American Pain Society
RESEARCH
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TREATMENT
ADVOCACY
PUBLISHED BY ELSEVIER

The Journal of Pain, Vol 13, No 6 (June), 2012: pp 590-597
Available online at www.jpain.org and www.sciencedirect.com

Procedural Pain and Oxidative Stress in Premature Neonates

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Abstract: Preterm neonates exposed to painful procedures in the neonatal intensive care unit exhibit increased pain scores and alterations in oxygenation and heart rate. It is unclear whether these physiological responses increase the risk of oxidative stress. Using a prospective study design, we examined the relationship between a tissue-damaging procedure (TDP; tape removal during discontinuation of an indwelling central arterial or venous catheter) and oxidative stress in 80 preterm neonates. Oxidative stress was quantified by measuring uric acid (UA) and malondialdehyde (MDA) concentration in plasma before and after neonates (n = 38) experienced a TDP compared to those not experiencing any TDP (control group, n = 42). Pain was measured before and during the TDP using the Premature Infant Pain Profile (PIPP). We found that pain scores were higher in the TDP group compared to the control group (median scores, 11 and 5, respectively; $P < .001$). UA significantly decreased over time in control neonates but remained stable in TDP neonates (132.76 to 123.23 μM versus 140.50 to 138.9 μM; $P = .002$). MDA levels decreased over time in control neonates but increased in TDP neonates (2.07 to 1.81 μM versus 2.07 to 2.21 μM, $P = .01$). We found significant positive correlations between PIPP scores and MDA. Our data suggest a significant relationship between procedural pain and oxidative stress in preterm neonates.

***Perspective:** This article presents data describing a significant relationship between physiological markers of neonatal pain and oxidative stress. The method described in this paper can potentially be used to assess the direct cellular effects of procedural pain as well the effectiveness of interventions performed to decrease pain.*

***Key words:** Neonate, procedural pain, uric acid, malondialdehyde.*

During a typical stay in the neonatal intensive care unit (NICU), a newborn often experiences numerous painful procedures in the course of monitoring and treatment. Contrary to previously held beliefs, premature neonates are able to perceive pain[4] as demonstrated by several pain scoring methods.[34] Such methods, however, tend to rely on a neonate's alertness and ability to react expressively to painful experiences. Consequently, caregivers are reluctant to prevent or treat pain if there appears to be no clear demonstration that it is occurring or if no immediate untoward effects are observed.[41] An approach that involves measurement of a systemic biochemical reaction to a painful stimulus has the potential benefit of providing objective means of evaluating the presence and degree of pain as well as the effectiveness of its treatment.

It is well documented that exposure to painful procedures often results in reductions in oxygen saturation and tachycardia, which increases energy expenditure and oxygen consumption.[7] However, there are currently few data that quantify the effects of increased pain and oxygen consumption on adenosine triphosphate (ATP) metabolism. Theoretically, an increase in oxygen consumption should increase the utilization and degradation of ATP to its purine byproducts, specifically uric acid (UA) (Fig 1). We made this observation in a pilot study from our laboratory in which we reported increased UA concentration in rabbit kits subjected to a single heel lance.[32] Because the process of purine degradation can result in the production of hydrogen peroxide, markers of oxidative stress can theoretically increase. This, in fact, was observed by Bellieni et al,[8]

Received November 10, 2011; Revised March 2, 2012; Accepted March 13, 2012.
Funded by a grant from the National Institute of Nursing Research (NR010407).
The authors have no conflict of interest to report.
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1526-5900/$36.00

