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Postnatal Gender-dependent Maturation of Cellular Cysteine Uptake

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Background. In view of the functional capacity of glutathione synthesis in premature infants, and because the availability of cysteine is one the rate limiting steps in glutathione synthesis, we hypothesized that the low glutathione levels in premature infants may be due to immaturity of the active cellular uptake of cysteine.
Objective. To document in cells from newborn infants the effect of maturity and gender on cysteine uptake and consequently on glutathione levels.
Methods. Incorporation of L-[^{35}S] cysteine was measured in leukocytes from cord blood and from tracheal aspirates (TAC) of newborn infants of varying (gestational as well as postnatal) ages and gender. Cysteine uptake was correlated with glutathione in TAC.
Results. The maturity of newborn girls positively influences cysteine uptake, which is responsible for 78% of the variation in their glutathione content. However, in newborn boys, gestational and postnatal ages did not influence the cysteine uptake.
Discussion. Cysteine uptake appears to be the limiting step explaining the reported gender-related differences in glutathione as well as the low levels of this central antioxidant found in premature infants. The immature cysteine uptake found in cells from premature infants raises questions about the bioavailability of this conditionally essential amino acid in regimens of parenteral nutrition for human neonates.

Keywords: Antioxidant defense; Cysteine uptake; Gender; Glutathione; Newborn; Parenteral nutrition

INTRODUCTION

Premature newborn infants are at risk of complications associated with an imbalance between their immature antioxidant defenses[1] and excessive oxidant loads.[2–4] Activities of antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase and catalase increase with gestational age.[1] Similarly, the level of glutathione, a central antioxidant, is low in premature infants.[5] This tripeptide is a cornerstone in the defense against oxidants,[6] as it has antiperoxide as well as antiradical properties. While glutathione is a cofactor of GPx, it can also react directly with radicals and regenerate other antiradical molecules such as vitamin C and vitamin E.[7,8] In this process, the reduced form of glutathione (GSH) is oxidized into GSSG, which in turn may be recycled to GSH by glutathione reductase (GSSG-R). To sustain normal cell function a tight control is maintained over the redox ratio GSH/GSSG. Therefore, when the activity of GPx surpasses the recycling capacity of glutathione, cellular accumulation of GSSG is prevented by its active export.[9,10] This protective mechanism explains the increased concentration of glutathione observed in the eluate of vessels perfused with *tert*-butyl hydroperoxide.[11] The ensuing lower GSH level will result in a lower capacity of the enzyme to reduce peroxides, as the normal cellular concentration of glutathione corresponds to the K_m of GPx.[12] Therefore, a new synthesis of glutathione becomes essential to compensate for the loss of this tripeptide in response to a peroxide load.[13]

The formation of glutathione depends on the availability of three amino acids: glycine, glutamate and cysteine. The normal cellular concentration of

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cysteine corresponds to the K_m of γ-glutamylcysteine synthetase,[14] the first enzyme involved in the formation of GSH, suggesting that a low cellular level of cysteine will affect the synthesis of glutathione. Hence, cysteine availability is a limiting step for glutathione synthesis.[15]

