Original Article

An Observational Study on Effect of High Human Milk Intake on Oxidative Status of VLBW Infants

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Background: Preterm infants are at high risk of oxidative stress injuries from various conditions such as low antioxidant capacity, relatively hyperoxia state outside utero, and possible immaturity of many organs. Human milk is an ideal nutrition and medication for these infants. It has antioxidative effects from both enzymatic and non-enzymatic constituents.

Objective: To evaluate the beneficial effects of human milk on oxidative status and antioxidant capacity in very-low birth weight infants.

Materials and Methods: The present research was a prospective cohort study conducted between January and December 2017. Preterm birthweight less than 1,500 grams were enrolled. Infants were divided into two groups based on breastmilk intake proportion, more than 50% or less than 50% of milk intake. Oxidative status was assessed using plasma malondialdehyde (MDA), and total antioxidant status (TAS) was assessed using Trolox equivalent antioxidant capacity (TEAC).

Results: Nineteen (19) VLBW infants were enrolled. Six infants (32%) were in low breastmilk intake group and 13 infants (68%) received breastmilk 50% or more of total enteral intake. Plasma MDA at D1, D7, and D28 were 6.49±5.76, 9.49±6.77, and 6.96±4.84 μM in the low breastmilk intake group, and 8.68±4.44, 8.82±5.01, and 5.71±2.74 in the high breastmilk intake group ($p>0.05$). Plasma TAS at D1, D7, and D28 were 4.06±0.87, 6.73±2.07, and 7.59±2.03 mmol of Trolox equivalence/L in the low breastmilk intake group, and 8.2±1.55, 7.4±2.29, and 6.58±1.9 mmol of Trolox equivalence/L in the high breastmilk intake group ($p>0.05$). There was no difference in patient characteristics regarding gender, gestational age, birthweight, mode of delivery, surfactant use, duration of mechanical ventilation, or duration of oxygen supplementation.

Conclusion: The present study was unable to demonstrate the differences of plasma MDA and total antioxidant capacity in VLBW infants. Therefore, further studies using more sensitive markers were suggested.

Keywords: VLBW, Very low birth weight, Preterm, Oxidative stress, Human milk

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Oxidative stress is an imbalance between production of reactive oxygen species (ROS) such as superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2), and antioxidant defenses. Antioxidant mechanisms of aerobic species include enzymatic systems like superoxide dismutase, glutathione peroxidase, and catalase, as well as non-enzymatic antioxidant factors such as vitamin A, E, C, and glutathione. ROS, when produced in physiologic concentration, play as important mediators in many cellular functions, like immune response and energy production. Although, oxygen has many beneficial effects to human life, too much oxygen can cause many negative effects to humans. In neonatal period, abrupt increase in oxygen availability during fetal to neonatal transition causes generation of oxidative stress and ROS. Premature babies are even more susceptible to oxidative stress injuries that can result in oxidative stress related diseases such as retinopathy of prematurity (ROP), bronchopulmonary dysplasia (BPD), periventricular leukomalacia (PVL), and punctate white matter lesion (PWML)[1]. Antioxidants in premature babies are incompletely developed and deficient[2]. Moreover, many conditions in preterm like hypoxia, infection, inflammation, and mitochondrial dysfunction predispose premature babies to oxidative stress injuries.

Human milk is considered an ideal nutrition for infants. It has distinguished properties to promote growth and health in all infants. In preterm babies, human milk is even more important. Data show that feeding preterm babies with human milk can

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promote brain volumes, better IQ in later life, and less metabolic disease[3,4]. Moreover, feeding human milk can reduce development of oxidative related disease in preterm such as necrotizing enterocolitis (NEC) and ROP[5,6]. Human milk contains a variety of antioxidant properties. It not only contains antioxidant enzymes like catalase, glutathione peroxidase, and superoxide dismutase, but it also contains other constituents important to enzyme functions like copper, zinc, uric acid, and erythropoietin. Although, formula contains significant amounts of antioxidative molecules like vitamin A, C, and E, studies have shown that human milk provides better antioxidant capacity than infant formula[7]. However, there is little evidence regarding oxidative stress status and antioxidant capacity of very low birth weight infants based on human milk intake volume. Therefore, the present study was aimed to assess the effect of human milk intake on oxidative stress and total antioxidant capacity in these infants.

