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## Urinary allantoin is elevated in severe intraventricular hemorrhage in the preterm newborn

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### Abstract

**Background**—Germinal matrix intraventricular hemorrhage (IVH) is the most common type of intracranial hemorrhage observed in preterm neonates. It is a precursor of poor neurocognitive development, cerebral palsy and death. The pathophysiology is not well defined, but damage to the fragile germinal matrix vasculature may be due to free radicals generated during inflammation and as a consequence of ischemia followed by reperfusion. Assessment of the oxidative stress status in these infants is therefore important. Urinary allantoin concentration was measured in preterm neonates as a marker of oxidative stress associated with IVH.

**Study design**—Urine was collected from 44 preterm neonates at four time points between 24 and 72 hours of life (HOL) and the allantoin content was determined by gas chromatography mass spectrometry (GCMS). Records were retrospectively reviewed and the incidence and severity of IVH was categorized as follows: no IVH (n=24), mild (grade 1-2) IVH (n=13) and severe (grade 3-4) IVH (n=7).

**Results**—Neonates with severe IVH showed significantly elevated allantoin levels vs subjects with no IVH from 36 HOL ( $0.098 \pm 0.013 \mu\text{mol}$  and  $0.043 \pm 0.007 \mu\text{mol}$ , respectively,  $p=.002$ ). The allantoin concentration remained elevated even at 72 HOL ( $0.079 \pm 0.014 \mu\text{mol}$  and  $0.033 \pm 0.008 \mu\text{mol}$ , respectively,  $p=.021$ ). There were no significant differences in allantoin levels in the no IVH and mild IVH groups. IVH was diagnosed by head imaging on average at about 11<sup>th</sup> post-natal day.

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#### Conflict of Interest

Ijeoma Esiaba, Danilyn M. Angeles, Megan S. Holden, John B.C. Tan, Yayesh Asmerom, Gerald Gollin and Danilo S. Boskovic declare that they have no conflict of interest.

#### Compliance with Ethics Requirements

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from the parents of all patients for their inclusion in the study.

**Conclusion**—Urinary allantoin levels were significantly elevated during the first 3 days of life in the neonates subsequently diagnosed with severe IVH, suggesting that oxidative stress might be a crucial factor in IVH pathogenesis. Further studies are needed to assess the usefulness of urinary allantoin in early identification of preterm infants at risk for or with severe IVH, and monitoring of the response to interventions designed to prevent or treat it.

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## INTRODUCTION

About 12% of live births in the United States in 2008 were classified as premature and 20-25% of premature babies develop intraventricular hemorrhage (IVH) each year [1]. IVH is the most common type of intracranial hemorrhage that occurs within the ventricular cistern in premature babies < 32 weeks gestation weighing < 1500g [2]. It usually develops during the first week of life, with about 75% of the cases occurring within the first 72 hours and 90% being diagnosed by the end of the first postnatal week [3, 4]. Predisposing risk factors include early gestational age, low birth weight, infection, inflammation and respiratory distress [5, 6]. IVH has neurological and developmental sequelae that include injuries to the white matter, hydrocephalus, poor motor and cognitive development, cerebral palsy and poor executive function [7, 8]. This makes IVH a major pediatric public health problem [9].

The mechanism and pathophysiology of IVH is not well understood. However, three major factors may be involved in the development of this condition: 1) very fragile germinal matrix microvasculature, 2) poorly regulated cerebral blood flow and 3) low response capacity of the hemostatic system characterized by relatively low platelets and coagulation factors. Hemorrhage of immature, fragile germinal matrix microvasculature is thought to occur in part due to fluctuations in cerebral blood flow. Cerebral blood flow is known to increase in response to several conditions including hypotension, acidosis, hypoxemia or hypercapnia [1, 10].

In spite of marked improvements in the quality of perinatal care, the incidence of IVH has remained undiminished over the past several decades [7]. While this may be partially due to the multifactorial nature of the disorder, attempted interventions and study are further complicated by the fragile nature of this patient population. Moreover, the availability of blood samples from this cohort for research purposes is necessarily very limited and generally reserved for already well established and clinically essential assays. Consequently, previous studies investigating oxidative status in premature infants employed single point assays using cord blood [11, 12]. As a result information is lacking about the changes in oxidative stress markers over time. Furthermore, there are currently no appropriate biochemical markers that could be used to identify infants with elevated risk of IVH, with IVH or that would be helpful in monitoring the effectiveness of preventive or therapeutic interventions. For this reason, attempts are being made to develop minimally invasive, potentially useful diagnostic tools that do not rely on further blood sampling, hence the use of urine in this study.