Because glutathione synthetic capacity is functional in cells from premature infants,[16] it was speculated that the cellular availability of cysteine might account for the low glutathione levels found in those infants.[5] This conditionally essential amino acid[17] may be incorporated in cells by three major pathways. The first one entails a Na^+-dependent active import of extracellular cysteine by the neutral amino acid transporter ASC.[18–21] The second pathway involves the oxidized form of cysteine, cystine, which crosses the cellular membrane by the Na^+-independent anionic amino acid transport system x_c^-[18–20] and is reduced to cysteine by the glutathione–glutathione reductase system. In plasma the concentration of cystine is 10 times greater than that of cysteine.[19] However, lymphocytes[19,20] and hepatocytes[18,20] do not transport cystine, but they have a strong uptake activity for cysteine.[20] The third pathway requires the activity of the exoenzyme γ-glutamyltransferase (γ-GT) which transfers the γ-glutamyl moiety of circulating glutathione to another amino acid, and the two dipeptides formed are incorporated in the cell where a dipeptidase releases cysteine. Cystine is also a substrate for cellular cysteine through the action of γ-GT on extracellular glutathione using cystine as γ-glutamyl-acceptor.[22] γ-GT is responsible for 50% of cystine uptake by different cell types.[22,23] The activity of this enzyme increases rapidly in the first few days of life[16] suggesting that the cellular availability of cystine is not limiting even in cells with a less active cystine uptake. Although the activity of γ-GT matures rapidly over a few days after birth,[16] the intracellular glutathione concentration remains low.[5] We hypothesized that this low glutathione level observed in premature infants is a consequence of a low intracellular substrate availability related to a deficient activity of cysteine uptake.

The aim of the present study was to document in cells from newborn infants if maturity modulated cysteine uptake and consequently glutathione levels. Because the level of glutathione is higher in tissues from newborn girls compared to boys,[5] cysteine uptake was analyzed as a function of gestational and postnatal age, as well as gender.

MATERIALS AND METHODS

NaCl, KCl, KH_2PO_4, Na_2HPO_4, glucose, $CaCl_2$, $MgCl_2$, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Aldrich Chemical, Milwaukee, WI. EDTA, TRIS, L-serine, Na borate, 1,4-dithiothreitol (DTT), NADPH, glutathione, glutathione reductase, were obtained from Boehringer Mannheim, Ville St-Pierre, Que., Canada. L-cysteine was bought from Sigma Chemical, St-Louis, MO. 0.9 μCi L-[^{35}S] cysteine and Ficoll–Paque™ were provided by Amersham Pharmacia Biotech, Piscataway, NJ. Travasol Blend C was obtained from Clintec-Baxter, Mississauga, Ont., Canada. Multi-12 Peadiatric® was purchased from Sabex Inc, Boucherville, Que., Canada.

Protocol

Cysteine uptake was studied in leukocytes from cord blood and from tracheal aspirates (TAC), available human material as used previously to study the glutathione metabolism in newborn infants.[5,16] The institutional Research Ethics Committee approved the study, and parental consent was obtained for each sample.

To differentiate the effect of gestational age from postnatal age, leukocytes were isolated from cord blood derived from infants (gestational age: 35 ± 1 weeks, range 27–40; born either by vaginal delivery or cesarean section. Samples were divided in four groups, preterm (<37 weeks) versus term neonates (≥37 weeks) according to gender. Cord blood leukocytes were isolated as described previously.[16] Briefly, fresh cord blood was collected on EDTA and centrifuged at 4°C, 3000 rpm for 10 min within 30 min following delivery. The buffy coat was mixed with 3 ml PBS and layered on top of 8 ml Ficoll–Paque™ gradient (70, 90 and 100%). The leukocyte band was collected after centrifugation at 15°C, 1400 rpm for 30 min and washed in 5 ml PBS. 0.25×10^6 cells suspended in PBS were immediately used for the cysteine uptake.

The effect of postnatal age was studied in cells derived from TAC obtained from infants (gestational age: 30 ± 1 weeks, range 24–36) who were intubated, ventilated and supplemented with oxygen for respiratory distress syndrome. Fraction of inspired oxygen (FiO_2) was adjusted to maintain their oxygen saturation between 90 and 95%, as measured by pulse oxymetry. As part of routine airway management of intubated infants, endotracheal secretions are cleared by aspiration every 3–4 h. In a convenience population of intubated infants, the isolation of TAC was performed as reported previously.[5,16] After centrifugation at 5000 rpm for 10 min, the cell pellet was suspended in 500 μl PBS. The cells were counted and 0.2×10^6 cells were used for the determination of cysteine uptake. Number of cells allowing, an aliquot of TAC was diluted in buffer A (100 mM TRIS, 100 μM EDTA, 10 mM L-serine, 10 mM Na Borate, pH 7.4) and stored at −80°C until glutathione determination. Samples of TAC were divided into six groups according to

