth in weight or height, anemia, zidovudine exposure, acute illness, maternal age, and maternal CD4 cell percent.

Materials and Methods

Subjects. This study used samples from children born to HIV-positive mothers and followed from birth at the University of Puerto Rico as participants in the Women and Infants Transmission Study (WITS). Infants born to these mothers are followed at birth and 1, 2, 4, 6, 9, 12, 15, 18, and every six months thereafter. Each visit consists of history and physical and laboratory examinations, which includes plasma for storage. Infants with documented or suspected HIV infection based on clinical, immunologic or culture criteria were excluded from analysis. Specifically, HIV-negative was defined for these infants as follows: all HIV cultures must be negative, of which there must be at least two, including one at 6 months of age or older; there must be two negative ELISA assays and no clinical or immunological abnormalities suggesting HIV infection. Children with at least three available stored specimens from different visits with sufficient volume for analysis were chosen. A total of 170 samples from 44 children were analyzed. Anemia was defined as hemoglobin less than 2 standard deviations below the age-adjusted mean. Maternal CD4 cell percentages were measured at delivery. The clinical protocol was approved by our Institutional Review Board. All parents or guardians signed an informed consent form before study entry.

Analytical methods. Samples were selected as described above. Plasma samples were collected in heparin containing tubes, centrifuged, and stored at -80°C. Plasma samples with any evidence of hemolysis were excluded from analysis. Determination of total plasma glutathione concentration was performed using a HPLC method described previously (9). Briefly, 100 μL of plasma were incubated with dithiothreitol followed by the addition of 1.0 M HClO₄ and centrifugation for 2 minutes. The supernatant was neutralized with bicarbonate and Tris HCl (pH 8.0). The sample was then derivatized with 100 mM monobromobimane in the dark at room temperature. The reaction was stopped with HClO₄ and the sample was placed in an HPLC vial for automatic injection (25 μL). GSH was separated on a quaternary HPLC system (HP 1050) with a Hypersil C₁₈ column (Hewlett-Packard) and re-injection occurred after 11 minutes. GSH retention time was 5.5 minutes. Our limit of quantitation for total plasma glutathione concentrations was 0.56 μM, and the intra- and inter-day coefficient of variations were 2% and 10%, respectively.

Statistics. Statistical analysis was performed using Statview® (SAS® Institute Inc., 1998). The Kolmogorov-Smirnov test was used to test for normality of distribution. Plasma GSH concentrations were not normally distributed, but log₁₀ transformation resulted in normal distribution. Plasma GSH concentrations for all children and appropriate sub-groups were therefore described using the mean, standard error, and median of the log₁₀ plasma GSH concentration hereafter referred to as log GSH. The geometric mean for all samples and appropriate sub-groups was calculated by taking the antilog of log transformed data. Z-scores for weight for age (WAZ), height for age (HAZ), and weight for height (WHZ) were obtained using Epi-Info anthropometric software. Comparisons of log plasma GSH concentrations among study subgroups defined by age group, sex, zidovudine therapy, WHZ, WAZ, and anemia were performed using Analysis of variance (ANOVA) and appropriate two sample t-tests. Homogeneity of variances used among groups was determined using Bartlett's test. Univariate linear regression analysis was performed to study the relation between age, weight, height, WAZ, HAZ, WHZ, maternal CD4 cell percent, and log GSH. A p value of < 0.05 was considered significant.

Results

Overall, the mean (SEM) plasma GSH concentration was 2.79 (0.18) μmoles/L with a range from 0.08 to 14.06 μmoles/L. As mentioned above, the distribution of GSH concentrations was not normal; logarithmic transformation did demonstrate normal distribution and was used for statistical comparisons. The geometric mean
for all samples analyzed was 1.94 (1.06) μmoles/L. There was a developmental effect of age with lower values seen in the younger infants. The geometric mean plasma concentrations at each age are depicted graphically in Figure 1 with the corresponding best-fit model: plasma GSH concentration = 1.07 log (age in weeks) + 1.05, r²=0.79. The model of GSH increasingly linearly with age rather than logarithmically produced a substantially poorer fit, with r²=0.49. ANOVA test resulted in p <0.0001. Fisher’s PLSD of mean log GSH at different ages revealed that mean log GSH at 0-2 wks., 1 mos. of age were significantly lower than mean log GSH at ages 4-18 mos. Additionally, mean log GSH was lower at 0-2 wks. of age than at 1 or 2 mos. of age, but mean log GSH at 1 and 2 mos. were not significantly different from each other. Nor were differences between log GSH at 4 to 18 mos. of age significant. There was also no significant effect of gender, the geometric mean GSH for boys was 2.13 (1.09) μmoles/L, n=98 vs. 1.71 (1.11) μmoles/L for girls, n=72; p=0.11. Gender had no significant effect at any age (see Table 1).