Allantoin is usually produced in humans by the non-enzymatic oxidation of uric acid by free radicals [13, 14] following oxidative stress [15], and by other agents associated with

inflammation [16, 17]. It was identified as a marker of oxidative stress in adults and validated by other known markers of oxidative stress [18-21, 14, 22, 23]. It was shown to be elevated in ischemic stroke [24], smoking [25], during intense exercise [26], ozone challenge [15], and injection of doxorubicin to breast cancer patients [25]. In neonates, elevated allantoin concentrations were linked to increased ATP utilization [27] and necrotizing enterocolitis [28]. Allantoin concentration is not affected by storage at  $-80^{\circ}\text{C}$  or by the freeze/thaw cycle [29, 30, 13]. The reported detection limit for allantoin is about 2 pmol [31, 15].

Our hypothesis is that oxidative and inflammatory reactions occurring prior to or during IVH will lead to the increased production of allantoin that will be excreted in the urine over time. Therefore, urinary allantoin was measured in preterm newborns at 12 hour intervals from 24 to 72 hours of life (HOL) to determine whether it might be a non-invasive marker for impending or ongoing IVH.

## METHODS

### Subject enrolment and sample collection

The Loma Linda University Institutional Review Board approved the study protocol and informed consent documents. Infants of less than 34 weeks gestational age, who were admitted to the Loma Linda University Children's Hospital Neonatal Intensive Care Unit (NICU) between October 2008 and December 2011, were identified as study candidates. Infants with congenital anomalies were excluded from the study.

After informed consent was obtained from a legal guardian, the investigators collaborated with the clinical staff to obtain urine samples from the subjects. Samples were collected by placing cotton balls over the urethral meatus. Urine-soaked cotton balls were removed from the diaper with every change of diaper and stored at  $4^{\circ}\text{C}$ . Samples that were free of stool were combined into 12-hour aliquots from 24 – 72 HOL. Urine was extracted from the cotton ball by applying pressure, filtered through a syringe filter and stored at  $-80^{\circ}\text{C}$ .

### Screening for IVH and classification of subjects

The medical records were reviewed to identify subjects in whom IVH was diagnosed and to determine the grade of the hemorrhage. IVH was diagnosed by head ultrasound and/or magnetic resonance imaging (MRI). The infants were grouped according to Papile's classification of IVH [32]. Cases in which imaging showed no signs of bleeding were classified in the no IVH group. The infants with grades 1 and 2 IVH, who had hemorrhage that was still contained within the germinal matrix and ventricles without dilatations of the ventricles, were grouped as mild IVH. The severe IVH group included those infants with hemorrhage within or beyond the ventricles with ventriculomegaly and/or parenchymal involvement.

### Measurement of allantoin

Allantoin was measured in the urine by the adaptation of the method developed by Gruber [15, 13]. In summary, 25  $\mu\text{L}$  of urine was spiked with 400  $\mu\text{L}$  of 10  $\mu\text{M}$  internal standard

(DL-allantoin- 5-<sup>13</sup>C;1-<sup>15</sup>N). The spiked samples were simultaneously deproteinized and extracted with 100 µL acetonitrile, vortexed and centrifuged at 20 000 g, 4°C, for 5 minutes. The supernatant was dried using the speed vacuum drier and derivatized with 50 µL of MTBSTFA (i.e. N-methyl-N-tert-butyl-dimethyl-silyltrifluoroacetamide) in pyridine (1:1 vol/vol). The derivatization process was facilitated by incubation at 50°C for 2 hours. Analysis was performed on Agilent 6890N Network GC System connected to an Agilent 5973 Inert Mass Selective Detector (both Agilent Technologies, Inc, Santa Clara, California). Separation was performed using an Agilent 122-5532G capillary column (25.7 m length, 0.25 mm internal diameter). Allantoin was quantified using selected ion monitoring mode with the 398.00 m/z ion being monitored for allantoin and the 400.00 m/z for DL-allantoin- 5-<sup>13</sup>C;1-<sup>15</sup>N. The ion abundance ratios (398.00/ 400.00) were converted to micromolar concentrations by use of a standard curve.

### Statistical Analyses

Demographic data for categorical variables were analyzed using one-way ANOVA analysis, Chi-square and Mann-Whitney U tests. Repeated measures ANOVA was used to compare the urinary allantoin concentration between the groups and over time. All statistical analyses were performed using IBM SPSS for windows version 22. Differences were considered significant at  $P < 0.05$ .

