ORIGINAL ARTICLE

Effects of exclusive formula or breast milk feeding on oxidative stress in healthy preterm infants

O Korchazhkina, E Jones, M Czauderna, S A Spencer

Arch Dis Child 2006;91:327-329. doi: 10.1136/adc.2005.084798

See end of article for authors' affiliations

Correspondence to:
Dr S A Spencer, Institute
for Science and
Technology in Medicine,
School of Medicine,
University of Keele,
Thornburrow Drive,
Hartshill, Stoke-on-Trent
ST4 7QB, UK; andy.
spencer@uhns.nhs.uk

Accepted 12 January 2006
Published Online First
20 January 2006

Background: Compared to formula, breast milk is considered to have superior antioxidant properties and consequently may reduce the occurrence of a number of diseases of prematurity associated with oxidative stress.

Aims: To test whether the antioxidant properties of breast milk in healthy premature infants are different to those of formula milk by comparing vitamin E levels in milk and determining the excretion of malondialdehyde (MDA) in urine.

Methods: Vitamin E was measured in the breast milk of 20 mothers who had given birth prematurely. Urinary MDA was measured in 10 exclusively breast milk fed and 10 exclusively formula fed healthy preterm infants receiving no vitamin supplements. MDA was measured after derivatisation with 2,4-dinitrophenylhydrazine and consecutive HPLC with UV detection.

Results: Urinary MDA concentrations were consistently very low ($0.074 \pm 0.033 \mu\text{M}/\text{mM Cr}$ and $0.078 \pm 0.026 \mu\text{M}/\text{mM Cr}$ in breast and formula fed infants respectively) and not significantly different between healthy breast milk and formula fed infants. Both breast and formula milk contained satisfactory levels ($0.3\text{--}3.0 \text{ mg}/100 \text{ ml}$) of vitamin E.

Conclusion: Antioxidant properties of both breast milk and formulae are sufficient to prevent significant lipid peroxidation in healthy premature infants.

Oxidative stress occurs when the formation of reactive oxygen species is not counteracted by an adequate antioxidant defence system. Preterm infants are at greatly increased risk of oxidative stress because the enzymatic free radicals defence systems (superoxide dismutase, catalase, glutathione peroxidase) are not sufficiently developed.¹⁻⁴ Blood levels of substances with antioxidant effects (α -tocopherol, ascorbic acid, β -carotene) are also low as transfer across the placenta is incomplete.¹⁻³ Furthermore these infants are often exposed to high concentrations of inspired oxygen as part of the respiratory management of premature lungs. This increased susceptibility to oxygen derived free radical damage has been implicated in the occurrence of a number of diseases of prematurity, including retinopathy of prematurity, necrotising enterocolitis, and bronchopulmonary dysplasia.⁶⁻¹⁰

Oxygen derived free radicals react with polyunsaturated fatty acids of various cellular membranes. This gives rise to a variety of lipid peroxidation products, including malondialdehyde (MDA).¹¹⁻¹²

Urinary MDA has been widely used in animal models and in humans as a non-invasive biomarker of lipid peroxidation induced by oxidative stress.¹³⁻¹⁴ Existing literature on the urinary and plasma levels of MDA in neonates is based on an assay with 2-thiobarbituric acid (TBARS).⁸⁻¹⁵⁻¹⁶ Unfortunately the TBARS assay is intrinsically non-specific towards MDA.¹⁷ Additionally, MDA can be produced as an artefact during the assay, therefore overestimating the real levels by approximately 10-fold.¹⁷⁻¹⁸ We have developed and validated a new method of measuring MDA in human urine based on the derivatisation with 2,4-dinitrophenylhydrazine and consecutive high performance liquid chromatography (HPLC) with ultraviolet (UV) detection.¹⁸ This DNPH based method was used to measure the level of MDA in urine to evaluate lipid peroxidation in preterm infants.