Materials and Methods

The present study was a prospective cohort study. It was done in a university-based hospital in suburban Bangkok, Thailand, between January and December 2017. Infants whose birth weight was less than 1,500 grams were enrolled if they were admitted into the author's neonatal unit within seven days of life. Exclusion criteria were severe congenital malformation, congenital infection, renal failure, or any syndromic or genetic diseases. Based on a previous study[7], total antioxidant capacity (TAC) in breast-fed and formula-fed preterm infants were 2.27±0.21 and 2.02±0.25 mmol Trolox equivalences/L, respectively. With an alpha and beta value of 0.1 and 0.2, a sample of 11 infants per group were required. The protocol was approved by the Ethic Committee of Thammasat University, MTU-EC-PE-6-020/59. The participant information sheets and consent forms were given to all parents of participants.

Gender, birthweight, Apgar score 1 and 5 minutes, RDS requiring surfactant replacement therapy, duration of mechanical ventilation, oxygen support, and hospital stay were recorded for each infant. Complications of prematurity such as BPD, ROP, and NEC were also recorded. BPD was diagnosed in infants requiring oxygen support for more than 28 days. NEC was diagnosed based on clinical and radiographic findings according to Bell's staging criteria. All infants received parenteral nutrition support within 24 to 48 hours of life, which was comprised of dextrose, proteins, lipid and parenteral vitamin (OMVI®), and mineral supplementation (Peditrace®). Enteral feeding was introduced as soon as possible, feeding was advanced per attending staff consideration until reaching 130 to 160 mL/kg/day. Most of the infants received enteral vitamin supplementation in the second week of life, multivim drop® 1 mL/day with vitamin A 2000 IU, vitamin D 400 IU, vitamin C 40 mg, dexpanthenol 3.5 mg. Iron supplementation was started during second and third week of life ranging from 2 to 4 mg/kg/day.

Human milk intake

In the study period, there was no donor milk available in the present unit. Therefore, the author used only mother's own milk (MOM). Percentages of human milk intake were calculated based on the amount of MOM intake or total milk intake during the first 28 days of life. High human milk intake group (high group) was defined as infants who received MOM more than 50% of total milk intake. Low human milk intake group (low group) was defined as infants who received formula less than 50% of total milk intake.

Oxidative stress and antioxidant capacity measurement

Oxidative stress status was assessed using lipid peroxidation product, malondialdehyde (MDA), using the thiobarbituric acid-reactive substances (TBARS). In summary, 100 µL of plasma was added to 750 µL of orthophosphoric acid, and vortexed and left at room temperature for 10 minutes. Then 250 µL of thiobutyric acid (TBA) were added and vortexed. After 30 minutes of 100°C incubation in water bath, samples were cooled down in ice for 10 minutes. The mixture was centrifuged at 4,000 rpm for 10 minutes, supernatant OD was measured at 532 nm. The concentrations of TBA (mmol/L) were estimated based on standard curve.

Total antioxidant status (TAS) was assessed using Trolox Equivalence Antioxidant Capacity (TEAC). The assay measures the antioxidant ability to decolorized ABTS [2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)] at 734 nm. The concentrations of TAS were estimated based on standard curve, as mmol Trolox equivalence/L.

Plasma MDA and TAS were measured three times, at 24 hours, 7 days, and 28 days of life.

Statistical analysis

Patients' characteristics are described in mean ± standard deviation (SD) and percentage. Plasma MDA and TAS were described in mean ± SD. Continuous

Table 1. Patient characteristics

	Low group (n=6); mean±SD	High group (n=13); mean±SD	p-value
GA (weeks)	31.8±4	30.9±2	0.498
Birthweight (g)	1,066±332	1,277±236	0.128
Maternal hypertension; n (%)	2 (33.3)	6 (46.1)	0.620
%BM intake*; median (min, max)	27.7 (2.42, 47.9)	90.9 (72.4, 132)	0.001
ADDHM (mL/kg/day); median (min, max)	17 (2.56, 47.3)	82.8 (54.3, 98.3)	0.001
Apgar score at 1 minute	7.1±2.8	7.7±1.7	0.588
Apgar score at 5 minutes	9±2	9.5±1.4	0.550
Ventilator days*; median (min, max)	11.5 (0, 73)	7 (1, 30)	0.114
Iron supplementation (mg/kg/day)	1.04±0.64	3.09±1.16	0.001
Oxygen days; median (min, max)	23.5 (1, 148)	34 (1, 89)	0.660
RDS received surfactant replacement; n (%)	3 (50.0)	5 (38.5)	1.0

