Can urinary excretion rate of malondialdehyde, uric acid and protein predict the severity and impending death in perinatal asphyxia?

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Abstract

Background: Perinatal asphyxia (PA) associated with multi-organ damage is a leading cause of neonatal mortality and morbidity. We evaluated if urinary malondialdehyde:creatinine (UMDA:Cr), uric acid:creatinine (UUA:Cr) and protein:creatinine (UP:Cr) vary with the severity of PA and if these parameters can predict the impending death in PA.

Methods: Study included 20 asphyxiated and 20 healthy newborn males. Hypoxic-ischemic encephalopathy (HIE) staging, APGAR (activity, pulse, grimace, appearance and respiration) score and urinary protein, uric acid, creatinine and MDA were evaluated.

Results: UMDA:Cr, UUA:Cr and UP:Cr were significantly higher and correlated with APGAR and HIE in PA. By regression analysis also, urinary parameters were found to have significant association with HIE stage and APGAR in PA. Receiver operating characteristics (ROC) curve of UP:Cr, UUA:Cr and UMDA:Cr showed area under curve of 0.896 (p=0.003), 0.859 (p=0.008) and 0.849 (p=0.010) with cut-off value of 9.04 mg, 2.34 mg and 3.49 µg/mg of creatinine respectively that can optimally predict the impending death in PA. SDS-PAGE of unconcentrated urine detected both high (73 kDa and 68 kDa) and low molecular weight proteins (52 kDa, 47 kDa, 25 kDa and 20 kDa) in PA but not in controls.

Conclusion: urinary excretion rate of uric acid, MDA and proteins is higher and has potential to act as biochemical markers for severity evaluation and death prediction in PA.

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Keywords: Malondialdehyde; Oxidative stress; Perinatal asphyxia; Proteinuria; Uric acid

Introduction

Perinatal asphyxia (PA) is an insult to the fetus or the newborn due to lack of oxygen (hypoxia) and/or lack of perfusion (ischemia) to various organs of sufficient magnitude and duration to produce more than fleeting functional and/or biochemical changes. Neonatal morbidity and mortality is higher in these cases [1] and PA is the single most important cause of stillbirths (45.1%) and neonatal deaths (28.8%). Overall incidence of asphyxia is reported to vary from 1 to 1.5% [2]. Hypoxic-ischemic encephalopathy (HIE) is common in extramural births with PA that contributes to 15%–20% of death during the neonatal period and 30% of those who survive suffer from neuro-developmental disorders, such as cerebral palsy and mental retardation [3]. Perinatal asphyxia can result in multisystem organ damage in a neonate. It has been reported that hypoxia and ischemia can cause damage to various organs like kidneys (50%), central nervous system (28%), cardiovascular system (25%) and lungs (23%) [4]. Moreover, kidneys are very sensitive to oxygen deprivation and irreversible cortical necrosis may occur due to prolonged renal insufficiency of hypoxic-ischemic episode. Perinatal asphyxia contributes to most of the neonatal renal failure [5]. It may cause alterations in urinary protein excretion [6–8].

Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) are reported to accumulate in asphyxia, which cause accumulation of adenosine, inosine and hypoxanthine [9]. These substrates are channelised to purine catabolism leading to generation of uric acid. As a result there is increased urinary...
excretion of uric acid. In the re-oxygenation period, free radicals are produced parallel to uric acid formation, which is claimed as an indicator of the severity of perinatal asphyxia [10].

The severity of birth asphyxia is commonly assessed using APGAR score and neurological damage is evaluated by HIE staging after delivery [11,12]. Perinatal asphyxia and its neurologic manifestations (hypoxic-ischemic encephalopathy) is the most important cause of brain injury and neurologic sequelae in full-term infants [13].

Longitudinal S100B protein, retinol binding protein, myoglobin and activin A level in urine have been claimed to predict the risk of hypoxic-ischemic encephalopathy and its possible neurologic sequelae soon after birth of asphyxiated infants [6–8]. Most of the reported markers except a few like urinary longitudinal S100B protein and activin A were seen in blood. Chu et al. hinted about some potential urinary markers for good and poor outcome of asphyxiated neonates from his metabolomic analysis [14]. There is hardly any ideal urinary biochemical marker to predict the severity of perinatal asphyxia and its associated brain damage. Proteinuria and hematuria were reported in asphyxiated neonates and those babies with HIE I and II had urinary anomalies [15]. If the evaluation of urinary protein excretion rate in first few days of birth can predict the neurological sequelae in asphyxiated neonates is not known.