The effects of current or recent acute illness on GSH concentrations could not be assessed, as too few infants for comparison were in these categories at any given age. Overall, anemic children were found to have lower geometric mean GSH concentrations, but age was a confounding factor with a higher percentage of children at younger ages demonstrating anemia. When compared to their non-anemic counterparts at the same age, anemic infants did not have significantly different plasma GSH concentrations (data not shown).

Table 1. Geometric mean plasma glutathione concentration for different age groups of non-infected HIV infants born from HIV infected mothers split by gender.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Mean age/ weeks</th>
<th>Geometric mean plasma GSH/μM</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>0.5±0.1</td>
<td>0.84 ± 1.16</td>
<td>0.747</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>0.92 ± 1.34</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4.1±0.2</td>
<td>1.52 ± 1.19</td>
<td>0.811</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>1.41 ± 1.26</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9.4±0.2</td>
<td>1.68 ± 1.17</td>
<td>0.874</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>1.75 ± 1.25</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18.2±0.3</td>
<td>3.17 ± 1.16</td>
<td>0.232</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>1.93 ± 1.59</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>26.8±0.3</td>
<td>2.97 ± 1.20</td>
<td>0.211</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>2.08 ± 1.21</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39.7±0.2</td>
<td>4.04 ± 1.16</td>
<td>0.456</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>3.10 ± 1.38</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>52.8±0.4</td>
<td>3.34 ± 1.27</td>
<td>0.162</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>1.84 ± 1.40</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>79.4±0.4</td>
<td>3.05 ± 1.45</td>
<td>0.746</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>2.52 ± 1.47</td>
<td></td>
</tr>
</tbody>
</table>

*Two sample t-test
*SEM

**Geometric mean GSH concentrations were analyzed according to growth parameters: weight, WAZ, HAZ, and WHZ. Linear regression revealed a very weak correlation between log GSH and weight, r²=0.137, but this was confounded by age. Linear regression revealed essentially no correlation between log GSH and WAZ (r²=0.006), HAZ (r²=0.001), or WHZ (r²=0.005). Similarly, when children were divided categorically into those with WAZ < -1 (low), -1 < WAZ < +1 (normal), and WAZ > +1 (high), a trend was seen towards increasing geometric mean GSH concentration, but this was not significant; 1.96 (1.28) μmoles/L vs. 2.22 (1.08) μmoles/L vs. 2.23 (1.22) μmoles/L, p=0.86. A similar trend with statistically differences was observed when WHZ was analyzed 1.19 (1.38) μmoles/L vs. 2.32 (1.09) μmoles/L vs. 2.67 (1.13) μmoles/L, p=0.04. Fisher’s PLSD showed that differences were between low vs. normal (p=0.025) and low vs. high (p=0.011). No difference was observed between normal vs. high (p=0.389).**

The effect of prophylactic zidovudine (ZDV) use by the mother prenatally and the infant for the first six weeks of life on geometric mean GSH concentrations was also assessed. There was not a significant effect seen at the 0-2 week visit, when the infants who received ZDV had a geometric mean GSH of 0.97 (1.20) μmoles/L, n=22 vs. those who did not receive ZDV 0.60 (1.27) μmoles/L, n=9; p=0.14. Similar results were obtained at the 1 month visit 1.55 (1.19) μmoles/L, n=18 vs. 1.31 (1.47) μmoles/L, n=6; p=0.71.
Lastly, the effects of maternal age and disease status as measured by CD4 cell percent at delivery on GSH concentrations at the newborn visit were examined by linear regression analysis. There was a trend towards increasing geometric mean GSH concentration with increasing maternal age, \( r^2=0.073 \), but this was not significant, \( p=0.14 \). Nor was there a significant correlation between geometric mean GSH and maternal CD4 cell percent, \( r^2=0.007 \); \( p=0.41 \).

