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Abstract

Background: Premature infants are susceptible to oxidative stress, increasing the risk of serious morbidities. High-dose human milk (HM) feedings decrease morbidity risks and may reduce oxidative stress in this population. The purpose of this study was to compare oxidative stress using serial urinary F_2-isoprostane concentrations in predominantly HM and preterm formula (PF) fed premature infants over the first 21 days of life (DOL), while controlling for perinatal oxidative stress exposures including bovine-based human milk fortifier (HMF) or PF introduction to predominantly HM-fed infants.

Methods: This was a quasi-experimental design that categorized 22 premature infants into mutually-exclusive comparison groups based on exposure to HM and PF. Serial urine samples (pre- and post-first feeding, and DOL 7, 14, and 21) were used to determine urine F_2-isoprostane concentrations measured by ELISA. We analyzed data using Mann Whitney U, Wilcoxon rank test and multilevel models.

Results: Comparing the predominantly HM-fed and predominantly PF-fed groups over time, median F_2-isoprostane concentrations decreased significantly in the predominantly HM group ($p = 0.003$) and increased significantly in the predominantly PF group ($p=0.01$). Perinatal oxidant exposures and the introduction of HMF did not affect results.

Conclusion: Our results demonstrate that predominantly HM feedings were associated with decreased oxidative stress while PF feedings increased oxidative stress in premature infants, even after controlling for perinatal oxidant exposures of HMF or PF introduction.

Clinical Relevancy Statement

Premature infants experience higher oxidative stress compared to term infants and they are particularly susceptible to perinatal and postnatal oxidative stress due to reduced antioxidant defenses. We observed a dose-dependent reduction in oxidative stress with human milk feeding.

Introduction

Premature infants are particularly susceptible to oxidative stress or excessive free radical accumulation from accelerated production or reduced antioxidant activity early in life.[1,2] Oxidative stress in the premature infant is a function of pregnancy and birth complications as well as elements in the neonatal intensive care unit (NICU) environment that affect the infant's immature body organs and pathways. Specifically, elevated maternal oxidative stress from premature rupture of membranes (PROM), chorioamnionitis, the use of high oxygen concentrations during delivery room resuscitation, and type of enteral feeding have been associated with increased postnatal oxidative stress in premature infants.[3-7] Oxidative stress has also been associated with increased risks of serious prematurity-associated morbidities, including necrotizing enterocolitis (NEC), late onset sepsis, bronchopulmonary dysplasia (BPD) and neurodevelopmental problems.[9,10] Thus, a priority in improving outcomes in premature infants is to identify practices that prevent and/or mitigate oxidative stress.

High-dose human milk (HM; milk from the infant's own mother) feedings during the NICU hospitalization decrease the risk of NEC, sepsis, BPD and neurodevelopmental problems in premature infants, but the mechanisms of protection are not fully elucidated.[11-14] One potential mechanism is the array of bioactive components in HM that may mitigate oxidative stress in this population, including antioxidants, growth factors, anti-inflammatory cytokines, mother-specific commensal bacteria, oligosaccharides and pattern-recognition receptors that facilitate bacterial-enterocyte crosstalk.[15] A small study from 2004 by Shoji et al. reported lower concentrations of oxidative stress biomarkers in 29 very low birthweight (VLBW; <1500 g birthweight) infants fed predominantly HM

than VLBW infants fed predominantly preterm formula (PF).[7] However, this study did not measure the impact of bovine-based human milk fortifier (HMF), which is standard of care for extremely preterm infants (<32 weeks gestation).[7,8] In a later study by Friel et al., HMF was associated with a dose-dependent increase in oxidative stress biomarkers in this population of 65 preterm infants.[6]

Thus, determining whether oxidative stress in premature infants is modifiable by early diet is a research priority. However, this area of inquiry has been limited due to the paucity of valid, reliable and non-invasive biomarkers of oxidative stress in this vulnerable population. Urinary F_2-isoprostanes is a product of radical-catalyzed polyunsaturated fatty acid peroxidation that has been used as a non-invasive biomarker of oxidative stress status *in vivo*[2], and is considered a reliable and valid measure of oxidative stress in premature infants.[2,6] Oxidative stress has been shown to be negatively correlated with gestational age, thus putting premature infants at higher risk for oxidative stress.[16] Thus, serial changes in this biomarker can be used to compare the differences in the oxidative stress response as a consequence of exposure to HM and PF feedings in this population.