TABLE I Characterization of studied population

	Gender	Group: 0–2 days (F: $n = 5$; M: $n = 5$)	Group: 3–4 days (F: $n = 6$; M: $n = 4$)	Group: 5–10 days (F: $n = 4$; M: $n = 7$)
Postnatal age (days)	F	1.6 ± 0.2	3.5 ± 0.2**	8.0 ± 1.2**
	M	1.6 ± 0.2	3.8 ± 0.3**	5.6 ± 0.4**
Gestational age (weeks)	F	31 ± 1	30 ± 2	27 ± 2
	M	31 ± 2	30 ± 3	29 ± 2
FiO_2 (%)	F	27 ± 4	30 ± 3	35 ± 5*
	M	36 ± 3	25 ± 2	28 ± 3
Amino acids intake (g/kg/day)	F	1.3 ± 0.3	1.9 ± 0.4	2.5 ± 0.1*
	M	0.7 ± 0.4	1.9 ± 0.6	2.2 ± 0.2
Multivitamins supplementation (% v/v)	F	1.8 ± 0.6	1.4 ± 0.3	1.8 ± 0.1
	M	1.7 ± 1.3	1.1 ± 0.3	1.5 ± 0.4

a *$p < 0.05$, **$p < 0.01$ in comparison to the other two groups ([0–2 days versus 3–4 days] versus 5–10 days) for the same gender.

gender and postnatal age: 0–2, 3–4 and 5–10 days as shown in Table I. Cells consisted of >90% of leukocytes.[16]

The nutritional state and particularly the provision of amino acids modulates glutathione levels,[24,25] and oxygenation as well as peroxides generated in multivitamins modify the redox balance of the cell.[13,26] These factors could influence cysteine uptake. Therefore, the following variables were compared between genders: FiO_2 (range: 0.21–0.45), intakes of amino acids (0–2.7 g/kg/day: estimated from the concentration of Travasol Blend C® in the TPN regimen plus the volume of milk/formula received), as well as the concentration (0–4% v/v) of the multivitamin preparation (Multi 12 Peadiatric®) added to TPN. The concentration of peroxides measured in photo-protected TPN bags containing 2.5% (v/v) Multi 12 Peadiatric® was 95 ± 9 μM ($n = 4$) after 24 h of use at the bed side.

Cysteine Uptake

For uptake experiments,[27–29] leukocytes were pre-incubated for 60 min with 10 volumes of buffer B (137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 5 mM DTT, 0.01% $CaCl_2$, 0.01% $MgCl_2$, 0.1% glucose) at 37°C. The cells were then washed once with buffer C (buffer B without glucose) and incubated in the same buffer containing 500 μM L-cysteine, 0.9 μCi L-[^{35}S] cysteine for 15 min at 37°C followed rapidly by a centrifugation and three washes with 1 ml of ice-cold PBS. The pellet was suspended in 250 μl PBS and frozen at −80°C to lyse cells. The radioactivity of the supernatant was determined. TAC were incubated in a similar fashion. Under those experimental conditions the cysteine uptake was found to be linear up to 30 min.[27–29] Each experiment was done in triplicate with a blank at time zero.

Glutathione Determination

Glutathione concentrations in cells derived from TAC were measured according to Griffith *et al.*.[30] Briefly, 0.6 mM DTNB, 0.2 mM NADPH and 10 μg of glutathione reductase were added to samples in a 1 ml total volume. The increased absorbance read at 412 nm was compared to those obtained with different concentrations of GSSG. Results were reported as GSH equivalent by 10^6 cells.