**Discussion**

These results reveal a marked increase in the plasma glutathione concentrations over the first few months of infancy. The developmental pattern of GSH depicted in Figure 1 is remarkably similar to that previously described for whole blood GSH in newborn cockerels over the first weeks of life (12). To our knowledge, the only report of GSH concentrations in plasma in newborns reports a relative deficiency of GSH in preterm vs. term infants, demonstrating the deficiency directly correlates with the degree of prematurity (13). The latter study also described a depletion on day 2 compared to day 1 which was restored by day 7 in the less severely premature infants. Development of GSH concentrations in newborns beyond day 2 was not described.

Erythrocyte and leukocyte glutathione concentrations in older children have also been described; GSH concentrations were significantly lower in older children (ages 5-12 yrs.) compared to adults 25-40 years old, but were similar to concentrations in aging adults (65-83 yrs.) (14). The mean plasma concentrations of GSH described in the current study in infants are significantly lower than those previously found by our group in healthy children (6.62 ± 0.58 μmol/L) whom were older with a mean age of 6 years (9). This suggests that plasma GSH concentrations continue to rise during early childhood. In fact, five patients in the current study were again analyzed when they were older (mean age=6 yrs.), and their mean total plasma GSH values were similar (6.06 ± 0.50 μmol/L) to healthy children values(9).

It has been demonstrated that there is a wide range of plasma concentrations in healthy adults and that this varies by demographic factors such as sex, age, and possibly race as well as lifestyle factors such as diet and exercise (15). Diets that have been associated with increased GSH include vegetarian diets, and diets replete vs. deficient in ascorbate (15). The effect of age varies with sex; several investigators have postulated an effect of sex hormones on GSH transport and metabolism (16). Estrogen in itself is known to scavenge free radicals. In infants, we did not see a significant effect of gender; however, sex hormone concentrations in infants are significantly less than they are in adults, with no differences between genders prepubertally.

The correlations we found in these infants between growth parameters of WAZ, HAZ, WHZ, and GSH concentrations were extremely weak. Nevertheless, an apparent trend to increasing GSH was observed when analyzing children with low vs. high WHZ; \( p=0.011 \) and low vs. normal (p=0.025). Previous animal studies have inconsistently demonstrated this association, perhaps because of its weakness. The study by Owens et al. demonstrated significantly higher GSH concentrations in poultry lineages chosen for high vs. low weight (12). Obesity in human adults has also been associated with higher plasma GSH concentrations (11). Growth failure (abnormally low HAZ) in HIV-infected children has previously been associated with a more significant GSH deficiency than that seen in HIV-infection without growth failure (9,17); however in these children, GSH concentration was most weakly associated with HAZ of the three growth parameters.

The findings of low plasma glutathione concentrations in newborn infants with significant increases in the following weeks are not surprising. It is understood that the liver is the primary source of systemic glutathione, although it has been documented experimentally in rats that extrahepatic tissues are also able to secrete reduced glutathione into plasma (18). It is well established that liver function in newborns is immature, with relatively rapid development over the first weeks of life. Specifically, with respect to synthetic function, the fetal liver has synthetic capability for all major plasma proteins after three months of gestation, but concentrations of most proteins in fetal plasma are low (19). Levels of different proteins approach that of the adult at different ages, e.g. albumin reaches adult levels at several months, lipoproteins increase over the first week of age to levels maintained until puberty, and complement values increase to mature levels during the first year. (19). Most hepatic microsomal enzymes responsible for reductive metabolism are also not mature by birth. They are undetectable or minimally active in utero, but rapidly mature after birth (19).