doi:10.1016/j.jpain.2012.03.010

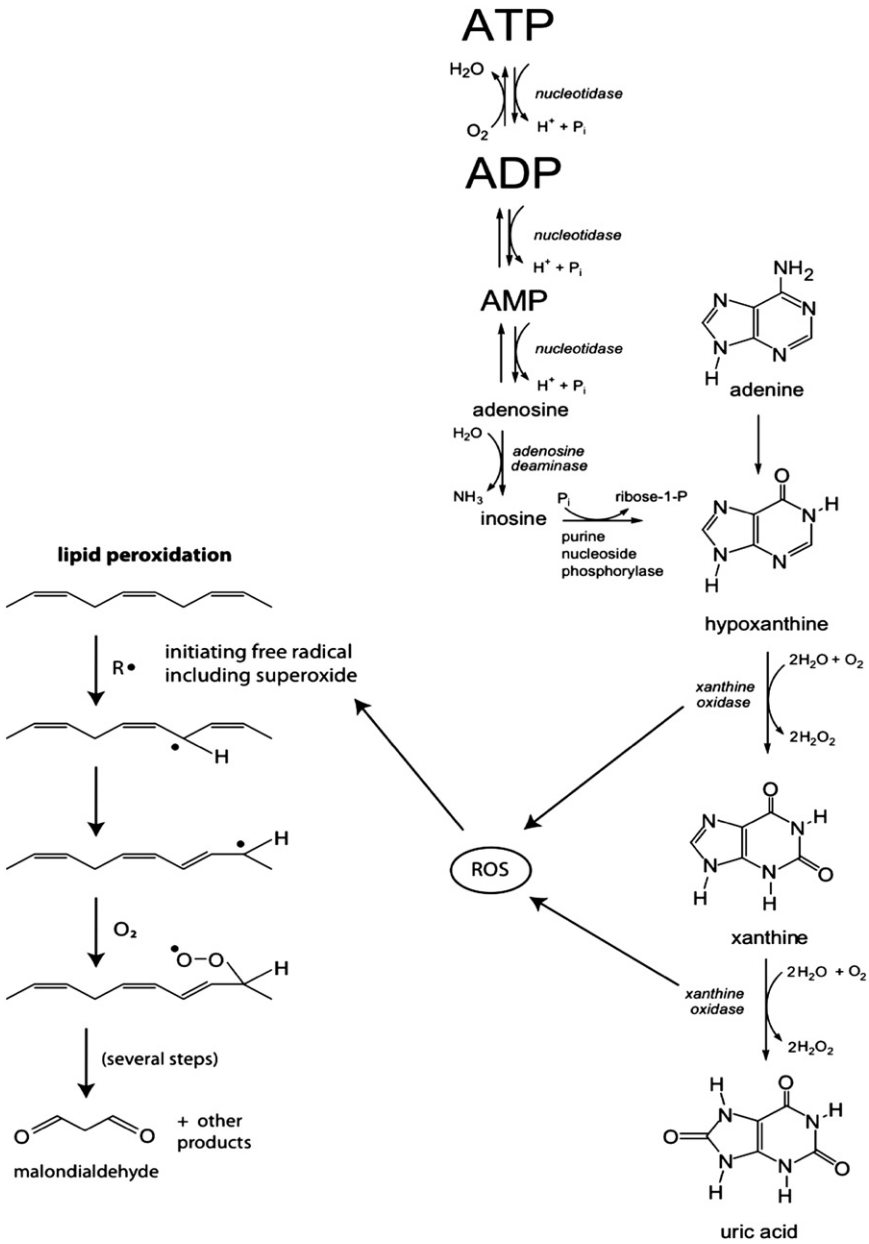

Figure 1. Pathway from ATP to UA and MDA.

who showed increased markers of oxidative stress in the plasma of premature neonates experiencing a single heel lance. However, no studies to date have examined the relationship between pain scores that reflect behavioral and physiological markers of pain, and plasma markers of ATP utilization and oxidative stress. Specifically, do markers of ATP utilization and oxidative stress increase as pain scores increase?