Statistical Analysis

For leukocytes derived from cord blood, the effect of gestation was treated by ANOVA for gender and maturity (preterm versus term). The effect of postnatal age, was analyzed by ANCOVA for gender and the three postnatal age groups, with gestational age as covariable. FiO_2 and nutrient intakes were compared between groups by ANOVA. To evaluate the effect of cysteine uptake on cellular glutathione content, a correlation was sought between these two parameters. The Bartlett's Chi square tested homogeneity of variances. All data are reported as mean ± SEM and are orthogonally compared. The level of significance was set at $p < 0.05$.

RESULTS

In leukocytes derived from cord blood, cysteine uptake was significantly ($F_{(1,21)} = 10.4$, $p < 0.01$) higher in term compared to preterm girls (Fig. 1). There was no effect of maturity on cysteine uptake ($F_{(1,21)} = 0.04$) in cells derived from boys.

In TAC, postnatal age had a positive effect ($F_{(1,24)} = 5.8$, $p < 0.05$) on cysteine uptake in cells derived from girls (Fig. 2). However, it was not affected ($F_{(1,24)} = 0.7$) by gestational age which varied from 24 to 36 weeks. In TAC derived from girls, the correlation between cysteine uptake and glutathione content was significant ($r^2 = 0.78$, $p < 0.01$, Fig. 3), while in TAC derived from boys no significant correlation was found ($r^2 = 0.0$, Fig. 4). As amino acid intake[24] as well as infused multivitamins[3] and oxygenation[31] influence glutathione metabolism,

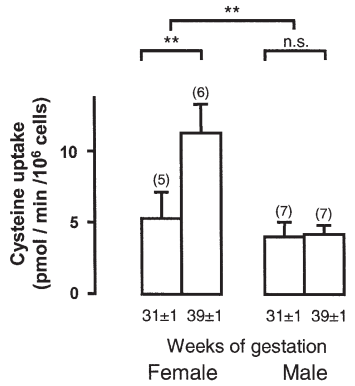

FIGURE 1 Cysteine uptake by leukocytes derived from cord blood of preterm and term infants. Data represent the mean ± SEM; sample size is shown at the top of each bar (n). In cells derived from females, uptake was lower (**$p < 0.01$) in preterm infants. While in cells derived from boys there was no increase in uptake with gestational age.

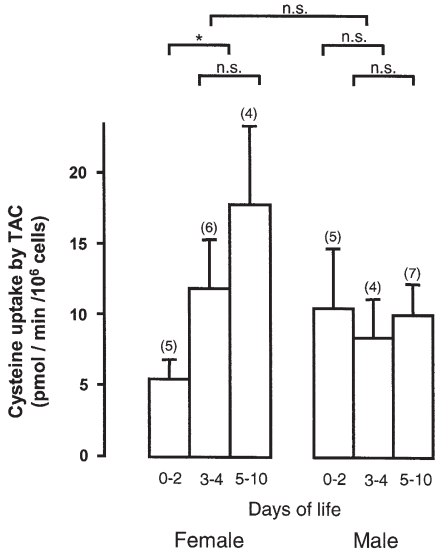

FIGURE 2 Cysteine uptake by TAC (tracheal aspirate cells) derived from intubated and ventilated infants, according to postnatal age. Data represent the mean ± SEM; sample size is shown at the top of each bar (n). Postnatal age was associated with a significant (*$p < 0.05$) increase in uptake in cells derived from female, while it did not affected uptake in cells from boys.

these parameters were compared between gender. There were no gender-related differences ($F_{(1,25)} < 0.8$).

DISCUSSION

Because of the neonatal immaturity of cystathionase activity[32,33] which contributes to limit the conversion of methionine to cysteine,[17] it has been advocated that this essential amino acid be added to TPN regimens. However, this has not been shown to result in a beneficial effect on nitrogen balance in parenterally fed human neonates.[34,35] These findings could be explained by the gender-related

