Importance of Monitoring Glutathione Level as a Marker for Oxidative Stress in Urea Cycle Disorders

Ketki V Kudalkar*, Anil B Jalan, Debjani Dasgupta

Abstract

Background: Oxidative stress (OS) is a major factor affecting pathogenicity in various inborn errors of metabolism. There is a possible role of free radical generation in the pathophysiology of neurodegenerative disorders, including some urea cycle disorders (UCDs).

Aim: To analyze the reduced level of blood glutathione (GSH) in neonates with UCDs as a parameter to monitor the effect of OS in them

Materials and Methods: The GSH level was analyzed in 24 neonates with UCDs: 1 neonate with carbamoyl phosphate synthetase deficiency (CPSD)/N-acetylglutamate synthetase deficiency (NAGSD), 4 with ornithine transcarbamylase deficiency (OTCD), 15 with argininosuccinate synthetase deficiency (ASSD), 3 with argininosuccinate lyase deficiency (ASLD), and 1 with arginase deficiency (ARGD).

Results and Discussion: This study demonstrated a lower level of GSH in neonates with UCDs (24.28 ± 14.97 nmol/mg Hb; n = 24) compared with normal controls (46.64 ± 17.51 nmol/mg Hb; n = 54). A considerably lower level of GSH was observed in neonates with CPSD/NAGSD (19.61 nmol/mg Hb; n = 1), OTCD (22.15 ± 6.59 nmol/mg Hb; n = 4), ASSD (24.42 ± 14.98 nmol/mg Hb; n = 15), and ASLD (24.11 ± 3.91 nmol/mg Hb; n = 3). The GSH level in a neonate with ARGD was comparable to that in the controls (33.91 nmol/mg Hb). Neonates who succumbed in the
Introduction

Urea cycle disorders (UCDs) constitute a group of rare congenital disorders caused by a deficiency of the enzymes or transport proteins required to eliminate ammonia from the body. The result of these disorders is hyperammonemia. Excess ammonia enters the central nervous system (CNS) and exerts its toxic effects. The morbidity and mortality in these disorders depend on the age of onset and the duration and severity of hyperammonemia.1-4

The brain is vulnerable to the harmful effects of ammonia, which may lead to cognitive impairment, seizures, cerebral palsy, and cerebral edema.5 Ammonia exposure alters several amino acid pathways and neurotransmitter systems and affects nitric oxide synthesis, signal transduction, cerebral energy metabolism, and oxidative stress (OS), which may ultimately lead to brain energy deficit and neuronal death.6-8

OS has been proposed as an important pathogenic feature in various inborn errors of metabolism (IEMs), including organic aciduria, aminoaciduria, and UCDs.9-12 OS associated with the presence of an increased concentration of ammonia in the brain has been proposed as a possible mechanism involved in ammonia toxicity in UCDs. OS reflects exposure to free radicals that are not neutralized by the antioxidant defences of the cell. Free radical generation resulting in OS is a sign of mitochondrial dysfunction.13

The exact mechanism of OS in UCDs is unknown, although production of free radicals and reduced levels of antioxidants such as GSH are likely the contributors of disease pathogenesis.14 Garcia et al15 proposed that the association of OS and an elevated concentration of ammonia in the brain is a possible mechanism of ammonia toxicity in the brain. Hyperammonemia was shown to diminish the activity of antioxidant enzymes, for example, superoxide dismutase, and was associated with the depletion of antioxidants, for example, GSH.16 Prestes et al17 have shown that accumulation of citrulline and ammonia (citrullinemia) reduces the antioxidant capacity of rat brain cells. This diminished activity of antioxidant mechanisms and increased superoxide radical could lead to cell damage.

neonatal period had a lower level of GSH compared with others in the same group (16.24 ± 13.33 nmol/mg Hb; n = 11), indicating that antioxidant mechanisms could be severely impaired in critically ill patients. The GSH level was found to vary inversely with ammonia level in a neonate with ASSD (P < .01). There was a significant improvement in GSH level upon controlling the ammonia level.

Conclusion: Patients with UCDs have a considerably reduced level of GSH, indicating increased OS. Thus, estimation of the reduced GSH level in these patients may serve as a useful indicator of their OS.