To explore this question, we studied 2 groups of preterm neonates: 1 group experiencing a common tissue-damaging procedure (TDP group), and 1 group not experiencing any TDP (control group). The TDP was tape removal associated with the discontinuation of a central arterial or venous catheter. Pain was measured before and during the TDP using the Premature Infant Pain Profile (PIPP), whose components include physiological (gestational age, heart rate, oxygen saturation) and behavioral (state and facial expressions) markers. ATP degradation was quantified by measuring UA levels, and oxidative stress was quantified by measuring malondialdehyde (MDA) in plasma obtained before and after the TDP. UA is a well-known end product of purine metabolism whose concentration is associated with increased ATP utilization, hypoxia, ischemia, or increased reactive oxygen species (ROS)[21] (Fig 1). MDA is a thiobarbituric acid-reacting substance that is formed by the action of ROS on lipid membranes[29,38] (Fig 1). Its use as a marker is relevant because although the study of oxidative stress and pain in neonates is relatively recent, the association of pain with elevated MDA is not novel and has been reported in adults. Elevated MDA concentrations were observed in adult patients with vascular-related pain,[36] young women with dysmenorrheal pain,[19,43] adults with neuropathic pain,[14] and adult patients with acute abdominal pain.[15] More recently, MDA and 8-

hydroxyguanosine were reported to significantly correlate with pain intensity in patients with temporomandibular joint disease.[35]

Evaluation of the effects of pain in premature neonates is challenging. We present biochemical evidence that a single painful procedure is associated with a systemwide perturbation in purine metabolism and oxidative stress. Furthermore, we show that a single TDP correlates with elevated MDA levels and PIPP scores, suggesting distant effects in the form of oxidative damage to lipid membranes.

Methods

We conducted a prospective cohort study at the Loma Linda University Children's Hospital NICU. The Loma Linda University Institutional Review Board approved our study protocol and informed consent documents. Families were approached for consent as soon as possible after birth.

Preterm infants less than 37 weeks gestation that met the following inclusion criteria were considered for enrollment: 1) weight of more than 1,000 g at time of enrollment; 2) arterial or central venous catheter in place; 3) no signs or symptoms of hypoglycemia, hypovolemia, hypoperfusion, hyperbilirubinemia, clinical sepsis, pallor, or moderate-to-severe respiratory distress; 4) absence of intraventricular hemorrhage grade 3 or higher; and 5) parental consent. Exclusion criteria included 1) multiple congenital abnormalities; 2) facial malformation; 3) complex congenital heart disease; 4) receiving analgesia or sedation; and 5) endotracheal intubation.

After parental consent was obtained, investigators collaborated with the clinical staff to obtain from a central catheter approximately .8 mL of blood before and 30 minutes after the TDP to measure UA and MDA levels. In control neonates, similar samples were obtained at 0 and 30 minutes from baseline. The time period of 30 minutes after TDP for blood sample collection was based on previous investigations that showed plasma levels of MDA significantly increasing 15 to 30 minutes after ischemia-reperfusion[30,44] and remaining elevated up to 2 hours later.[23] Our pilot study in rabbit kits also showed elevations of UA 30 minutes after a single heel lance.[32] To isolate the effects of TDP, subjects were given at least 1 hour of quiet time in which no TDPs were performed before baseline blood samples were drawn, and no additional TDPs were performed during the study period. Samples were centrifuged within 5 minutes to separate cells from plasma, which was then stored at −80°C. All stored plasma samples were analyzed within 1 week of acquisition.

Pain Assessment

To assess pain, we used the PIPP, an instrument that was designed to assess acute pain in preterm neonates.[40] The scoring system includes 7 items, each graded from 0 to 3. Two items describe baseline characteristics of the neonate (gestational age and behavioral state), 2 items are derived from physiological measurements (heart rate and oxygen saturation), and 3 items describe facial actions (brow bulge, eye squeeze, and nasolabial furrow). Gestational age, behavioral state, heart rate, and oxygen saturation were assessed and recorded by a trained research nurse (L.S.) at the bedside. Facial actions were assessed and scored by a neonatologist who was blinded to group assignment (K.H.), who had undergone training in the use of the PIPP and was experienced in observing and quantifying facial actions. Previous work on validation of the PIPP score showed an ability to differentiate painful from nonpainful or baseline events ($F = 48$; $P = .0001$), with interrater reliability coefficients of .93 to .96 and intrarater reliability coefficients of .94 to .98.[5,12,40]