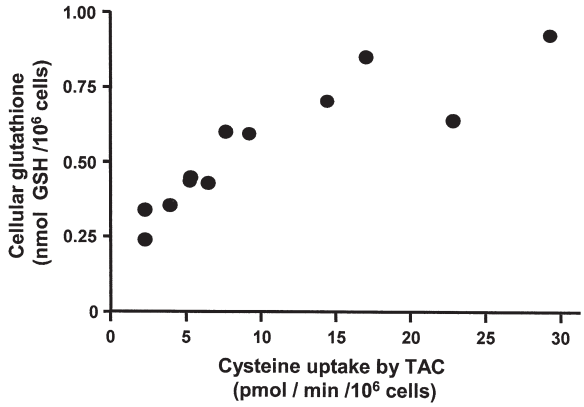

FIGURE 3 Correlation between intracellular glutathione and cysteine uptake in TAC (tracheal aspirate cells) derived from female infants. The linear correlation ($r^2 = 0.78$, $p < 0.01$) documents that in girls cysteine uptake accounts for 78% of the variability in cellular glutathione content.

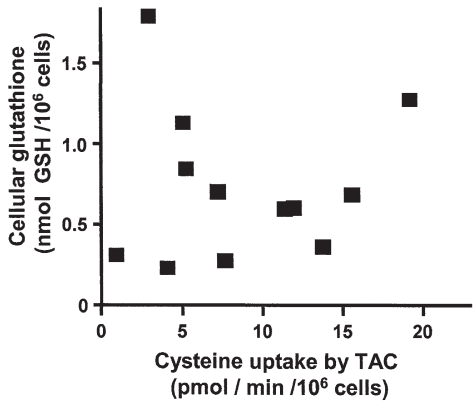

FIGURE 4 Correlation between intracellular glutathione and cysteine uptake in TAC (tracheal aspirate cells) derived from male infants. No correlation was demonstrated.

differences in maturation of cellular cysteine uptake as documented in the present study. In accordance with the aim of the study, cysteine uptake appears to be the rate limiting step accounting for the reported gender-related differences in glutathione metabolism as well as the low levels of this tripeptide found in premature infants.[5]

As glutathione synthesis is self-regulated,[36,37] the effect of cysteine uptake on cellular glutathione level will be observed when GSH consumption occurs. In response to a peroxide load, the glutathione loss from endothelial cells derived from boys is greater than in cells from girls.[11] Consequently, we would expect to observe a correlation between cysteine uptake activity and GSH cellular levels in boys when such a correlation is observed in girls. Indeed, the results show that the maturation of baby girls influences cysteine uptake, which is responsible for 78% ($r^2 = 0.78$) of the variation in glutathione content. However, cysteine uptake did not explain variations in glutathione content in samples from boys. A further variable affecting glutathione

consumption is the level as well as the source of oxidant stress. Peroxides generated in TPN have been associated to the consumption of glutathione,[3] while oxygen supplementation induces glutathione synthesis[14,31,38] leading to increased GSH levels. It is unlikely that the gender-related variations in correlation between cellular cysteine uptake and glutathione concentrations found in this study were related to differences in treatment, as nutritional support and oxygenation did not differ between genders. Therefore, factors other than availability or uptake of substrate might account for the absence of correlation noted in specimens from boys.

The role of maturation on cysteine uptake was documented by evaluating the effect of gestational age and postnatal age in leukocytes. But these cells were derived from separate sources, namely cord blood and TAC. Changes in cytology with gestational age or postnatal age could account for the modifications in cysteine uptake observed in cells derived from girls (Figs. 1 and 2). However, the activity measured in leukocytes from cord blood or in TAC derived from boys suggests that differences in cytology did not influence the activity of cysteine uptake. In TAC, neutrophils represent the main leukocytes in the first four days of life, whereas macrophages are prominent by day eight.[39–41] In cord blood leukocyte count increases with gestation. The differential count of white blood cells varies with gestational age. While lymphocytes predominate until 37–38 weeks gestation, the proportion of neutrophils increases from 32 weeks to become the commonest leukocyte at term.[42] But in children, there is no difference in leukocyte counts related to gender.[43] Therefore, it is unlikely that observed differences were related to changes in cytology. The effect of gestational age observed in cells from cord blood (Fig. 1) was not found in TAC. This discrepancy could be explained by a maturation of cysteine uptake occurring in the last month of gestation, as the range of gestational age of infants from whom TAC were sampled, was limited from 24 to 36 weeks. Also, because cysteine availability is a limiting step in glutathione synthesis, variables known to influence glutathione metabolism such as postnatal events and environmental factors might have a bearing on cysteine uptake in TAC, thereby masking the effect of gestational age.