The purpose of this study was to leverage urine samples from a prospective observational cohort study of premature infant to compare F_2-isoprostane concentrations as a function of exposure to HM and PF feedings, while controlling for potential confounders. We hypothesized that HM feedings would reduce oxidative stress whereas PF would not reduce and may increase oxidative stress.

Methods

Subjects: This study included 55 premature infants from a larger prospective observational study conducted at Rush University Medical Center NICU during the years 2009 – 2010.[17] Approximately five urine samples were collected from each infant for serial measurement of F_2-isoprostane concentrations: pre-first feed (prior to the introduction of enteral feeds); post-first feed (12 – 24 hours after the introduction of enteral feeds); and on days of life (DOL) 7, 14, and 21. Infants were excluded from this analysis if they did not have both pre- and post- enteral feeding urine samples or had received antibiotics for >48 hours immediately after birth, yielding a total of 22 infants with 91 urine samples for this study. The study was approved by the Rush University Medical Center and the University of Illinois at Chicago Institutional Review Boards, and written informed consent was obtained from a parent or guardian.

Design: A quasi-experimental design was used in which infants were categorized into mutually-exclusive comparison groups depending upon their exposure to HM and PF. Laboratory measures were completed without knowledge of comparison group categorization because categorization was based on these completed measures.

Data Collected: Data were prospectively collected and included the following.

Infant demographic data: birth gestational age, birth weight, birth weight *z*-score[18] and sex. Additional clinical data that might have affected the primary outcome were collected and included as potential confounders: a diagnosis of PROM and/or chorioamnionitis, use of ≥50% oxygen for infant resuscitation at delivery, and indomethacin use. Occurrences of neonatal morbidities related to oxidative stress were collected: NEC (stage ≥2), late onset sepsis (positive blood culture with clinical

symptoms and ≥5 days of antibiotics), and BPD (oxygen or positive pressure requirement at 36 weeks postmenstrual age).

Feeding data: Daily feeding type (HM and/or PF) and volume of each was collected prospectively for each infant. Only HM from the infant's own mother (e.g., no donor HM) was used and fresh HM was prioritized over frozen HM when available. Per unit policy, HMF was added to HM when enteral feeding volume = 140 mL/kg/d had been achieved. HM dose was calculated daily for each infant as the percentage of total enteral feedings equal to HM as follows: (mLs HM / [mLs HM + mLs PF] X 100) as described by Bigger et al.[19] The dose of HM was used to classify infants into dichotomous and mutually-exclusive groups for comparison purposes as follows. *First feeding type* comparisons categorized infants into *HM* or *PF* groups based on enteral feeding type during the first 24 hours after the introduction of enteral feedings. For first feeding type, the HM group received 100% (exclusive) HM (n = 13), whereas the PF group received any PF (< 100% HM; n = 9). HM group infants did not receive HMF during this short time period.

To compare the effect of HM dose over time, two additional groups were created based on the predominant type of feeding received by the infant over the first 21 DOL. For these serial *predominant feeding type* comparisons, two groups were created: the HM group received ≥50% HM (n = 16), and the PF group received <50% HM (n = 6). Powdered intact protein bovine HMF (there was an institution semi-annual rotation between two products at the time of the study) was added to all HM feedings upon achievement of enteral feeding volume = 140 mL/kg/day. The DOL of HMF introduction

was recorded. There was minimal HM displacement with the addition of powdered HMF, and fortification never exceeded the standard of 24 kcal/oz.