Measurement of MDA

Plasma MDA levels were determined using an adaptation of the selected ion-monitoring gas chromatography-mass spectrometry (GC-MS) (Agilent, Santa Clara, CA) analysis of phenylhydrazine-derivatized plasma, as described by Cighetti et al.[17] Specifically, the sample was prepared by the mixture of .1 mL plasma, .26 nmol methyl malondialdehyde (MMDA), 5 nmol butylated hydroxytoluene (10 μL of .5 mM) (Sigma-Aldrich, St. Louis, MO), .2 mL citrate buffer (.4 M, pH 4.0), and deionized water, up to a final volume of 480 μL. Then, 20 μL of 50 mM phenylhydrazine (1 μmol) (Sigma-Aldrich) was added as the derivatizing agent. After 30 minutes' incubation at 25°C, the samples were extracted with 1 mL hexane, vortexed for 1 minute, and centrifuged (3,000 *g*, 10 minutes) at 25°C. The organic phase was removed, concentrated by nitrogen stream to 100 μL, and analyzed by GC-MS, in selected ion monitoring mode (injection volume of 2 μL). Ion 144.00 was monitored for MDA, and ion 158.00 for MMDA. The ion abundance ratios were converted to micromolar concentrations by use of a standard curve. All measurements were performed in triplicate. Values with coefficients of variation of less than 10% were included in the final analyses.

The internal standard, MMDA, was synthesized using a modified method of Paroni et al.[31] Briefly, 2-methyl-3-ethoxyprop-2-enal (Sigma-Aldrich) was suspended in 7 M NaOH and stirred for 150 minutes. This was diluted with 5 mL of water and extracted with three 5-mL volumes of CH_2Cl_2. Water was then evaporated from the aqueous layer. The residue was crystallized once from 5 mL ethanol and 5 mL benzene and then 3 times from 5 mL ethanol and 5 mL diisopropyl ether. The resulting white powder was diluted in water, filtered, and stored at −80°C.

Measurement of Uric Acid

Plasma was transferred to separate Eppendorf tubes and immediately centrifuged within 5 minutes in a Microfuge 22R (Beckman Coulter Inc, Brea, CA) for 30 minutes at 18,000 *g*, 4°C. The supernatant was immediately transferred to Microcon centrifugal filter devices (Millipore Corp, Bedford, MA), 200 μL per device, and spun for 90

minutes at 14,000 *g*, 4°C. Filtrate was removed, and 150 μL was transferred to an Eppendorf tube containing 1×10^{-7} mol of 2-aminopurine (Sigma-Aldrich) as the internal standard. Analysis was done the same day using high-performance liquid chromatography (HPLC) (Waters 996 PDA, 715 Ultra Wisp Sample Processor; Millipore Corp) or the tubes were frozen at −80°C until analysis could be performed. Previous HPLC analysis demonstrated that UA values remained stable despite freezing.

Three 45-μL injections were used for each sample. Samples were injected onto a Supelcosil LC-18-S 15 cm × 4.6 mm, 5-μm column (Supelco; Sigma-Aldrich), with the following isocratic conditions: 50 mM ammonium formate buffer, pH 5.5, flow rate 1.0 mL/minute. UA concentration was quantitated by first obtaining integrated peak areas for UA and for 2-aminopurine at appropriate retention times and wavelengths, as described by our laboratory.[11] Then, the peak area ratios of UA to 2-aminopurine were determined and converted to micromolar concentrations using standard curves. Samples were analyzed in triplicate, and values with coefficients of variation of less than 10% were included in the final analyses. The limit of detection for UA was 5.0 μM.

