Blood Glutathione and Cysteine Concentrations in Twin Children

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Glutathione and cysteine are major antioxidants in blood that are associated with health and longevity. To ensure their measurement, careful attention to avoid auto-oxidation is necessary to stabilize the samples. Since no report of these compounds has been reported in children, our goal was to determine their levels of reduced and oxidized glutathione (GSH and GSSG) and cysteine (Cys and CSSC), To this end, 140 healthy children, ages 2 to 9 years from the Louisville Twin Study were studied. Blood samples were collected and analyzed for GSH, GSSG, Cys, and CSSC by our HPLC dual electrochemical method. The results showed that GSH and total GSH (GSH + GSSG) levels for monozygotic (MZ) twins were significantly higher ($P < 0.001$) than levels for dizygotic (DZ) twins. However, the opposite occurred for Cys and total Cys (Cys + CSSC) in that the levels were significantly higher for DZ twins than for MZ twins. ($P < 0.005$–0.013). In spite of this marked difference in zygosity, the within-pair correlations for twin pairs used for estimating heritability suggested that there was a major environmental influence for total GSH and total Cys. Finally. GSH levels were significantly lower for young (2–9 years) children than adults ($P < 0.001$).

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Our research is on biomedical aspects of aging with emphasis on tissue reduced glutathione (GSH) status during the life span. Previously, we studied human aging using animal models like the mosquito, mouse, and rat. A series of findings indicated that a marked GSH deficiency occurred during senescence of these organisms. Of particular interest was that the blood GSH deficiency in the aged C57BL/6J mouse (1) was paralleled by GSH decreases in liver, kidney, heart, spleen, and brain (2–4). These results suggested that the blood level could serve as an overall body index. Later findings in the mosquito showed that its GSH deficiency of aging was linked to a cysteine (Cys) decrease. Correction of this decrease by administration of magnesium thiazolidine-4-carboxylic acid, a precursor of Cys, also corrected the GSH deficiency and increased the life span by about 40% (5–8).

We are unaware of reports of blood GSH and Cys levels for children. Therefore, the present study, employing our method (9) for determining specific amounts of GSH and oxidized glutathione (GSSG) and CYS and oxidized cysteine (CSSC), was undertaken with healthy twin children as subjects. This study will extend our knowledge of glutathione levels to the early years of the life span, and by using twins, will estimate the degree of genetic influence upon children's glutathione and cysteine levels.

Materials and Methods

The subjects were 140 healthy twin children between the ages of 2 and 9 years, reared together, and participating in the Louisville Twin Study. After their parents had given informed consent, blood samples were obtained from the twins. Because our previous studies of humans had shown no difference in GSH levels for fasted compared with unfasted subjects, the twins were not required to fast. The sample was obtained by venipuncture with an EDTA evacuated tube, and a portion of the sample was removed for erythrocyte count, hemoglobin concentration, and hematocrit. The remainder was quickly chilled in crushed ice and was deproteinized by the addition of 1 volume of blood to 4 volumes of freshly prepared 5% metaphosphoric acid. After 15 min, the specimen was centrifuged at 13,000*g* for 2 min. The resultant supernatant was removed and quantified for GSH, GSSG, Cys, and CSSC with an HPLC-dual electrochemical method (9). This procedure was routinely validated for proportionality and recovery of spiked samples (9, 10).

Our previous study showed that over 99.5% of GSH in blood was localized in the erythrocytes, and 97% of Cys was in plasma (11). For these reasons we used the bases of expression, GSH per 10^{10} erythrocytes and Cys per milliliter plasma. Both GSH and Cys data were also expressed as per milliliter blood for comparison with each other.

Statistical analyses utilized Student's *t* test, the Mann-Whitney nonparametric test, correlations, and methods for

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comparing correlations (12, 13), and all were available on SPSS 9.0 for Windows. Estimates for heritability were obtained by Falconer's method for deriving broad-sense heritability (14). The number of subjects varied slightly for analyses of the hematological measures because of missing data.