UCDs are associated with increased OS, and it may be associated with the neurologic outcomes; hence, OS should be monitored, and therapies that improve the redox imbalance may help in improving the outcome of these patients.

Key Words: Glutathione, oxidative stress, urea cycle disorders, inborn errors of metabolism, ammonia, neurologic
OS is also related to nitric oxide imbalance in UCDs. Some studies have linked energy deficit with hyperammonemia. It has also been shown to increase lipid peroxidation and membrane damage. Metabolism of ammonia to glutamine in the brain is proposed to cause osmotic disturbance in the brain. Glutamine also mediates the production of reactive oxygen species (ROS) and increases mitochondrial permeability, which is initiated by ROS. Presence of OS in the brain without suitable defences may aggravate the CNS condition.

GSH, the most abundant low-molecular-weight thiol compound synthesized in the cells, plays an important role in protecting the cells from oxidative damage by neutralizing many ROS. During this process, it is transformed to its oxidized form (GSSG). In individuals with IEMs such as mitochondrial disorders, organic acidemia, and UCDs, there is an increased generation of ROS; hence, it is likely that the utilization of GSH to neutralize the excess ROS is higher in those with IEMs than in individuals with normal mitochondrial function, which thus results in deficiency of GSH in the IEM population. In times of metabolic crisis, ROS production is increased, which could lead to fast exhaustion of GSH stores and consequently reduced cellular capacity to neutralize these intermediates. This may explain why genetic disorders that affect mitochondrial function or GSH homeostasis rapidly worsen in times of intercurrent catabolic illness that may result in overproduction of oxidants.

Despite this relationship of OS in the progression of metabolic disorders, very few reports have correlated the GSH level in patients with UCDs. With these considerations in mind, this study analyzed blood samples of newborns with UCDs, speculating that increased OS in them would result in a low GSH level. Lower level of GSH was indeed seen in patients with all UCDs, especially ornithine transcarbamylase deficiency (OTCD), argininosuccinate synthetase deficiency (ASSD), and argininosuccinate lyase deficiency (ASLD). This correlation between GSH level and disease etiology offers a unique outlook to measure OS in IEMs.

**Aim**
To analyze the reduced level of blood GSH in neonates with UCDs as a parameter to monitor the effect of OS in them

**Materials and Methods**
To assess the redox status of neonates with UCDs, the level of GSH in erythrocytes of these neonates and in normal controls was analyzed.

The study population consisted of 24 neonates with UCDs (age, 2 d–9 mo; mean age: 6.55 ± 3.67 mo), 24 normal children (mean age: 4.02 ± 3.95 mo), and 54 adult controls (mean age: 33.33 ± 16.25 y). The patient group consisted of neonates diagnosed with UCDs: 1 neonate with carbamoyl phosphate synthetase deficiency (CPSD)/N-acetylglutamate synthetase deficiency (NAGSD), 4 with OTCD, 15 with ASSD, 3 with ASLD, and 1 with arginase deficiency (ARGD). The diagnosis was based on clinical signs and symptoms as well as biochemical analyses of their blood and urine. All the controls were healthy children or adults with no known symptom associated with any IEM. None of the neonates or controls was taking any kind of antioxidant supplements at the time of analysis. Informed consents were obtained from the parents of the neonates with UCDs and the controls.

Estimation of reduced GSH in the peripheral blood samples from these neonates was done as an integral part of their basic investigations. The erythrocytes were separated and hemolyzed immediately, and the protein fraction was precipitated with metaphosphoric acid. The supernatants were stored until analysis. Biochemical colorimetric analysis for quantification of GSH was performed by enzymatic recycling method, using GSH reductase and Ellman reagent. The Ellman reagent (5,5’-dithio-bis-2-[nitrobenzoic acid], ie, DTNB) reacts with the sulfhydryl group of GSH to produce a yellow derivative 5-thio-2-nitrobenzoic acid, that is, TNB, which can be estimated by measuring the absorbance at 405 to 414 nm. The level of GSH was calculated in nmol/mg Hb of the hemolysate for each sample. The GSH level in neonates was compared with that of the controls. Statistical analyses were performed using Student t test and Mann–Whitney U test.
Results