Statistics

At the time our study was planned, there were no studies that had examined the relationship between pain and oxidative stress in premature neonates. We based our sample-size calculation on our pilot study that compared MDA and UA concentration in neonates born by elective cesarean section to those born by vaginal birth.[11] Based on that calculation, 35 subjects per group were required to demonstrate a difference between groups with 80% power and α = .05 (Sample Power 2.0 [SPSS Inc, Chicago, IL]). We recruited additional subjects per group to account for data-collection errors. The total sample size was 80 infants (Control n = 42 and TDP n = 38).

Assumptions of normality and equal variance were assessed. Demographic data for categorical variables were analyzed using chi-square test. Repeated measures analysis of variance for 1 between-subject factor (group) and 1 within-subject factor (time) were assessed to evaluate the effect of the procedure (control versus TDP) on plasma UA and MDA concentrations over time. Interaction terms in the General Linear Model were used for this purpose. Percent change in UA, as well as MDA, between baseline and 30-minute values were calculated as follows:

$$(([UA_{30min}] - [UA_{0min}])/[UA_{0min}]) \times 100$$

Correlations between UA, MDA, and biobehavioral markers (PIPP) were examined using Spearman's rho. All statistical analyses were performed using SPSS Statistics for Windows version 17. Differences were considered significant at $P < .05$.

Results

General Results

Subject recruitment occurred from July 2007 to August 2009. After obtaining parental consent, samples were obtained from 80 subjects that met study criteria. As described in Table 1, no significant differences in demographics were found between the control and TDP groups. The clinical characteristics and the environmental condition of subjects at the time of sampling were also compared (Table 2). At the time of sampling, no significant differences were observed in mode of ventilation, baseline oxygen saturation, postnatal age, fraction of inspired oxygen (FiO_2), hemoglobin, acuity (as measured by Score of Neonatal Acute Physiology–Perinatal Extension-II [SNAPPE-II]), environmental noise, kidney function (as measured by blood urea nitrogen and creatinine), and number of exposure to TDPs (Table 2).

Procedural Pain Score as Measured by PIPP

As expected, neonates in the TDP group had significantly higher procedural pain scores than the control group (Table 3). Median procedural pain scores were as follows: control group, 5 (min–max, 0–11); and TDP group, 11 (min–max, 3–19), $P < .001$ (Table 3).

Table 1. Subject Demographics

	No Procedure	*TDP*	P *value**
Number of patients	42	38	
Birth weight (g)	1,644 ± 486	1,637 ± 499	NS
Estimated gestational age (weeks)	31.2 ± 3	31.2 ± 3	NS
Gender (%)	Male–54.1	Male–54.1	NS†
Apgar–1 minute	6 ± 2.0	6 ± 3.0	NS
Apgar–5 minute	8 ± 2	7 ± 3	NS
Race (%)	Caucasian–52.4 Hispanic–23.8 African American–14.3 Asian–7.1 Not documented–2.4	Caucasian–39.5 Hispanic–44.7 African American–10.5 Asian–5.3 Not documented–0	NS†

Abbreviation: NS, not significant.
NOTE. Mean ± SD.
*Independent samples t-test.
†Chi-square test.

Table 2. Physiological and Environmental Conditions at Time of Sampling

	No Procedure (n = 42)	*TDP (n = 38)*	P *value**
FiO_2	.24 ± .05	.24 ± .07	NS
Mode of ventilation	Room air = 24 (57.1%) Nasal cannula = 9 (21.4%) Nasal CPAP = 6 (14.3%) NIPPV = 3 (7.1%)	Room air = 26 (68.4%) Nasal cannula = 4 (10.5%) Nasal CPAP = 5 (13.1%) NIPPV = 3 (7.9%)	NS
Baseline oxygen saturation (%)	97 ± 2.8	97 ± 3.6	NS
Postnatal age (days)	20 ± 12	20 ± 15	NS
Weight (g)	1,850 ± 515	1,890 ± 568	NS
Hemoglobin (mg/dL)	12.7 ± 2.2	13.2 ± 2.2	NS
SNAPPE-II score	7.9 ± 3.6	7.6 ± 5.5	NS
Blood urea nitrogen (mg/dL)	16.8 ± 10.6	14.8 ± 8.3	NS
Creatinine (mg/dL)	.47 ± .19	.46 ± .18	NS
No. of TDPs from birth to time of sampling	95 ± 69	107 ± 96	NS
Environmental noise (dB)	51 ± 5	53 ± 4	NS