Biosamples and Laboratory Measures: Urine samples were collected, snap frozen, and stored at -80°C until analyses for this study were performed. Urinary F_2-isoprostane concentrations were measured using enzyme-linked immunosorbent assays (ELISA; F2-isoprostane, Cayman Chemical, Ann Arbor, Michigan). Urine creatinine concentrations were measured using colorimetric assay (Cayman Chemical, Ann Arbor, Michigan) to standardize F_2-isoprostane concentrations according to renal function: (F_2-isoprostane or I-FABP / creatinine).

Statistical Analyses: Data were entered and analyzed using SPSS (IBM Corp. Released 2013. Version 22.0. Armonk, NY). Descriptive analyses were conducted for all data and measures of central tendency were determined. Data were non-normally distributed, so the Mann Whitney U was used to compare differences in gestational age, birth weight, and birth weight *z*-scores between infant feeding groups. Mann Whitney U was also used to compare differences in F_2-isoprostane concentrations between subjects with and without the following variables: diagnosis of PROM, use of ≥50% oxygen at delivery, presence of chorioamnionitis, and indomethacin use. Wilcoxon signed-rank test was used to compare differences in F_2-isoprostane concentrations for the HM and PF groups as a function of first feeding type.

For multilevel model analyses, F_2-isoprostane concentrations were log transformed to normalize the data. In the primary analyses, multilevel models were used to compare rate of change in F_2-isoprostane concentrations between the HM and PF groups accounting for individual responses. Predominant feeding groups were modeled

as an infant-level fixed effect with F_2-isoprostane concentrations as dependent variables measured at the five serial time points with random effects for both intercept and time. The models adjusted for gestational age and birth weight. Secondary analyses considered percentage of HM as a time-varying covariate. In these analyses, F_2-isoprostane concentrations from four time points were used in the model (post-first-feeding, DOL7, DOL14, DOL21) with percentage of HM received at four time periods (first feeding, DOL1-6, DOL7-13, and DOL14-20) as the covariate. Multilevel models were conducted using SAS (Version 9.3 of the SAS System for Windows).

Results

Study population: The median gestational age and median birth weight *z*-score for the cohort (n = 22) were 30 weeks (IQR 27.8, 32.7) and -0.24 (IQR -0.81, 0.44), respectively (Table 1). There were no statistical differences in sex, birth gestational age, birth weight, birth weight *z*-scores, PROM, ≥50% oxygen use at delivery, indomethacin use, or duration of parenteral nutrition between the groups, both when categorized as first feeding groups (HM group n = 13; PF group n = 9) and predominant feeding groups (HM group n = 16; PF group n = 6; three infants crossed to HM group from PF group) (Table 1). No infants developed NEC or sepsis during the study period. Three (14%) infants developed BPD.

Effect of first feeding type: Thirteen infants received HM and nine infants received PF as first feeding type. The median pre-feed concentrations of F_2-isoprostane were not statistically different between the two feeding groups (p = 0.06). From pre-feed to post-feed, the median F_2-isoprostane concentration tended to decrease in the HM group

while remaining stable in the PF group with no statistically significant differences (Table 1).

Effect of predominant feeding type over time: Three infants from the first feeding type PF group were categorized into the predominantly HM fed group based on HM dose received over DOL 1-21. There were no additional group crossovers in the remaining infants.

Over time, median F_2-isoprostane concentrations decreased significantly in the predominantly HM group ($p = 0.003$), whereas median F_2-isoprostane concentrations increased significantly for the predominantly PF group ($p=0.01$). The slopes that characterize these F_2-isoprostane patterns for the two predominant feeding groups were statistically different starting on DOL 12 through to DOL 21 (Figure 1 and Table 2). A dose-response relationship was noted between the percentage of HM and F_2-isoprostane concentrations, such that each 10% increase in percentage of HM was associated with a 4.9% decrease in F2-isoprostane concentration ($p=0.003$). Introducing the variables of "time to reach full feeding" and the “time of HMF and/or PF introduction" into the multilevel model did not affect the time-dependent changes in F_2-isoprostane concentrations. In fact, F_2-isoprostane concentrations decreased significantly in the predominantly HM group between the pre-and post-introduction time points for HMF and/or PF (143 (89, 250) vs. 79 (61, 115), respectively; $p=0.02$). Chorioamnionitis was diagnosed for only one subject, thus it was not included in additional analyses. The presence of PROM, ≥50% oxygen use at delivery, and indomethacin use did not affect F_2-isoprostane concentrations when added as covariates into the models.