GA=gestational age; BM=breastmilk; ADDHM=average daily intake of human milk; RDS=respiratory distress syndrome; SD=standard deviation

* Data were used Mann-Whitney U test (non-normal distribution)

Table 2. Plasma MDA and TAS

	Low group (n=6); median (min, max)	High group (n=13); median (min, max)	p-value
MDA_{D1}* (mmol/L)	8.44 (0, 11)	8.68 (4.39, 16.5)	0.796
MDA_{D7}* (mmol/L)	8.16 (2.69, 22.4)	7.96 (1.94, 20)	0.930
MDA_{D28}* (mmol/L)	6.38 (1.01, 13.1)	5.93 (1.65, 9.11)	0.723
$^{\Delta}$ MDA_{D28-1}* (%)	–40.8 (–72.8, –8.89)	–36.3 (–76.7, 90.2)	0.739
TAS_{D1}	4.06±0.87	8.2±1.55	0.014
TAS_{D7}	6.73±2.07	7.4±2.29	0.546
TAS_{D28}	7.59±2.03	6.58±1.9	0.348
$^{\Delta}$ TAS_{D28-1}	79.6 (53.6, 105.6)	–12.3 (–59.9, 44.9)	0.027

MDA=malondialdehyde; TAS=total anti-oxidant status

* Data were used Mann-Whitney U test (non-normal distribution)

variables were analyzed using independent t-test and Mann-Whitney U test were used in normally distributed and non-normally distributed data, respectively. Categorical variables were analyzed using Fisher's exact test; Stata, version 14 (StataCorp LP, College Station, TX, USA).

Results

There were 19 infants enrolled in the present study. Gestational age ranged from 27 to 36 weeks. Birth weight ranged from 604 to 1,474 g. Most of mothers (82%) received antenatal steroid. Six infants were in low human milk intake group with mean GA of 31.8±4 weeks, and mean birthweight 1,066±332 g. Thirteen infants were in high group with mean GA of 30.9±2 weeks and mean birthweight of 1,277±236 g. Infants' baseline characteristics are presented in Table 1. There was no statistical difference in gestational age, birthweight, Apgar score, and proportion of infants receiving surfactant replacement therapy.

Plasma MDA and TAS are shown in Table 2. There was no difference in plasma MDA at DOL 1, 7, and 28. Infants in high group has TAS higher than infants in low group at the first day of life. TAS were not statistically different at DOL 7 and 28. However, there were significant differences in percentage change of TAS from D1 to D28. Infants in high BM intake had decreased TAS, while infants in low BM intake had increased TAS.

In term of clinical outcomes, none of the infants in the present study had NEC or ROP. Only one infant in high group has central line-associated blood stream infection (CLABSI). There was no statistical difference in term of BPD, days of mechanical ventilation or duration of oxygen supplementation,

Table 3. Clinical outcomes of infants

	Low group (n=6); median (min, max)	High group (n=13); median (min, max)	p-value
BPD; n (%)	3 (50.0)	6 (54.55)	1.00
Ventilator day*	11.5 (0, 73)	7 (1, 30)	0.114
Oxygen day*	23.5 (1, 148)	34 (1, 89)	0.660
LOS (Days)*	41 (19, 176)	41 (12, 176)	0.759
CLABSI; n (%)	0 (0.0)	1 (7.69)	1.00

BPD=bronchopulmonary dysplasia; LOS=length of hospital stay; CLABSI=central line-associated blood stream infection

* Data were used Mann-Whitney U test (non-normal distribution)

and length of hospital stay as shown in Table 3.