Reperfusion in perinatal asphyxia is known to be associated with oxidative stress [16,17]. Oxidative stress leading to carbonylation, fragmentation, cross-linking and loss of thiol groups and nitration of proteins is observed in perinatal asphyxia [18]. Oxidation of fat cells produces residues called thiobarbituric acid reactive substances (TBARS) such as malondialdehyde. The measurement of MDA in urine is a well-documented method for evaluating oxidative damage [19].

A systematic study to evaluate the significance of proteinuria in asphyxia cases has not been done. Taking into account of all the above, the present study was designed to evaluate if the severity of perinatal asphyxia and its associated brain damage in male newborn babies can be assessed from urinary protein creatinine ratio, TBARS creatinine ratio, uric acid creatinine ratio and whether any of these parameters could predict the impending death in PA.

### Materials and methods

#### Study subjects

The study recruited 20 normal healthy male babies (without birth asphyxia) and 20 male neonates with perinatal asphyxia (as evidenced by any 2 or more of the following conditions like cord blood of pH < 7.1, APGAR score of ≤ 7 immediately after 5 min of birth, meconium stained liquor, abnormalities of fetal heart rate and clinical evidence of HIE) [20] from Neonatal Intensive Care Unit of Department of Pediatrics, JIPMER, Puducherry, India. The degree of HIE was evaluated with Sarnat and Sarnat’s clinical staging system as mild (stage I), moderate (stage II) and severe (stage III) [21]. We excluded neonates of mothers with chronic maternal ill health or any other major complications like pregnancy induced hypertension, gestational diabetes etc. Neonates with intra-uterine infections, malformations or intra-uterine growth retardation were also excluded from this study. This study was approved by institute human ethics committee and research council. Informed consent was obtained from the mothers of all neonatal subjects included in the study.

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (N=20)</th>
<th>Perinatal asphyxia Total (N=20)</th>
<th>Perinatal asphyxia Survived cases (N=12)</th>
<th>Perinatal asphyxia Dead cases (N=08)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (kg)</td>
<td>2.95±</td>
<td>2.54±</td>
<td>2.41±</td>
<td>2.62±</td>
</tr>
<tr>
<td></td>
<td>0.19±</td>
<td>0.31±</td>
<td>0.31±</td>
<td>0.33±</td>
</tr>
<tr>
<td></td>
<td>(2.6–3.33)</td>
<td>(1.92–3.0)</td>
<td>(1.92–3.0)</td>
<td>(2.04–3.0)</td>
</tr>
<tr>
<td>APGAR score at 5 min of birth</td>
<td>8.4±</td>
<td>4.20±</td>
<td>5.42±</td>
<td>2.50±</td>
</tr>
<tr>
<td></td>
<td>(7–10)</td>
<td>(1–7)</td>
<td>(2–7)</td>
<td>(1–5)</td>
</tr>
<tr>
<td>Urinary protein creatinine ratio (mg protein/mg of creatinine)</td>
<td>1.61±</td>
<td>7.74±</td>
<td>5.25±</td>
<td>11.49±</td>
</tr>
<tr>
<td></td>
<td>(1.06–2.81)</td>
<td>(2.99–12.99)</td>
<td>(2.9–10.5)</td>
<td>(7.5–12.9)</td>
</tr>
<tr>
<td>Urinary uric acid creatinine ratio (mg uric acid/mg of creatinine)</td>
<td>0.91±</td>
<td>2.30±</td>
<td>1.90±</td>
<td>2.87±</td>
</tr>
<tr>
<td></td>
<td>(0.63–1.33)</td>
<td>(0.63–3.65)</td>
<td>(0.63–3.11)</td>
<td>(1.60–3.65)</td>
</tr>
<tr>
<td>Urinary MDA creatinine ratio (µg of MDA/mg of creatinine)</td>
<td>1.32±</td>
<td>3.05±</td>
<td>2.28±</td>
<td>4.06±</td>
</tr>
<tr>
<td></td>
<td>(0.71–1.94)</td>
<td>(0.99–3.52)</td>
<td>(0.99–1.83)</td>
<td>(2.49–5.22)</td>
</tr>
</tbody>
</table>

*Correlation coefficient (ρ) is significant at ρ<0.01.