In the clinical conditions under which the TAC were sampled, factors such as nutrient intake and oxidant stress could have influenced glutathione metabolism and accounted for some of the gender-related variability in cysteine uptake observed with postnatal age. Indeed, the provision of amino acids as well as oxygen supplementation are known to stimulate glutathione synthesis,[31] while peroxides inhibit it.[3,13] Although the present study was not designed for that purpose, the influence of such factors could not be shown. The higher level of oxygenation received by boys in the 0–2 days group and in the 5–10 days group for girls (Table I) is not consistent with the observed gender-related differences.

The gender-related differences in cysteine uptake or glutathione metabolism are not limited to cells from cord blood or TAC. In response to an oxidant challenge, endothelial cells from girls exhibited a greater stimulation of the activity of glutathione reductase associated with a modulation of vasoreactive mediators.[5,11] These gender-related differences may underline physiologic variations in the maturity of newborn infants as reported for human lung maturation.[44] The curve of glutathione levels in function of gestational age[5] suggests a slower maturation in boys. In view of reported gender-related differences in morbidity[45–47] and developmental IQ[48] in favor of preterm girls, the present observation opens new fields of investigation into protein metabolism and antioxidant defenses.

For a comparable gestational age we have previously[5,16] reported a lower glutathione content in TAC (0.15–0.2 nmol/10^6 TAC) than what was measured in the present study (between 0.2 and 1.5 nmol/10^6 TAC). This could be explained by a modification in the source of parenteral multivitamins received by these infants, as the peroxides generated in multivitamins produce a drop in glutathione in endothelial cells[13] as well as in lungs of guinea pigs.[3] In the previous reports, infants received MVI Pediatric (Rhone-Poulenc) generating $141 \pm 13\,\mu M$ peroxides in the photoprotected TPN bag (before the addition of lipids),[4] while in the present study they received Multi-12 pediatric (Sabex) which generates $95 \pm 9\,\mu M$ ($n = 4$) peroxides under the same conditions, and multivitamin concentration. In both of these studies the same cysteine-free amino acid solution was used. Because of the immaturity of the conversion of methionine to cysteine[17] it is questionable whether we would have found similar results if the infused TPN regimen contained cysteine.

With the provision of cysteine in the parenteral nutrition regimen, plasma cysteine concentrations were 50% higher in preterm compared to term infants.[49] This raises questions about the bioavailability of infused cysteine in preterm infants and supports the notion of a maturational process of cellular cysteine uptake. On the other hand, parenteral cysteine supplementation had a positive effect on glutathione levels in various newborn animal models[50,51] suggesting bioavailability of this essential amino acid. The discrepancy between results obtained in those animal models and human neonates could be related to the immaturity of cellular cysteine uptake in humans.

From the results of the present study it appears that the postnatal pattern of maturation of cysteine uptake in cells isolated from female infants is comparable to that of the γ-GT system.[16] But the activity of γ-GT in TAC derived from 6-day-old infants is about 300–600 times higher than the activity of cysteine uptake (6000 versus 10–18 pmol/min/10^6 cells). Therefore, supplementing parenteral nutrition with glutathione rather than with cysteine should be envisaged to respond to increased demands for cysteine[52] and to boost glutathione mediated antioxidant defenses in premature infants. This is supported by a recent report by Stabler *et al*.[53] documenting that systemic cysteine concentrations were higher when the parenteral regimen fed to a primate model contained glutathione instead of cysteine.

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