It has been reported recently¹⁹ that compared to formula, breast milk can suppress the oxidative DNA damage in

premature (approx 29 weeks of gestation) and very low birth weight infants (VLBW, less than 1500 g). Human milk contains various bioactive compounds, including enzymes, numerous immunological factors, hormones, growth factors, and various antioxidants.²⁰⁻²² Superoxide dismutase and glutathione peroxidase are two important antioxidant enzymes found in human milk.²³ Whether these compounds in milk have any beneficial effect on the infant is not yet established.

Major antioxidants in commercially available brands of formula milk are vitamins E and A which are added in relatively high amounts, depending on the manufacturer. Vitamin E is a major antioxidant in milk, which may impact on the results. Therefore the levels in breast milk were measured and those in formula checked against the manufacturers' stated values.

The hypothesis that healthy premature infants fed exclusively breast milk receive more efficient antioxidant protection than formula fed counterparts was tested. To evaluate lipid peroxidation that might occur in infants, we measured the levels of MDA in urine. As MDA can be generated in vitro from oxidation of milk lipids, levels in milk were also measured.

METHODS

Patients

Recruitment into this study was performed in accordance with the approval of the North Staffordshire Local Research Ethics Committee. Infants born prematurely at the University Hospital of North Staffordshire were eligible for this study if they fulfilled all of the following inclusion criteria: no oxygen or phototherapy; no complications of prematurity; no history of parenteral nutrition, on total enteral feeding (volume of feed minimum 150 ml/kg); no vitamin supplementation; and

Abbreviations: MDA, malondialdehyde; TBARS, 2-thiobarbituric acid; VLBW, very low birth weight

exclusively breast milk or formula fed. Written informed parental consent was obtained. Breast milk was expressed on the unit using an electric breast pump (Lactina, Symphony, Medela, UK) and fed through the tube in the same manner as formula milk. The formula milk used in the study was SMA Gold (Wyeth).

Collection and analysis of urine and breast milk specimens

Spot urine samples were collected once from each infant. Collection of urine specimens was performed non-invasively by placing a cotton wool pad onto the perineal area of an infant inside an ordinary nappy. Following urination the saturated pads were removed, placed into a syringe (capacity 20 ml), and urine was squeezed into a sterile container. Any stool contaminated specimens were discarded and the collection repeated. In a preliminary experiment with urine spiked with MDA, we established that the levels of MDA measured before and after soaking urine into a pad for up to 30 minutes and recovering using a syringe were the same. After collection the specimens were placed on ice and transported to the laboratory for measurements, which were performed on the same day. MDA was measured after the derivatisation with 2,4-dinitrophenylhydrazine and consecutive HPLC with UV detection as described in detail previously.¹⁸ Urine creatinine was measured using HPLC²⁴ and was used to express MDA excretion as μM of MDA per mM of creatinine ($\mu\text{M}/\text{mM Cr}$).

Milk assigned for measurement of MDA and vitamin E was expressed into sterile plastic containers using the above hospital grade electric breast pump. Each milk specimen represented a complete expression of one breast. After collection milk was immediately frozen and kept in the freezer at -40°C until analysis. Analyses of vitamin E (α , β , and γ tocopherol, and tocopherol acetate) were performed within 10 days of collection according to the method described previously.²⁵ MDA in breast milk and formula was measured using the method described previously¹⁸ with a small modification. As MDA is water soluble it was measured in the whey component after separation of fat by centrifugation.

Statistical analysis

A sample size calculation was applied to the primary outcome measure, which was MDA excretion in urine. In order to detect a large effect size of 1, with a power exceeding 80% for two sided significance level of 5%, a sample size of $n = 20$ in each group was required.

Two way analysis of variance was used to compare means (Minitab version 13). The difference was considered significant for $p < 0.05$. Means and standard deviations are presented.

RESULTS

MDA in urine

An interim analysis of urine results in 10 patients in each group showed that all urine MDA levels were negligible. Consequently urine collection was discontinued at this point.