### Table 2

Correlation coefficient (ρ) among APGAR score, HIE staging, urinary protein creatinine ratio, urinary uric acid creatinine ratio and urinary TBARS creatinine ratio in perinatal asphyxia cases

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HIE staging</th>
<th>Urinary protein creatinine ratio (mg protein/mg of creatinine)</th>
<th>Urinary uric acid creatinine ratio (mg uric acid/mg of creatinine)</th>
<th>Urinary MDA creatinine ratio (µg of MDA/mg of creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APGAR score at 5 min</td>
<td>−0.899*</td>
<td>−0.723*</td>
<td>−0.661*</td>
<td>−0.706*</td>
</tr>
<tr>
<td>HIE staging</td>
<td>0.811*</td>
<td>0.789*</td>
<td>0.901*</td>
<td>0.796*</td>
</tr>
<tr>
<td>Urinary protein creatinine ratio</td>
<td>0.692*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary uric acid creatinine ratio</td>
<td>0.862*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Correlation coefficient (ρ) is significant at ρ<0.01.
Urine samples were collected from the normal healthy neonates and neonates with PA by using urine continence bag on day one of birth. It was the first and/or second void urine. We included only male neonates as collection of uncontaminated urine samples using continence bag was easier. Approximately 20–25 mL of urine sample was collected and mixed with 1% sodium azide and centrifuged at 2500 g for 5 min. The supernatant was collected and stored at −50 °C until analysis.

**Determination of urinary protein excretion**

Urinary protein was evaluated with pyrogallol red binding assay [22] using commercial kit from Randox laboratories limited (Crumlin, UK) adapted to RX Imola random access autoanalyser (Randox laboratories limited, Crumlin, UK). Urinary creatinine was assayed by Jaffe’s method [23] using kit from Randox laboratories limited which was adapted to the same autoanalyser. The ratio of urinary protein (mg/dL) and creatinine (mg/dL) was used to determine daily protein excretion [24,25].

**Measurement of urinary uric acid**

Urinary uric acid was estimated by uricase-peroxidase enzymatic method [26] by using commercial kit purchased from Randox laboratories limited (Crumlin, UK) adapted to RX Imola autoanalyser.

**Measurement of urinary MDA**

Urinary MDA was determined by thiobarbituric acid method [27]. In brief, aliquots of 500 µL of urine were mixed with 500 µL thiobarbituric acid (1% w/v, pH 1.5) and boiled for 30 min. After cooling at room temperature, it was centrifuged at 3000 rpm for 5 min and then the absorbance of supernatant was measured at 540 nm (Systronics UV-Vis Spectrophotometer 117, Kerala, India). Concentration of MDA was calculated using molar extinction coefficient (1.56 × 10^5 M\(^{-1}\) L cm\(^{-1}\)) and molecular weight of MDA and expressed in term of microgram/deciliter (µg/dL). The rate of MDA excretion in urine was expressed in µg of MDA/mg of creatinine.

**Determination of molecular weight**

Urinary proteins were separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis from unconcentrated urine and the molecular weight of proteins was determined [28].

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**Sample collection**

Urine samples were collected from the normal healthy neonates and neonates with PA by using urine continence bag on day one of birth. It was the first and/or second void urine. We included only male neonates as collection of uncontaminated urine samples using continence bag was easier. Approximately 20–25 mL of urine sample was collected and mixed with 1% sodium azide and centrifuged at 2500 g for 5 min. The supernatant was collected and stored at −50 °C until analysis.

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**Fig. 1. Receiver operating characteristic curve of urinary (A) malondialdehyde: creatinine ratio, (B) uric acid: creatinine ratio and (C) protein: creatinine ratio with area under curve of 0.849 (p=0.010), 0.859 (p=0.008) and 0.896 (p=0.003) respectively in asphyxia cases.**

**Fig. 2. SDS-PAGE pattern of urinary proteins in neonates with perinatal asphyxia (Lanes 2–5) and healthy control babies. (Lanes 6–7). Lane 1 represents the markers. SDS-PAGE pattern of urinary proteins in neonates with perinatal asphyxia and healthy control babies shows that in neonates with perinatal asphyxia, both high molecular weight proteins (73 kDa and 68 kDa) and low molecular weight proteins (52 kDa, 47 kDa, 25 kDa and 20 kDa) were excreted. This SDS-PAGE pattern also shows that there were hardly any proteins detectable in healthy control babies. This figure is representative of all the subjects included in the study.**
used 10% polyacrylamide gel for separation and the separated proteins in the gels were stained by silver staining method [29]. The bands were scanned using ‘Quantity 1’ software with the ‘Gel documentation’ system (Bio-Rad, Segrate, Milan, Italy).