The GSH level in the peripheral blood samples from neonates with UCDs was measured. The GSH level in them (24.28 ± 14.97 nmol/mg Hb; n = 24) was significantly reduced compared with the level in normal adult controls (46.64 ± 17.51 nmol/mg Hb; n = 54) and normal children (30.89 ± 15.35 nmol/mg Hb; n = 24) (Table 1 and Figure 1). A considerably lower level of GSH was observed in neonates with CPSD/NAGSD (19.61 nmol/mg Hb; n = 1), OTCD (22.15 ± 6.59 nmol/mg Hb; n = 4), ASSD (24.42 ± 14.98 nmol/mg Hb; n = 15) (Figure 2), and ASLD (24.11 ± 3.91 nmol/mg Hb; n = 3) (Figure 3). One patient with ARGD was included in this study, in whom the reduced level of GSH was comparable to that in the controls (33.91 nmol/mg Hb). The results given in Table 1 indicate that the level of GSH in neonates with UCDs is significantly lower compared with that in normal controls.

Table 1. GSH Level in Controls and Neonates With UCDs

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Individuals, n</th>
<th>Mean Level of GSH, Mean ± SD, nmol/mg Hb</th>
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<tbody>
<tr>
<td>Normal Adults</td>
<td>54</td>
<td>46.64 ± 17.51</td>
</tr>
<tr>
<td>Normal Children</td>
<td>24</td>
<td>30.89 ± 15.35</td>
</tr>
<tr>
<td>Neonates With UCDs</td>
<td>24</td>
<td>24.28 ± 14.97</td>
</tr>
<tr>
<td>CPSD/NAGSD</td>
<td>1</td>
<td>19.61</td>
</tr>
<tr>
<td>OTCD</td>
<td>4</td>
<td>22.15 ± 6.59</td>
</tr>
<tr>
<td>ASSD</td>
<td>15</td>
<td>24.42 ± 14.98</td>
</tr>
<tr>
<td>ASLD</td>
<td>3</td>
<td>24.11 ± 3.91</td>
</tr>
<tr>
<td>ARGD</td>
<td>1</td>
<td>33.91</td>
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</tbody>
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ARGD, arginase deficiency; ASLD, argininosuccinate lyase deficiency; ASSD, argininosuccinate synthetase deficiency; CPSD, carbamoyl phosphate synthetase deficiency; GSH, glutathione; NAGSD, N-acetylglutamate synthetase deficiency; OTCD, ornithine transcarbamylase deficiency; UCD, urea cycle disorder.

Discussion

OS plays an important role in the pathogenesis of many diseases. Mitochondrial disorders, including the respiratory chain defects or defects in branched chain amino acid metabolism (organic acidemias) and UCDs, have been shown to be associated with diminished redox balance. Dysfunction of the respiratory
chain decreases ATP production and increases the production of intracellular ROS and reactive nitrogen species (RNS).\textsuperscript{33} Respiratory chain abnormalities have also been documented in various organic acidemias, such as methylmalonic acidemia and propionic acidemia.\textsuperscript{34-36} OS associated with an increased concentration of ammonia in the brain in individuals with UCDs has been proposed as a probable mechanism involved in ammonia toxicity in UCDs. The exact mechanism of OS in UCDs is not known, but the production of free radicals and decreased intracellular GSH level are probable contributors to disease pathogenesis.\textsuperscript{37}

The extremely transient nature of ROS and RNS makes their estimation in routine testing complicated. However, increased OS can be indirectly identified from the decrease in the GSH level, thus making GSH measurement a more stable index of cellular redox status.\textsuperscript{38} As GSH is the main antioxidant in mammalian cells, a decrease in its intracellular level, regardless of the mechanism, indicates chronic OS in patients with UCDs.

UCDs occur because of the inability to detoxify nitrogen and are characterized by severe hyperammonemia, respiratory alkalosis, and clinical symptoms such as altered sensorium, refusal to feed, vomiting, and failure to thrive. In this study, in neonates with UCDs, the GSH level decreased with an increase in the ammonia level. The 15 neonates with ASSD presented with lethargy, vomiting, convulsions, and refusal to feed in the early neonatal life. Of these, 4 survived, while the rest succumbed to life-threatening episodes, such as infections, seizures, and coma, during the neonatal period. The neonates who survived were followed up on a regular basis, and various clinical and biochemical parameters were monitored, including citrulline, ammonia, and reduced GSH level. The main objective of this study was to evaluate the importance of GSH measurement in patients with UCDs. As the GSH level in these patients would indicate their cellular redox status, we hypothesize that the decreased GSH level and hence increased OS in these patients would affect the disease prognosis.