Abbreviations: CPAP, continuous positive airway pressure; NIPPV, noninvasive positive pressure ventilation; NS, not significant.
NOTE. Mean ± SD.
*Independent samples t-test.

Differences Between Baseline and Post-Procedure UA and MDA Levels in Control Versus TDP Groups

There were no significant differences in baseline UA and MDA levels between control and TDP groups. However, although UA levels decreased over time in the control group, UA levels remained unchanged in the TDP group (Fig 2). More importantly, we saw a significant increase in MDA over time in the TDP group that was not observed in the control group ($P = .02$) (Fig 2).

Correlation Between PIPP Pain Score and MDA

We found significant correlations between pain scores and MDA (Pearson correlation, .283, $P = .012$). As the pain score increased, MDA levels also increased. In addition, we found significant correlations between procedural heart rate and MDA (Pearson correlation .286, $P = .014$). We observed that as heart rate increased, MDA concentration also increased. More importantly, we also found a significant correlation between MDA and oxygen saturation; as oxygen saturation decreased, MDA concentration increased (Pearson correlation, .454, $P < .001$). We found no significant correlation between plasma UA levels and total PIPP pain scores.

Discussion

Premature neonates in the NICU have reduced endogenous substrate fuel stores and metabolic reserve.[33] Their ability to respond to acute stressors is limited due to their physiological immaturity, which impedes their ability to mobilize substrate during catabolic metabolism.[16] Their response to routine NICU procedures often includes significant alterations in transcutaneous oxygen levels (T_cPO_2) and heart rate.[18,39] Because neonates have fixed stroke volumes, acute changes in heart rate can potentially reduce cardiac output and cause tissue hypoperfusion, increasing the risk of ischemia before compensatory mechanisms take effect. Ischemia combined with decreased arterial oxygen tension can contribute to increased ATP utilization in the face of decreased ATP synthesis. This can lead to enhanced degradation of ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP).[24] Further degradation leads to dramatic increases in adenosine levels. In turn, adenosine is converted to inosine and subsequently to hypoxanthine, xanthine, and UA (Fig 1). During conditions of decreased ATP supply, the enzyme xanthine oxidase is activated, catalyzing the conversion of hypoxanthine to xanthine and xanthine to UA, while generating ROS.[37] In this context, there has been little research evaluating the link between painful procedures, increased ATP utilization, and oxidative stress in premature neonates.

Our data demonstrate a significant relationship between procedural pain and MDA, a well-accepted marker of oxidative stress. Specifically, we observed an increase in MDA in preterm neonates exposed to a single painful procedure compared to those who were not exposed to such a procedure. None of the neonates in this study had conditions that were shown to increase plasma MDA concentration such as moderate-to-severe

Table 3. Pain Score as Measured by PIPP

	No Procedure (n = 42)	*TDP (n = 38)*	P *value**
Baseline pain score (median)	3 (0–7)	3 (0–8)	NS
Procedural pain score (median)	5 (0–11)	11 (3–19)	<.001

Abbreviation: NS, not significant.
*Mann-Whitney U test.