Table 1 Clinical data of urine donors

	EBM fed	Formula fed
Birth weight, g	2694 $\pm$ 758	2611 $\pm$ 394
Gestational age, weeks	34.5 $\pm$ 1.5	35.6 $\pm$ 1
Age, days	6 $\pm$ 1.5	4 $\pm$ 1.7
Total n (male:female)	10 (4:6)	10 (6:4)

EBM, expressed breast milk.

The group characteristics of breast milk and formula milk fed infants are presented in table 1.

The levels of MDA in urine of healthy preterm 4–6 day old infants exclusively fed with expressed breast milk ($0.074 \pm 0.033 \mu\text{M}/\text{mM Cr}$, range 0.037–0.138) were not significantly different from those who were exclusively fed with formula milk ($0.078 \pm 0.026 \mu\text{M}/\text{mM Cr}$, range 0.051–0.125) and in both cases were very low indeed. In view of the possibility that urine creatinine may not give a reliable indicator of urine concentration in preterm infants, the absolute values are also presented. For breast milk fed infants the urine MDA was ($0.098 \pm 0.056 \mu\text{M}$), and for formula milk fed infants it was $0.109 \pm 0.028 \mu\text{M}$. The results between the two groups are similar.

MDA in breast milk and formula

Twenty mothers with a plentiful milk supply who had given birth prematurely were recruited into the study (table 2).

In both formula and freshly expressed breast milk, levels of MDA were below the detection limits (detection limit $0.06 \mu\text{mol/l}$). A preliminary study of breast milk specimens, which had been stored at -20°C for more than 12 months prior to analysis, measured a significant amount of MDA in milk (up to $5 \mu\text{mol/l}$). This was due to oxidation of milk fat, as a result of prolonged storage.

Vitamin E in breast milk and formula

Expressed milk from the same subjects (table 2) were also used for vitamin E analysis. The major forms of vitamin E found in human milk were α -tocopherol and γ -tocopherol. Tocopherol acetate was present in minor amounts in some milk. The levels of the major form of vitamin E (α -tocopherol) were variable within the range 0.3 – $1.5 \text{ mg}/100 \text{ ml}$ ($0.72 \pm 0.42 \text{ mg}/100 \text{ ml}$). Levels of vitamin E in formula milk were in an excellent agreement with the levels stated by manufacturers (1.1 – $3.0 \text{ mg}/100 \text{ ml}$), depending on the brand.

DISCUSSION

In this study there was no significant difference between values of urine MDA in healthy preterm infants solely fed breast milk or formula milk. Urine MDA was measured using the DNPH based method and was found to be about 10-fold lower than levels reported previously^{8,15} (range of values 0.05 – 0.3 versus 0.5 – $2.0 \mu\text{mol/l}$) using the TBARS method. This method has been shown to greatly overestimate the real values of MDA^{17,18} due to various artefacts of the assay. Therefore it is likely that our results represent the correct range of values.

One possible criticism of the use aldehydes²⁶ like MDA as indicators of oxidative stress is their susceptibility to influence by external factors such as energy status, physical activity, temperature of the environment, and a variable intake of peroxides in the diet. However, in this respect preterm infants are in a well controlled situation with limited physical activity, and a diet restricted to breast milk or formula. Furthermore levels of MDA in both freshly expressed breast milk and formula were below the limits of

Table 2 Clinical data of milk donors ($n = 20$)

Parameter	Average $\pm$ SD	Range
Age, years	28 $\pm$ 6	20–41
Gestation, weeks	32 $\pm$ 4.5	24–38
Milk volume per breast, ml	86 $\pm$ 32	35–170
Days since delivery	9 $\pm$ 2.5	3–13
Days expressing	8 $\pm$ 2.3	3–12

What is already known on this topic

- Preterm infants are at high risk of lipid peroxidation as a result of oxidative stress
- Excretion of MDA in urine can be used as a non-invasive marker of lipid peroxidation

detection. Therefore, the intake of MDA by infants from both groups was negligible.