Discussion

To our knowledge, this is the first study to compare oxidative stress as a function of specific doses and timing of HM and PF feedings in a contemporary cohort of premature infants while controlling for potential confounders. Using changes in F_2-isoprostane concentrations as a biomarker of oxidative stress, we compared HM and PF groups for two specific post-birth exposure periods: as a pre-post first feeding exposure and cumulatively over the first 21 days post-birth. For the first feeding exposure comparison, different trends in F_2-isoprostane concentrations were found between the HM and PF groups, with values decreasing post-feed in the HM group and increasing slightly in the PF group, although these differences did not reach statistical significance. However, this difference reached statistical significance between the predominantly HM group and the predominantly PF group through to 21 days post-birth. These differences were not affected by perinatal risk factors for oxidative stress, including PROM, ≥50% oxygen use at delivery, indomethacin use, or the introduction of HMF and/or PF. These data suggest that high-dose HM may downregulate oxidative stress responses in the early post-birth exposure period.

Our finding that HM feedings are associated with a *decrease* in oxidative stress in the early post-birth period in premature infants is consistent with previously published research by Shoji et al.[7] Similar to our study, Shoji compared the oxidative stress response in 15 predominantly HM-fed (>90% of feedings = HM) and 14 predominantly PF-fed (>90% of feedings = PF) VLBW infants at 2, 7, 14 and 28 days post-birth, using urinary 8-hydroxydeoxyguanosine (8-OHdG) as a biomarker for oxidative stress. Over these time points, the median concentrations of 8-OHdG in HM-fed but not PF-fed

infants decreased, with statistically significant differences noted between the groups for the 14 and 28-day time points. The time-effect differences observed in Shoji et al. is similar to our study where the difference between feeding groups was not significant after first feeding exposure. The most plausible explanation is that the total amount of HM feeding exposure after one feed was not yet high enough to exert an effect on oxidative stress in our study. A limitation of the Shoji et al data is that no information is provided about whether HMF was used during the study, and if so, its impact on oxidative stress was not addressed.

The effect of bovine-based HMF on oxidative stress in 65 VLBW infants was studied by Friel et al, using dose-response methodologies and F_2-isoprostane as a biomarker.[6] Two groups of infants were studied: a HM group (≥ 75% of all NICU feedings = HM) and a PF group (≥ 75% of all NICU feedings = PF). For the HM group, the exposure to HMF was measured by calculating the cumulative HM dose received by infants over the entire NICU stay and then categorizing infants into three groups based on the percentage of the cumulative HM dose that was mixed with HMF (0-19%, 20-49%, and ≥50%). Although F_2-isoprostane concentrations were measured every two weeks during the NICU hospitalization, no baseline pre-feed data were reported. Results revealed a dose-response relationship between cumulative exposure to HMF and oxidative stress. F_2-isoprostane concentrations in the HMF ≥50% group were higher than mean F_2-isoprostane concentrations in the PF group.[6]

In contrast, our findings did not reveal a significant effect of HMF on the oxidative stress response in HM-fed infants. Either HMF or PF was introduced to our predominantly HM feeding group at a median of DOL 8, and 100% of all HM feeds were