Statistical analysis

All data were analyzed by SPSS software version 13 for windows [SPSS Inc., Chicago, USA] and presented as mean± SD. Mann–Whitney U test was used for comparison of data and for finding association, Spearman’s correlation analysis was done. ROC curve was drawn to determine the area under curve and optimum cut-off value of the parameters in order to predict the severity and impending death in PA. A p<0.05 was considered as statistically significant.

Results

The mean of gestational age of 39.59±0.71 (range of 38.60–40.80) weeks in controls and 39.12±0.88 (range of 37.60–40.80) weeks in cases was not statistically different. Hypoxic-ischemic encephalopathy staging of perinatal asphyxia cases revealed that out of 20 participants, 2 had stage I, 10 had stage II and 8 had stage III.

Table 1 shows the mean and SD of APGAR score at the 5th minute after birth, urinary protein, uric acid and MDA creatinine ratios and their ranges in controls and asphyxiated neonates. The neonatal subjects had a mean birth weight of 2.95±0.19 (ranging from 2.6 to 3.33) and 2.53±0.31 (range of 1.92–3) kg in controls and cases respectively. There was no difference in birth weight between dead and survived PA cases.

Urinary protein creatinine ratio (UP:Cr), uric acid creatinine ratio (UUA:Cr) and MDA creatinine ratio (UMDA:Cr) were significantly higher in asphyxiated neonates as compared to controls. APGAR score was significantly lesser in cases as compared to control neonates. There were eight number of deaths registered among the participated cases. UP:Cr, UUA:Cr and UMDA:Cr were significantly higher and APGAR score was significantly lesser among the dead cases as compared to survived cases and controls.

Table 2 depicts the results of Spearman’s correlation analysis. UP:Cr, UUA:Cr and UMDA:Cr show a significant positive correlation with HIE staging and significant negative correlation with APGAR score. UP:Cr, UUA:Cr and UMDA:Cr also show a significant positive correlation with each others.

Receiver operating characteristics (ROC) curve of UP:Cr (Fig. 1(C)) showed area under curve (AUC) of 0.896 (p=0.003) with cut-off value of 9.04 mg/mg of creatinine (87.5% sensitivity and 91.7% specificity). ROC curve of UUA:Cr (Fig. 1(B)) and UMDA:Cr (Fig. 1(A)) shows AUC of 0.859 (p=0.008) and 0.849 (p=0.010) with cut-off value of 2.34 mg/mg of creatinine (87.5% sensitivity and 93.3% specificity) and 3.49 µg/mg of creatinine (87.5% sensitivity and 91.7% specificity) respectively to predict impending death in PA.

Fig. 2 showed the SDS-PAGE pattern of protein bands of unconcentrated urine of cases and control. Here we detected both high (73 kDa and 68 kDa) and low molecular weight proteins (52 kDa, 47 kDa, 25 kDa and 20 kDa) in PA but not in controls.

Discussion

The present study evaluated the severity of perinatal asphyxia in male newborns using APGAR score and HIE staging and attempted to explore if urinary MDA, uric acid and protein excretion rate differ between healthy controls and perinatal asphyxia and if these parameters can be used as markers to evaluate severity of PA and associated brain damage and if the same parameters can predict the impending death in asphyxiated cases. Although there was difference in birth weight of controls and PA cases, it was not different in dead and survived cases of PA (Table 1) and birth weight did not correlate with the measured urinary parameters (data not shown) indicating that birth weight was not a determinant of the variables and impending death in the present study.

Oxidative stress in perinatal asphyxia

In spite of non-specificity due to cross-reactivity with other substances, MDA as measured by thiobarbituric acid reactivity is a sensitive index of oxidative stress which is popular and clinically used [19]. Being water soluble, MDA appears in urine. Urinary MDA level is claimed to reflect the level of lipid peroxidation in vivo [29]. Urinary MDA creatinine ratio was significantly higher (p<0.01) in newborn babies with perinatal asphyxia as compared to healthy controls (Table 1). This indicates the association of perinatal asphyxia with oxidative stress. Our results are in agreement with the results of previous studies who demonstrated a rise in oxidative stress in perinatal asphyxia [30,31]. A significant negative correlation between urinary MDA creatinine ratio and APGAR score and a significant positive correlation between urinary MDA creatinine ratio and HIE staging (Table 2) indicate that oxidative stress level is dependant on severity of perinatal asphyxia and a high level of urinary MDA may be indicative of the extent of brain damage induced by perinatal asphyxia. As indicated by urinary MDA creatinine ratio (Table 1), oxidative stress level was significantly higher in neonates who died of PA as compared to the survived babies. An ROC curve (Fig. 1(A)) shows that urinary MDA creatinine ratio can be used to predict death in perinatal asphyxia. At the cut-off level of 3.495 µg of MDA/mg of creatinine, the sensitivity and specificity of predicting death in perinatal asphyxia were 87.5% and 91.7% respectively.