## RESULTS

Forty-four infants were enrolled and categorized based upon the presence and extent of IVH. The 1- and 5-minute Apgar scores were not obtained for four of the infants that were transported shortly after home delivery to the Loma Linda University Children's Hospital NICU. The subjects were homogenous for estimated gestational age (EGA), birth weight and 1-minute Apgar score. There were no significant differences in the incidences of respiratory distress syndrome (RDS) and necrotizing enterocolitis (NEC) and, requirement for mechanical ventilation support. IVH was diagnosed after about 17 and 11 postnatal days in neonates with mild and severe IVH, respectively. The 5-minute Apgar score for the severe IVH group ( $3.86 \pm 2.54$ ;  $p=0.002$ ) was significantly lower than the scores in the no IVH ( $6.82 \pm 1.59$ ) and mild IVH ( $6.27 \pm 1.56$ ) groups (Table 1).

The urinary allantoin concentration was similar in the three groups at 24-36 HOL. Between 36 and 72 HOL the concentration of allantoin in the no IVH and mild IVH groups remained relatively stable at  $0.043 \pm 0.007$  and  $0.037 \pm 0.01 \mu\text{mol}$ , respectively. A significant elevation in allantoin ( $0.098 \pm 0.013 \mu\text{mol}$ ) was observed between 36 – 48 HOL in neonates with severe IVH ( $p = .002$ ) compared to those with mild IVH or to controls. Allantoin remained significantly elevated ( $0.079 \pm 0.14 \mu\text{mol}$ ;  $p = .021$ ) in this group even at 72 HOL (Figure 1).

## DISCUSSION

The objective of this study was to evaluate changes in urinary allantoin, a marker for oxidative stress, in premature infants who developed IVH. We found that allantoin was significantly elevated in infants with severe IVH compared to those with no IVH or with mild IVH. This elevation was observed at 36 HOL and it persisted up to 72 HOL. This is

consistent with prior observations that neonates with poor outcomes experience increased oxidative stress shortly after birth [33, 12, 11].

Allantoin, a non-enzymatic product of purine and marker of oxidative stress [15, 22], is produced when uric acid is oxidized by reactive oxygen species [17]. The mechanism of allantoin production from uric acid by the action of free radicals is well documented [34, 16, 35]. Hellsten and coworkers observed a more than two-fold increase in plasma allantoin during recovery in adults who exercised [26]. This suggests that oxygen radicals generated during hyperoxia may be responsible for the production of allantoin from urate in humans [13]. Apart from this, oxidative stress induced by inflammation and toxicity has been shown to increase allantoin levels, making it a potential marker of oxidative stress [36, 14]. Based on these reports we can infer that oxidative stress is the likely cause of the elevated allantoin we observed in the neonates with severe IVH.

Our study documented urinary allantoin content over time in preterm neonates. The greatest increase in content was found at 36-48 HOL in neonates with severe IVH. Prior studies measured oxidative stress markers only at a single point in time in cord blood [11, 12]. This may be partially due to the difficulty associated with repeated blood sampling in this fragile population. We chose urinary allantoin because it can be repeatedly measured over time and could permit tracking of the progression of oxidative stress in infants with IVH. In addition, it may be helpful in following the effectiveness of any therapeutic interventions they may be receiving.

Most of the neonates (>79%) experienced some degree of respiratory distress shortly after birth and were placed on mechanical ventilation at some point during hospitalization (Table 1), however, the group that developed severe IVH appeared to have had a more difficult transition to extrauterine life and were possibly poorly perfused as indicated by the significantly lower Apgar score at 5 minutes. Severe IVH has been correlated to low 5-minute Apgar scores [37-39]. The incidence and severity of IVH has been partially linked to the timing, severity and intensity of hypoxia-ischemia, as well as the method of reperfusion employed to correct this [40]. Basu *et al.*, demonstrated a correlation between hyperoxemia and increased cerebral blood flow in neonates who developed IVH between 24 –48 HOL [41]. We observed a similar time course of the elevation of allantoin in the infants with severe IVH, whereby allantoin levels significantly increased at 36 HOL in this group. This timing further suggests that the rise in allantoin may be linked to perinatal events such as maternal/placental inflammation and ischemia-reperfusion. Therefore, urinary allantoin may serve in identifying which neonates are experiencing oxidative stress and need further monitoring for morbidities associated with prematurity.

Severe IVH is known to have poor neurological outcome. If urinary allantoin indicates increased free radical production and possible tissue injury, then the significantly higher allantoin levels presented here are consistent with reports associating severe IVH (grade 3-4) with more adverse neurological outcomes [3]. IVH as well as retinopathy of prematurity (ROP), necrotizing enterocolitis (NEC) and bronchopulmonary dysplasia (BPD) have been termed oxygen-radical diseases (ORD) of neonatology [42, 33]. Free radicals generated during hypoxia-ischemia and reperfusion are suspected to play a role in their pathogenesis in

the low-birth weight preterm newborn [43], but the resulting lesions present differently in different tissues [44]. Allantoin levels were found to be elevated in NEC [28] and BPD [45, 21]. Further studies are needed to better characterize such associations.