Results

The glutathione and cysteine levels of MZ and DZ twins are presented in Table I. The MZ twins were only 0.6 years older ($P < 0.037$) than the DZ twins. Red blood cell counts, hemoglobin concentrations, and hematocrits were not significantly different between the groups of MZ and DZ twins.

Statistical comparisons between the MZ and DZ twins utilized *t* tests and significant differences were confirmed with Mann-Whitney tests as shown in the right columns. The GSH and total GSH concentrations for the MZ twins were significantly higher than those for DZ twins, but GSSG levels for both groups were low and not significantly different. In an opposite manner, Cys, total Cys, and CSSC concentrations for DZ twins were significantly higher than for MZ twins.

GSH + GSSG was 19% higher for MZ twins compared with DZ twins, but there was no significant difference between the groups for Cys + CSSC.

Similarities of individuals within twin pairs were determined by intraclass or within-pair correlations calculated for MZ twin pairs and DZ twin pairs. Intraclass correlations for GSH + GSSG and Cys + CSSC were quite high (ranging from 0.74–0.92) for the pairs of MZ and DZ twins (Fig. 1), and there were no significant differences between the MZ and DZ types.

Estimates of heritability derived from these correlations indicate that 28% of the variation for GSH + GSSG can be attributed to genetic differences among the children, and 72% of the variation can be attributed to environmental differences. For Cys + CSSC, because the intraclass correlation for DZ twin pairs was slightly (but not significantly) higher than the intraclass correlation for MZ twin pairs, all (i.e., 100%) of the variation of Cys + CSSC among the children can be attributed to environmental differences.

The relations between children's age and GSH and Cys levels are given in Figure 2. The correlations indicate that there are significant systematic relations between the age of the twins, whether MZ or DZ, and GSH and Cys levels. It is of interest that the correlations between age and GSH are positive and the correlations between age and Cys are negative, but the explanation for these age-related contrasts is not known. In Figure 3 the GSH levels for the 2- to 9-year-old twins were compared with those for 40 young adults, 20- to 39-years-old, determined previously by us with the same analytical methods (15). The mean and SEM for 138 twins was 376 ± 9.73 μg GSH/10^{10} red blood cell, a value significantly lower ($P < 0.001$) than the value of 547 ± 8.46 for the adults. A comparison between CYS values for twin children and adults was not possible because CYS data for adults were not available.

The GSH differences were due to the amount of metabolite and not to the concentration expression as per milliliter of blood, per milliliter of plasma, or per 10^{10} red blood cells. These terms are based on hematocrit, red blood cell count, and hemoglobin content, which were the same for the twin children and the adults.

Discussion

The results indicate that the blood glutathione level for MZ twins was higher than the level for DZ twins. The blood glutathione level for twin children in general was lower than that for adults, and these levels increased with age. However, the opposite was found for the cysteine level. For DZ twins, the level was higher than for MZ twins, and the level for twins decreased systematically with age. These findings extend our information on blood GSH to almost the entire life span of healthy humans.

Table I. Blood Glutathione and Cysteine Content in Twin Children

	Monozygotic	Dizygotic	*P* value[a]	
			t test	Mann-Whitney
Age (years)	4.50 ± 0.17 (98)[b]	3.89 ± 0.21 (42)	0.037	–
RBC (10^6 cells/ml)	4.57 ± 0.034 (97)	4.57 ± 0.060 (42)	0.998	–
Hemoglobin (g/dl)	12.7 ± 0.13 (97)	12.7 ± 0.16 (42)	0.697	–
Hematocrit (%)	37.2 ± 0.36 (97)	37.1 ± 0.48 (42)	0.875	–
GSH (μmol/10^{10} RBC)	1.29 ± 0.036 (97)	1.07 ± 0.058 (42)	0.001	0.001
GSSG (μmol/10^{10} RBC)	0.062 ± 0.004 (97)	0.057 ± 0.005 (42)	0.457	–
GSH + GSSG (μeq/10^{10} RBC)	1.42 ± 0.038 (97)	1.18 ± 0.056 (42)	0.001	0.001
Cys (nmol/ml plasma)	4.28 ± 0.30 (94)	5.88 ± 0.49 (41)	0.005	0.006
CSSC (nmol/ml plasma)	34.0 ± 0.82 (94)	37.8 ± 1.69 (41)	0.024	0.021
Cys + CSSC (neq/ml plasma)	72.2 ± 1.80 (94)	81.5 ± 3.68 (41)	0.013	0.014
GSH + GSSG (neq/ml blood)	643 ± 17.4 (98)	540 ± 25.5 (42)	0.001	0.001
Cys + CSSC (neq/ml blood)	55.8 ± 3.46 (98)	60.6 ± 6.58 (42)	0.486	–