Uric acid excretion in perinatal asphyxia

A significantly higher (p<0.01) urinary uric acid creatinine ratio in asphyxiated newborns as compared to control babies (Table 1), a significant positive correlation between urinary uric acid creatinine ratio and HIE staging and a significant negative correlation between urinary uric acid creatinine ratio and APGAR score (Table 2) indicate that uric acid excretion rate in urine increases with severity of perinatal asphyxia. Such higher levels of urinary uric acid creatinine ratio and a correlation with HIE stage has been observed by others also [32]. Uric acid is a product of ATP catabolism which is known to be enhanced in perinatal
asphyxia due to enhanced xanthine oxidase activity on reperfu-
sion and this might contribute to oxidative stress in PA [10]. The
significant ($p=0.008$) area under curve (0.859) of ROC indicates
that this parameter can act as a good predictor of death in such
cases. At the cut-off level of 2.345 mg of uric acid/mg of
creatinine, the sensitivity and specificity of predicting death in
perinatal asphyxia were 87.5% and 83.3% respectively.

**Protein excretion in perinatal asphyxia**

A significantly higher ($p<0.01$) urinary protein excretion rate as
assessed from urinary protein creatinine ratio in perinatal asphyxia
cases as compared to controls (Table 1), a significant negative
correlation between urinary protein creatinine ratio and APGAR
score and significant positive correlation between urinary protein
creatinine ratios and HIE staging (Table 2) indicate that the urinary
protein excretion rate depends on the degree of renal impairment
that was associated with the severity of asphyxia [33]. A
significantly higher urinary protein creatinine ratio in cases who
died of PA than that of survived cases (Table 1) indicates more
severe renal damage in dead cases. Among the three parameters
evaluated in the present study, the urinary protein excretion is the
most affected by PA. The protein excretion did not overlap between
PA and control groups and between the two subgroups of PA i.e.,
survived and dead cases. The significant ($p=0.003$) area under
curve (0.896) of ROC indicates that this parameter can act as a good
predictor of death in PA cases. At the optimum cut-off level of
9.04 mg of protein/mg of creatinine, the sensitivity and specificity
of predicting death in perinatal asphyxia were found to be 87.5%
and 91.7% respectively. There are various factors like TNF-α and
other pro-inflammatory cytokines those are induced by oxidative
stress and are known to alter the glomerular permeability [34–36].
Oxidative stress appears to damage the podocytes of glomerular
membrane. Since there is no known mechanism for regeneration of
podocytes, its loss may result to irreversible kidney damage [37].
This free radical mediated renal damage and associated high TNF-
alpha and other pro-inflammatory cytokines might be the cause of
higher urinary protein excretion rate in perinatal asphyxia.

SDS-PAGE pattern of urinary proteins showed that both
high molecular weight proteins (73 kDa and 68 kDa) and
low molecular weight proteins (52 kDa, 47 kDa, 25 kDa and
20 kDa) were excreted in neonates with perinatal asphyxia
(Fig. 2). The 68 kDa protein band is predominantly due to
albumin. As PA is associated with oxidative stress [30,31]
which is evidenced from higher rate of urinary MDA excretion
by kidney. That may be the reason of appearance of proteins other than albumin in glomerular filtrate giving rise
to these extra bands. Impairment of tubular reabsorption due
ischemic damage might also contribute to the appearance
of proteins in urine [39,40]. It is worth investigating the me-
chanism involved in increased protein excretion in perinatal
asphyxia.

**Conclusion**

In conclusion, the present study reveals that oxidative stress
prevails in perinatal asphyxia. The excretion rate of urinary
proteins, urinary MDA and urinary uric acid increases with the
severity of perinatal asphyxia and associated brain damage and
hence these parameters have potential as severity evaluation and
prognostic markers. Urinary protein at cut-off level of 9.04 mg/
mg of creatinine, urinary uric acid at cut-off level of 2.59 mg/mg
of creatinine and urinary MDA at cut-off level of 2.195 μg/mg
of creatinine might be used to predict impending death in cases with
perinatal asphyxia. Because of convenience we included only
male babies. A similar study may also be conducted with female
neonates. A similar study with bigger sample size is warranted to
conclusively prove the clinical usefulness of these biomarkers to
evaluate HIE severity to device intervention strategy to limit the
negative neurological outcome and to know if their use should be
limited to very severe cases and as predictor of death.

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