mixed with HMF between DOL 8 – 21. However, median F_2-isoprostane concentrations continued to decrease significantly after the introduction of these bovine products when compared to our predominantly PF group. Several possibilities may explain the differences between our results and those of Friel et al., with the most logical being the potential cumulative impact of high-dose HMF, as noted over the 10-week period in the Friel et al study. Our study collected data only for a 21-day period, during which our infants would have been receiving only colostrum and transitional HM, both of which contain greater concentrations of protective proteins than HM produced later in lactation.[15,20] These compositional differences in early HM may be especially protective against oxidative stress. HM handling conditions within the NICU setting may also affect the antioxidant and related bioactivity of HM, especially in the presence of HMF.[21-23] These NICU-specific procedures include prioritization of fresh versus frozen HM feedings, thawing and warming procedures and whether HMF is added for a single feeding or for a 24-hour feeding volume. Friel et al did not provide information about these NICU-specific practices, so direct comparisons with our findings cannot be made. Since our policy was to delay the introduction of HMF until enteral feeding volume = 140 mL/kg/d had been achieved and we prioritized providing fresh over frozen HM, infants in our study may have received a longer duration of fresh colostrum and transitional HM feedings, a practice that has been linked to reduction in morbidities in this population.[24] However, subsequent clinical research in this field should report and/or control for these potential confounders that are commonplace in the NICU setting. Lastly, the HMF used by Friel et al contains higher iron content out of the two commonly used commercially available HMFs, whereas the HMFs used during our study period was a

mix of both HMFs on a rotation basis. The consistent use of higher iron containing HMF in the Friel cohort may have contributed towards the observed higher oxidative stress in infants who received more HMF.[23]

Although previous investigators demonstrated increased urinary F_2-isoprostane concentrations for up to 12 hours post-birth when infants were exposed to PROM or 100% oxygen for resuscitation at delivery[3-5], our findings revealed no impact from these associated risk factors over the 21-day post-birth study period. Since these previous studies measured oxidative stress only through to 12 hours post-birth, it is possible that high-dose HM feedings may partially neutralize these initial perinatal oxidation risks in the premature infant population. Friel et al adjusted their data for infant sex and supplemental oxygen exposure during the NICU stay, whereas Shoji did not report data about these perinatal risk factors.[6,7]

While we did not observe any incidences of NEC or sepsis during our study period, there were three cases of BPD, all of which were in the predominantly HM group. However, this difference did not yield statistical significance between the two study groups; this is likely due to the small sample size and this study was not powered to detect BPD as a primary outcome. Interestingly, Shoji et al found higher incidences of retinopathy of prematurity and NEC in their HM group compared with PF group, although these differences were also not statistically significant likely due to small sample size.[7] These are unexpected findings that will require additional investigation with a larger sample size with precise quantification and timing of HMF use.

This study has many strengths, including serial urine samples that allowed time-dependent comparisons of oxidative stress between HM and PF feeding groups. These

serial urine samples also permitted specific examination of the effect of HMF/PF introduction in the predominantly HM fed group, bridging the previous study findings of Shoji and Friel.[6,7] Unlike previous studies, these serial samples also included a baseline pre-feed measure of urinary F_2-isoprostane concentration to permit comparisons of the impact of first feeding type. An additional strength is the clinical database for subjects that informed the calculation of exact HM and PF doses for the time points of interest and the exclusion of infants receiving prolonged antibiotic therapy, thus mitigating the effect of presumed sepsis on oxidative stress. However, the primary limitation of this study is its pilot nature and relatively small sample size, which likely limited the power to detect significant differences for all comparisons. In addition, HMF was added when feeding volume reached 140 mL/kg/d in this study per our enteral feeding policy, which is later than many institutions, and may limit the generalizability of our results.

In summary, we found that predominantly HM feedings decreased oxidative stress and predominantly PF feedings increased oxidative stress in premature infants over the first 21 DOL. These findings were not affected by the timing of introduction of HMF or PF in the predominantly HM group, nor by perinatal risk exposures of PROM, the use of O_2 concentrations $\geq$ 50% for delivery room resuscitation or the use of indomethacin. We conclude that predominantly HM feedings in premature infants during the first 21-days post-birth reduces oxidative stress in this vulnerable population which can explain the underlying mechanism by which HM decreases risk of morbidities. Subsequent research should prioritize comparison studies that enroll a larger sample of premature infants, and include exact measures of HM and PF doses, the timing of HMF

introduction, adjust for perinatal risk factors, and include additional oxidative stress-related diseases such as late onset sepsis, BPD, and neurodevelopmental problems.

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