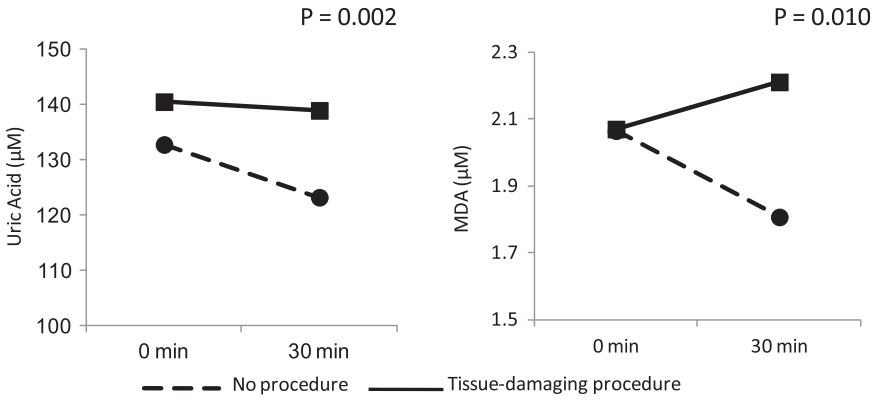

Figure 2. Plasma [UA] and [MDA] at baseline and 30 minutes post-TDP.

respiratory distress, elevated FiO_2 requirements, lipid infusions, hyperbilirubinemia,[6,26] or clinical signs of septicemia.[25] Instead, the elevated MDA is consistent with an increase in the production of ROS secondary to enhanced ATP degradation (Fig 1) in response to increased energy requirements and reduced oxygenation brought about by the painful procedure. Elevations in heart rate and reductions in oxygen saturation significantly correlated with elevated MDA levels. Further studies are required to investigate this mechanism as well as other possible mechanisms that may increase MDA such as pain-related tissue injury, inflammation, and cytokine production. To validate the relationship between procedural pain and oxidative stress, it will also be important to measure other oxidative stress markers, such as damage to DNA (8-hydroxy-2′-deoxyguanosine), lipids (4-hydroxy-2-noneal, isoprostanes, isofurans), and nitration of proteins (plasma nitroalbumin, peroxynitrite marker of nitrotyrosine 3-nitro-4-hydroxyphenylacetic acid, para-hydroxyphenylacetic acid) in plasma, and other biological fluids.[10,22,42]

Our findings have important implications for clinical practice. Because neonates are exposed to multiple painful procedures every day,[13,28] previous investigations have focused on examining and decreasing the painful effects of these procedures. A number of interventions, such as the use of sucrose,[1,9,27] pacifiers,[2,9] or morphine,[3,20] have been successful in decreasing signs of pain, but studies investigating the possible biochemical sequelae of painful procedures in neonates are needed. If exposure to multiple painful procedures is shown to contribute to oxidative stress, biochemical markers of such stress might be useful in evaluating mechanism-based interventions that could decrease the adverse effects of exposure to these procedures.

Although our findings are novel in the neonatal population, our study was performed without randomization. The painful procedure (tape removal) was a clinical requirement necessary in the normal course of care in the NICU setting, and no additional painful procedures were employed solely for the benefit of a randomized experimental protocol. However, as shown in Table 1, the demographic and clinical characteristics of subjects in both the control and TDP groups were not significantly different. All of the subjects were clinically stable premature neonates, with minimal oxygen requirements and similar clinical acuity status. Randomized trials that examine the biochemical effect of painful procedures in more acutely ill neonates with higher SNAPPE-II scores are needed.

Conclusion

Our data demonstrate an important relationship between exposure to a single painful procedure and oxidative stress. Because neonates are exposed to many painful procedures during hospitalization, it is important to further examine the effect of single, multiple, and accumulated TDPs in a randomized clinical trial. Mechanistic studies that will determine whether or not procedure-related increases in oxidative stress exacerbate preexisting pathological conditions or trigger development of new abnormalities would provide additional insight.

Acknowledgments

We acknowledge Michael Lockwood for his assistance in subject recruitment. We thank all the NICU nurses, nurse practitioners, and physicians for their assistance with study procedures.

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