In another study,¹⁹ 8-hydroxydeoxyguanosine (8-OHdG) was used as a marker of oxidative stress. Levels were found to be lower in VLBW breast fed infants compared to those who were formula fed. However, in this study infants were more premature (29 versus 35 weeks of gestation) and they had complications of prematurity requiring phototherapy, ventilation, oxygen therapy, and parenteral nutrition. Furthermore the differences in urine 8-OHdG levels between the two groups were significant only on the 14th and 28th days after birth, but not earlier,¹⁹ indicating that metabolites relating to oxidative stress may take time to appear.

The levels of vitamin E measured (0.3–1.5 mg/100 ml) in breast milk are in good agreement with those reported previously.^{21–22–27} MDA measurements in urine suggest that these levels are satisfactory in terms of preventing peroxidation of lipid in well preterm infants. The same is true of all the commercially available formulae for preterm infants that were tested, having high amounts of vitamin E (1.1–3.0 mg/100 ml) and other antioxidants, including vitamin C and selenium.

In conclusion, a new more accurate technique has been used for measuring MDA in urine of healthy preterm infants. This shows that the antioxidants present in both human milk and formula are sufficient to prevent significant lipid oxidation and the appearance of anything other than trivial amounts of MDA in urine. This technique should now be applied to sick preterm infants in a range of situations. An increase in MDA levels would be a useful indicator of inadequate protection from oxidative stress in these situations. This would present the opportunity to target antioxidant treatment in affected individuals, with the possibility of monitoring the effects and subsequently reducing serious complications.

Authors' affiliations

O Korchazhkina, S A Spencer, Institute for Science and Technology in Medicine, University of Keele, Stoke-on-Trent, UK

E Jones, Neonatal Unit, University Hospital of North Staffordshire NHS Trust, Stoke-on-Trent, UK

M Czauderna, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jablonna, Poland

Funding: this study was supported by funds of the North Staffordshire Medical Institute, the Institute for Science & Technology in Medicine, and the Royal Society