The low level of GSH in these neonates could indicate that they suffer from systemic OS during times of intercurrent metabolic decompensations. Some studies have suggested that redox imbalance plays a major role in the pathogenic effects seen in patients with metabolic diseases.\textsuperscript{39} These reports are supported by studies documenting an increased production of ROS, a decreased level of GSH, and a compensatory increase in antioxidant enzymes in blood samples from patients with UCDs.\textsuperscript{40,41} Our results support these observations that UCDs, except for ARGD, are probably associated with increased OS, which may lead to decreased levels of cellular antioxidants, especially the key antioxidant GSH. A decreased level of GSH may thus affect the outcome in these disorders.

The reduced GSH level in the neonates who could not survive the neonatal period was lower than that in the others in the group (16.24 ± 13.33 nmol/mg Hb; \( n = 11; \ P < .01 \)), indicating that antioxidant mechanisms could be severely impaired in critically ill neonates. A lower level of GSH implies a greater degree of OS in these neonates at the time of metabolic crisis (Table 2 and Figure 4). Increased OS has been implicated in the pathophysiology of UCDs such as ASSD, OTCD, and ASLD.

We also found the GSH level to vary inversely with the ammonia level, indicating OS with an increased level of this metabolite. One neonate presented in the neonatal period with hyperammonemia, failure to thrive, vomiting, and lethargy. Metabolic studies were performed on day 1 of life because of a family history of ASSD. This neonate was diagnosed with ASSD with significantly elevated levels of citrulline, ammonia, and orotic acid. Prompt treatment was started on day 2. The neonate was followed up on a regular basis, and his ammonia, citrulline, arginine, and GSH levels were analyzed.

<table>
<thead>
<tr>
<th>Table 2. GSH Level in Patients With ASSD</th>
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<tbody>
<tr>
<td><strong>Group</strong></td>
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<tr>
<td>Neonates With ASSD Who Died (( n = 11 ))</td>
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<tr>
<td>Neonates With ASSD Who Are Alive (( n = 4 ))</td>
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GSH level was significantly decreased in patients who did not survive.
ASSD, argininosuccinate synthetase deficiency; GSH, glutathione.
at each visit. His GSH level was very low at the first presentation. A significant and gradual increase in GSH level was observed upon improvement of ammonia status. Figure 5 shows the trend in the levels of GSH, ammonia, citrulline, and arginine over the 2-year follow-up of this neonate. He had frequent episodes of metabolic derangements and needed frequent hospitalizations. During these intercurrent metabolic crises, an increase in ammonia level and decrease in GSH level were observed, indicating increased OS during this period. The GSH level did not correlate significantly with citrulline and arginine levels, indicating that citrulline and arginine may not have an important role in inducing OS in these neonates as much as ammonia did.

There is growing interest in recognizing biomarkers of OS in human diseases. Our results indicate that GSH level may be a useful biomarker for assessing the degree of OS in UCDs and even analyzing the response to therapy in these patients. Because GSH deficiency may play a role in the pathogenesis of neurodegenerative diseases, it is also judicious to speculate that therapies that improve redox imbalance may be beneficial for cognitive and neurologic outcomes in UCDs. Presently, antioxidant supplements are often given to these patients, without the ability to monitor the therapeutic response. Assessing the degree of OS in these disorders using GSH level may thus be beneficial to improve the outcome. These findings thus provide the foundation to embark on further studies focusing on the relationship of factors such as age, specific diagnosis, disease severity, clinical status, and treatment of GSH deficiency.

Conclusions

UCDs are associated with increased OS. The reduced GSH level was seen to vary with toxic metabolites, and a low level of GSH was seen during times of intercurrent metabolic decompensation. The GSH level was significantly low in neonates who died. Hence, a reduced GSH level may be associated with the neurologic outcome. Thus, OS should be monitored in all IEMs including UCDs. Therapies that improve the redox imbalance may be neuroprotective and help in improving the survival of these patients.

References

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