Discussion

Even though, human milk provides better antioxidant capacity than infant formula, in the present study, there was no difference in plasma MDA, which reflects lipid peroxidation and total antioxidant capacity in these VLBW infants. Previously, several studies reported potential benefits of human milk on oxidative stress or antioxidant capacity of infants[7]. A study from Shoji et al demonstrated that VLBW infants who received breastmilk more than 90% of total intake had lower urinary 8-hydroxydeoxyguanosine (8-OHDG), a marker of DNA oxidative stress, at DOL 14 and 28 than infants who received formula. Another study from Shoji et al group showed that breastmilk can reduce H2O2-induced oxidative damage in intestinal epithelial cell lines[8-10]. Oveisi et al study showed that total antioxidant capacity was significantly higher in human breast milk than in formula[11].

There were several reasons why the present study findings could not differentiate any difference of lipid peroxidation and total antioxidant capacity in breastfeeding preterm infants. First, VLBW infants have immature antioxidant systems and inadequate ROS scavengers. Therefore, even though human milk has high antioxidative properties, it might not be enough to alleviate the oxidative stress related damage in these groups of patients. Second, the author's infants had higher plasma MDA than in previous studies[12,13], because the mean duration of oxygen supplementation was more than a month in both groups. In addition, eight infants (42%) in the present study had severe respiratory support, needed surfactant replacement therapy and mechanical ventilation, which predisposed infants to more oxidative stress injury. Generally, nutrition plays a role in protecting infants from oxidative stress injury, but oxidative related disease in preterm infants has multifactorial causes such as antenatal steroid, gestational age, intrauterine infection and inflammation, and hospital acquired infection. Human milk alone might not be sufficient to completely protect these infants from oxidative stress related disease. Because of the small sample group size, the author was unable to eliminate these confounding effects. In addition, the present study was not primarily designed to detect any difference in clinical outcome, hence it was unable to show the effect of human milk on clinical outcomes. In the present study, plasma MDA was assessed by TBARS. Assessment of MDA in lipid rich samples such as plasma or serum had some limitations, such as degree of hemolysis, sampling, and storage process. Moreover, TBARS could react with other carbonyl groups containing compounds in plasma[14]. To minimize preanalytical errors, whole-blood was centrifuged as soon as possible, and then serum was stored in –20°C until analyzed within one month. Surprisingly, the author found higher TAS at birth in high BM intake groups even though there were no differences in gestational age or maternal status. Serum TAS was not different at DOL7 and 28. There were reports of higher TAS in term infants fed human milk compared to formula fed infants[7]. According to development of antioxidant system, infant total antioxidant capacity is increased as infants get more maturity. The present subjects in both groups had the same gestational age, and similar clinical course so the author might not be able to demonstrate any difference in TAS between the two groups. Recent study from Pozzo et al, showed that TEAC properties of some preterm formula was not different from human milk. This might be attributed to the types of protein, vitamins, carotenoids, and flavonoids content in the formula[15]. However, percentage changes of TAS from D1 to D28 were significantly different between groups. In the present study, infants in high BM intake group had decreased TAS, contrast to low BM intake groups, which had

higher TAS. This finding might probably result from higher intake of iron supplementation in high BM group. Preterm infants are vulnerable to iron toxicity especially from non-transferrin-bound iron (NTBI) from high doses iron supplementation or recurrent transfusion. Regarding immature antioxidant system, preterm infants cannot eliminate iron excess from normal physiologic pathway result in cellular toxicity. Therefore, infants in high BM intake group have significantly decreased of TAS from D1 to D28[16].

Conclusion

The present study had found no difference in lipid peroxidation nor total antioxidant capacity in infants that received mainly breastmilk or preterm formula. The author's suggestion for future study is to use more specific and sensitive markers for detecting oxidative damage on lipid, protein or even DNA.

What is already known in this topic?

VLBW infants are at high risk of oxidative stress injury and oxidative stress related disease. Human milk is superior than formula in term of antioxidant properties.

What this study adds?

There was no difference in serum MDA using TBARs, and TAS using TEAC. Theoretically, infants that received more human milk should have better anti-oxidative status. However, only human milk might not be sufficient to alleviate all the oxidative injury to these infants.

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Conflicts of interest

The authors declare no conflict of interest.

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