Competing interests: none

REFERENCES

- Huertas JR, Palomino N, Ochoa JJ, *et al.* Lipid peroxidation and antioxidants in erythrocyte membranes of full term and preterm newborns. *BioFactors* 1998;**8**:133–7.
- Robles R, Palomino N, Robles A. Oxidative stress in the neonate. *Early Hum Dev* 2001;**65**:S75–81.
- Inder TE, Graham P, Sanderson K, *et al.* Lipid-peroxidation as a measure of oxygen-free radical damage in the very-low-birth-weight infant. *Arch Dis Child Fetal Neonatal Ed* 1994;**70**:F107–11.
- Georgeson GD, Szony BJ, Streitleman K, *et al.* Antioxidant enzyme activities are decreased in preterm infants and in neonates born via caesarean section. *Eur J Obstet Gynecol Reprod Biol* 2002;**103**:136–9.
- Baydas G, Karatas F, Gursu MF, *et al.* Antioxidant vitamin levels in term and preterm infants and their relation to maternal vitamin status. *Arch Med Res* 2002;**33**:276–80.
- Saugstad OD. Chronic lung disease: the role of oxidative stress. *Biol Neonate* 1998;**74**:21–8.
- Schock BC, Sweet DG, Halliday HL, *et al.* Oxidative stress in lavage fluid of preterm infants at risk of chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2001;**281**:L1386–91.
- Schlenzig JS, Bervoets K, von Loewenich V, *et al.* Urinary malondialdehyde concentration in preterm neonates—is there a relationship to disease entities of neonatal intensive-care. *Acta Paediatr* 1993;**82**:202–5.
- van Zoeren-Grobbe D, Lindeman JH, Houdkamp E, *et al.* Markers of oxidative stress and antioxidant activity in plasma and erythrocytes in neonatal respiratory distress syndrome. *Acta Paediatr* 1997;**86**:1356–62.
- Winterbourn CC, Chan T, Hendrikje B, *et al.* Protein carbonyls and lipid peroxidation products as oxidation markers in preterm infant plasma: associations with chronic lung disease and retinopathy and effects of selenium supplementation. *Pediatr Res* 2000;**48**:84–90.
- Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indexes of lipid-peroxidation and peroxidative tissue-injury. *Free Radic Biol Med* 1990;**9**:515–40.
- Esterbauer H, Benedetti A, Lang J, *et al.* Studies on the mechanism of formation of 4-hydroxynonenal during microsomal lipid-peroxidation. *Biochim Biophys Acta* 1986;**876**:154–66.
- Guichardant M, Valette-Talbi L, Cavadini C, *et al.* Malondialdehyde measurement in urine. *J Chromatogr B Biomed Appl* 1994;**655**:112–16.
- de Zwart LL, Meerman JH, Commandeur JN, *et al.* Biomarkers of free radical damage applications in experimental animals and in humans. *Free Radic Biol Med* 1999;**26**:202–26.
- Drury JA, Nycyk JA, Cooke RWI. Comparison of urinary and plasma malondialdehyde in preterm infants. *Clin Chim Acta* 1997;**263**:177–85.
- Granot E, Golan D, Rivkin, *et al.* Oxidative stress in healthy breast fed versus formula fed infants. *Nutr Res* 1999;**19**:869–79.
- Halliwel B, Chirico S. Lipid-peroxidation—its mechanism, measurement, and significance. *Am J Clin Nutr* 1993;**57**:S715–25.
- Korchazhkina O, Exley C, Spencer SA. Measurement by reversed-phase high-performance liquid chromatography of malondialdehyde in normal human urine following derivatisation with 2,4-dinitrophenylhydrazine. *J Chromatogr B Anal Technol Biomed Life Sci* 2003;**794**:353–62.
- Shoji H, Shimizu T, Shinohara K, *et al.* Suppressive effects of breast milk on oxidative DNA damage in very low birthweight infants. *Arch Dis Child Fetal Neonatal Ed* 2004;**89**:136–8.
- Bernt KM, Walker WA. Human milk as a carrier of biochemical messages. *Acta Paediatr* 1999;**88**:27–41.
- Boersma ER, Offringa PJ, Muskiet FA, *et al.* Vitamin-E, lipid fractions, and fatty-acid composition of colostrum, transitional milk, and mature milk—an international comparative study. *Am J Clin Nutr* 1991;**53**:1197–204.
- Chappell JE, Francis T, Clandinin MT. Vitamin-A and Vitamin-E content of human-milk at early stages of lactation. *Early Hum Dev* 1985;**11**:157–67.
- L'Abbe MR, Friel JK. Superoxide dismutase and glutathione peroxidase content of human milk from mothers of premature and full-term infants during the first 3 months of lactation. *J Pediatr Gastroenterol Nutr* 2000;**31**:270–4.
- Jen JF, Hsiao SL. Simultaneous determination of uric acid and creatinine in urine by an eco-friendly solvent-free high performance liquid chromatographic method. *Talanta* 2002;**58**:711–17.
- Paixao JA, Stamford TLM. Lactate matrices. A single guide for extraction and quantification of fat-soluble vitamins. *Journal of Liquid Chromatography & Related Technologies* 2002;**25**:217–39.
- Draper HH, Csallany AS, Hadley M. Urinary aldehydes as indicators of lipid peroxidation in vivo. *Free Radic Biol Med* 2000;**29**:1071–7.
- Chappell JE, Francis T, Clandinin MT. Simultaneous high-performance liquid-chromatography analysis of retinol ester and tocopherol isomers in human-milk. *Nutr Res* 1986;**6**:849–52.

What this study adds

- Normal levels of MDA in healthy preterm infants have been defined using an accurate validated method
- The antioxidant properties of both formula and expressed breast milk are sufficient to prevent lipid peroxidation in healthy preterm infants