[a] Dizygotic versus monozygotic.
[b] Mean ± SEM (number of subjects).

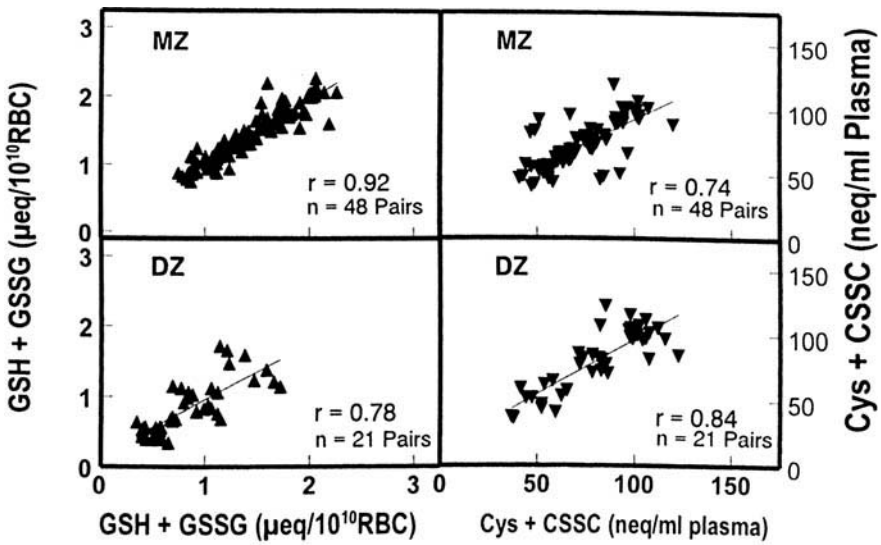

Figure 1. Intraclass correlations of MZ and DZ twin pairs.

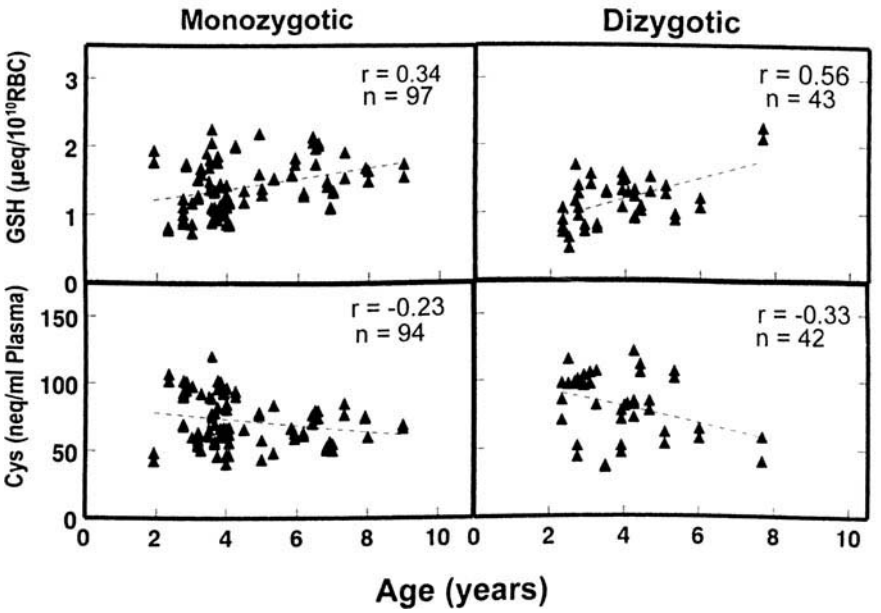

Figure 2. Correlation between GSH or Cys and age of twins.