It currently remains speculative the cause(s) of decreased GSH concentrations in newborn and young infants. As previously suggested for premature infants (13), likely the cause is multifactorial and may include, reduced secretion of GSH by the liver to plasma either because of decreased synthesis or increased intra-hepatic recycling, or increased GSH consumption because of increased oxidative stress. Whether or not the latter mechanism contributes to lesser GSH concentrations in
the newborn, the implications of decreased GSH in infancy likely include a lesser ability to functionally manage oxidative metabolites or exogenous substances utilizing GSH and related enzymes for metabolism (e.g. acetaminophen). Prophylactic therapy with ZDV did not significantly alter GSH concentrations in this cohort; ZDV is metabolized primarily by hepatic glucuronidation. It may be significant that these infants were also exposed to zidovudine in utero which may have induced an increased metabolic capacity.

It is also currently unknown what developmentally is the rate-limiting factor with respect to the reductive mechanism consisting of glutathione and related enzymes. One study found activity of glutathione-s-transferase (GST) to be directly related to GSH content in aging adults (2) suggesting that GSH concentrations may be rate-limiting. Post-natal development of glutathione-reductase, glutathione-peroxidase and GST have been described to a limited extent. GST utilizes glutathione to inactivate reactive molecules and has been found in fetal liver to have approximately two-thirds the level of activity found in adult liver. GST activity develops in the fetal liver during the first trimester and does not correlate with gestational age thereafter (20). Conflicting results have been seen with GST in aging adults (2, 20). Activity of glutathione-reductase, the only enzyme that reduces GSH, has been characterized as quite high at birth, rapidly reaching adult levels thereafter (21). Glutathione-peroxidase, responsible for reducing hydrogen peroxide, has been found to have lower activity in children than adults in New Zealand (22); however, this was attributed to low dietary selenium in children as opposed to a normal developmental pattern. Selenium supplementation has been shown to increase glutathione-peroxidase activity in some circumstance (23).

There are multiple ramifications of this study. As previously mentioned, oxidative cellular injury against which GSH and associated enzymes protect is postulated to be centrally involved in diverse processes including aging, cardiovascular and pulmonary diseases, cancer, immune dysfunction, and disease progression in HIV infection, among others. For example, HIV replication is enhanced in GSH depleted cells (24). Relative GSH deficiency in infancy could partially contribute to higher viral loads in HIV-infected infants compared to adults; clinical implications for therapy are apparent. Recognizing the normal ontogeny of this important reductive mechanism will allow better assessment of abnormal oxidative stress as well as improve prediction of the capacity to detoxify exogenous compounds that rely on these mechanisms.

Resumen

La glutatonia (GSH) es el antioxidante principal en humanos. Se ha postulado que el daño oxidativo celular está envuelto en diversos procesos incluyendo el envejecimiento, cancer, enfermedades cardiovasculares, y la progresión de la enfermedad con el Virus de Imunodeficiencia Humana (VIH). Concentraciones normales plasmáticas de GSH han sido caracterizadas en niños y adultos, pero no durante el desarrollo del infante. Los objetivos de este estudio son: (i) medir las concentraciones de GSH en plasma en infantes no infectados con VIH nacidos de madres infectadas, (ii) determinar las variaciones del desarrollo con relación a la edad y el género de los pacientes, y (iii) evaluar las posibles asociaciones con el crecimiento, anemia, y otras variables materno-infantiles. Ciento setenta (170) muestras de plasma fueron obtenidas de 44 infantes no infectados con VIH (desde el nacimiento a 18 meses) nacidos de madres infectadas del Estudio de Transmisión de Madre e Infante (WITS, Unidad de Puerto Rico). El promedio geométrico de la concentración de GSH en plasma para todas las muestras analizadas fue de 1.94 (1.06) μmoles/L. Un efecto del desarrollo con la edad fue observado con concentraciones menores de GSH en los infantes más pequeños (0-2 meses) que en los niños mayores (4-18 meses). No hubo efectos significativos con la edad, género, anemia, exposición a zidovudina, edad de la madre, porciento de CD4 celular de la madre, o crecimiento del infante, aunque se observó una tendencia a aumentar la concentración de GSH con la razón de peso/altura. Estos resultados pueden tener ramificaciones clínicas incluyendo la predicción para la capacidad de detoxificar oxidantes a diferentes edades, y la posible explicación de las altas cargas virales observadas en infantes infectados con VIH.

References