The exact timing of IVH development is not clear, but it is suspected to occur sometime during the first week of life [4]. The 5-minute Apgar indicated that the neonates who developed severe IVH were sicker and perhaps more poorly perfused. But IVH was not diagnosed in this group by the conventional head imaging until about the 11<sup>th</sup> post-natal day, on average. Preterm newborns do not usually undergo head imaging when they are unstable and possibly intubated. The observed elevated urinary allantoin from 36-72 HOL was associated with severe IVH. The timing of this elevated urinary allantoin may not give an indication of exact timing of IVH development, but it may indicate ongoing physiological changes prior to or during IVH. Urinary allantoin, probably in combination with low Apgar scores, may be helpful to the clinician toward earlier consideration of IVH risks.

In conclusion, elevated allantoin level at 36 HOL which persisted up to 72 HOL was associated with severe IVH, which was diagnosed on average at about the 11<sup>th</sup> day of life. These findings support previous work suggesting that oxidative stress plays an important role in the pathophysiology of IVH [11, 46, 47]. Further studies are needed to establish the possible usefulness of urinary allantoin, during the first 2-3 days of life, in identifying neonates at risk for severe IVH. It may also be helpful in monitoring of the effectiveness of therapeutic interventions designed to limit further progression of IVH and reduce the neurological sequelae.

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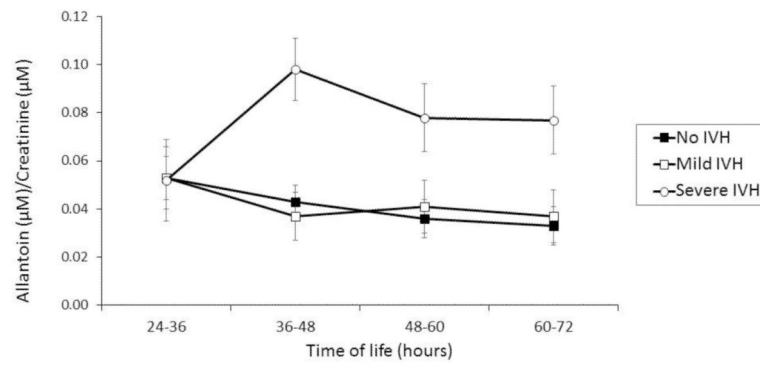
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**Figure 1.**

Urinary allantoin concentration between 24 and 72 hours of life in infants with no IVH (n=22), mild IVH (n=11) and severe IVH (n=7). The allantoin concentration in infants with severe IVH was significantly elevated at 36-48 hours (p=0.002), 48-60 hours (p=0.039) and 60-72 hours (p=0.021).

**Table 1**

## Subject Demographics

	No IVH (n=24)	Mild IVH (n=13)	Severe IVH (n=7)	P-value
EGA(weeks)	27.5±2.9	26.2±1.3	25.6±2.3	.112 <sup>†</sup>
Birth Weight (g)	1043±414	932±264	894±288	.550 <sup>†</sup>
1-Minute APGAR	4.64±2.38	4.09±2.91	2.43±2.30	.145 <sup>†</sup>
5-Minute APGAR	6.82±1.59	6.27±1.56	3.86±2.54	.002 <sup>a†</sup>
IVH Diagnosis, DOL Mean ± St.Dev. Median (Range)	NA	45.9±52.0 17 (2-158)	11.1±6.5 11 (3-21)	0.099 <sup>††</sup> 0.341 <sup>*</sup>
NEC	3 (12.5%)	2 (15.4%)	1 (14.3%)	.855 <sup>††</sup>
RDS	19 (79.2%)	12 (92.3%)	6 (85.7%)	.136 <sup>††</sup>
Mechanical Ventilation	21 (87.5%)	13 (100%)	7 (100%)	.146 <sup>††</sup>
Incomplete Data	2	2	0	

**Abbreviations:** EGA, estimated gestational age; DOL, day of life; NA, not applicable; NEC, necrotizing enterocolitis; RDS, respiratory distress syndrome. Data are mean ± standard deviation.

<sup>a</sup>Severe IVH significantly different from mild and no IVH

<sup>†</sup>ANOVA test

<sup>††</sup>Chi-Square test

<sup>\*</sup>Mann-Whitney U test.