The quantitative analysis of GSH and Cys in blood has been studied for over a decade. Unfortunately. plasma GSH was often measured instead of erythrocytic GSH, which we found constitutes 99.5% of the GSH in blood (11). Also GSSG, the disulfide or oxidized form, has been used as a measure of oxidative stress. In our experience with various blood thiols and disulfides of different animal and human populations, very little blood GSSG has been found, and oftentimes was undetectable. Thus, we believe that the presence of GSSG is often an experimental artifact due to hemolysis and auto-oxidation. The recent comprehensive work of Kleinman and Richie (10) elucidated this problem with time course data of blood GSH measured within 15 to 30 sec after drawing the blood sample. Thus, these data demonstrate that a major concern in blood GSH and Cys studies is sample collection, preparation, and storage.

The intraclass correlations calculated for MZ and DZ twin pairs were high for both types of twins. Although one

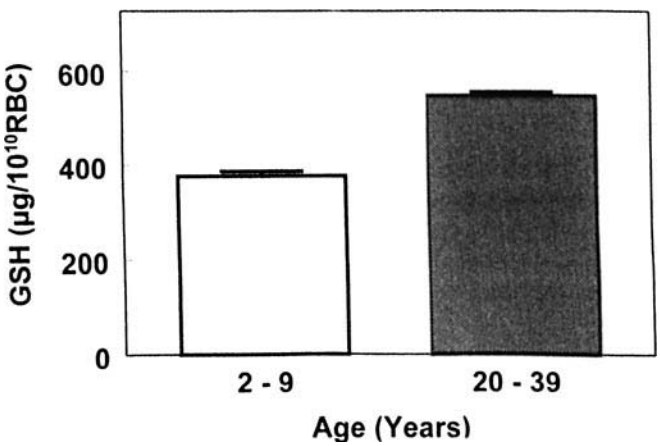

Figure 3. Blood GSH is lower in children than in young adults. Twin children were 2- to 9-years-old, young adults were 20- to 39-years-old. (Reproduced with permission from Ref. 15). $P < 0.001$.

might have expected that the intraclass correlations for MZ twin pairs would have been significantly higher than for DZ twin pairs, comparison between the two groups provided no significant differences. Therefore, from comparisons afforded by the correlations alone, the evidence is that the degree of genetic similarity is not a major contributor to the similarity of the level of GSH + GSSG or Cys + CSSC.

Estimates of heritability derived from Falcomer's method indicated that 72% of the variation of GSH + GSSG and 100% of the variation of Cys and CSSC could be attributed to differences among the children's environments. Because the twins within each twin pair were reared in the same household, we presume that the similarity within twin pairs for diet and nutrition is the source of environmental influence.

In both MZs and DZs the Cys levels were negatively correlated with age, while the GSH levels were positively correlated. The current study provides no answer, although one might expect that the maturation of children would be marked by positive correlations between age and both GSH and Cys, and the incorporation of Cys into GSH is presumably direct and not age-conditioned. However, this was not the case in this study, and additional research on longitudinal samples of children is needed.

There are several possible mechanisms for the GSH differences with age. During growth and development, GSH is used for synthesis, but after maturity, it is needed for maintenance and repair. Also, during the mature stage, GSH is needed for other areas such as defense against environmental toxicity and immunological protection. Finally, during senescence, GSH status drops, as we have found in various human and animal populations. This phenomenon may be due to suboptimal nutrition, insufficient exercise, and unhealthy and stressful conditions. GSH functions most likely shift depending on the current life style and environment. Further investigation of these factors is required for elucidation.

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