Microlipid-Induced Oxidative Stress in Human Breastmilk: *In Vitro* Effects on Intestinal Epithelial Cells

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ABSTRACT

Objectives: To (1) determine whether medium chain fatty acids (Microlipid[®]) added to human breastmilk generates reactive oxygen species (ROS), and (2) measure the physiological effect(s) of Microlipid[®](ML)-supplemented human breastmilk in an enterocyte cell culture bioassay.

Methods: ML was added to milk according to manufacturer's recommendations and total hydroperoxides measured at intervals with the FOX 2 and TBARS assays. Physiological effects of supplementation were measured using a human enterocyte cell line (Caco-2BBE) and/or a primary human fetal intestinal cell culture (FHS-74 Int). Endpoints included: intracellular oxidative stress, transepithelial electrical resistance (TEER), apoptosis, and interleukin (IL)-6 production.

Results: Immediately postsupplementation, ML did not significantly increase ROS, as determined by both the FOX 2 and TBARS assays. Further, storage of milk + ML at 4°C prevented significant increases in total hydroperoxides. However, by 4 hours postsupplementation at room temperature, both assays revealed significantly higher hydroperoxide and lipid peroxide levels. ML-supplemented milk stored at room temperature for 4 hours had the following effects in cell culture bioassays: elevated oxidative stress, increased rates of apoptosis, decreased transmembrane electrical resistance (TEER) values and, in both cell culture assays, significantly increased secretion of IL-6.

Conclusions: Based on our measurements of extracellular and intracellular ROS, milk supplemented with fresh ML does not induce significant oxidative stress. However, when stored for 4 hours at room temperature, ML induces significant levels of oxidative stress. Decreases in TEER and increases in apoptosis and IL-6 secretion are consistent with ML-induced oxidative stress. It therefore is likely that in clinical situations, if ML-supplemented milk is not administered quickly, the newborn may be placed at greater risk of oxidative stress.

INTRODUCTION

HUMAN MILK HAS LONG BEEN RECOGNIZED as the optimal form of nutrition in the newborn period.¹ However, there is evidence that human milk does not provide the optimum level of calories and nutrients for preterm infants.² For these infants, fortification of breastmilk and formula with a variety of additives, including N-3 and N-6 fatty acids, has been shown to enhance postnatal growth.³ With the initiation of enteral feeds, gastrointestinal

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disorders such as necrotizing enterocolitis can develop. The etiology of such disorders may be linked to dietary sources of oxidative stress.⁴ In particular, linoleic acid, a supplement added to both enteral and parenteral formulations, is known to form lipid peroxides in the latter.⁵

Peroxide formation in fortified breastmilk has not been previously studied, and clinical practice regarding the reconstitution, storage, and use of lipid-fortified breastmilk has the not been fully examined. As a result, there is a paucity of information on which to base certain clinical guidelines regarding the acceptable period of storage for milk-ML admixtures. An informal survey of neonatal intensive care and special care units has revealed little consensus on this issue (W. Diehl-Jones, unpublished). Furthermore, little is known about the physiological effects of such supplements on the infant gut. The intestinal mucosa of the neonate is the site of first contact between exogenous nutrients and macromolecules destined for internalization. At a time when the gut is changing from a sterile to a nonsterile luminal environment, and when intestinal permeability is high and defense systems low, the newborn intestine may be particularly susceptible to insult and injury from reactive oxygen species (ROS) and oxygen-free radicals.

Conceptually, there are two potential sources of oxidative stress in the neonate: ischemia/ reperfusion injury, as can occur in the pre- and postnatal periods, and enteral factors. In particular, lipid peroxides constitute a potent potential source of ROS, which may greatly perturb intestinal homeostasis in the newborn infant. We have investigated the contributions of ML, a widely used dietary supplement given to premature infants, to the generation of ROS and/or reactive oxygen and its physiological impact. The latter was monitored by means of *in vitro* human cell cultures which were used as a bioassay of the effects of ROS on enterocyte physiology.

MATERIALS AND METHODS

Milk collection and storage

Preterm milk samples were obtained after ethical approval and informed consent from women who had delivered between 28 and 32 weeks of gestation. Samples were collected by staff nurses after manual expression or via a breast pump, and stored in sterile 50-mL plastic specimen containers. After collection in the neonatal intensive care unit, samples were frozen at -20° C and protected from light exposure. Samples were thawed immediately before use, and were subsequently discarded. Pasteurized milk was obtained by heating aliquots at 85°C for 5 minutes. Samples which were photoprotected were wrapped in aluminum foil.

Experimental design

Milk samples were pooled from at least three different donors and were subsequently divided into two groups: the fortified group and nonfortified. ML (50% linoleic acid emulsion with 4.5 calories/mL) (Mead Johnson Nutraceuticals, Evansville, IN) was added to samples in the fortified group according to manufacturer's directions (5 parts ML:100 parts breastmilk). Milk samples were then incubated in the dark at either room temperature (22°C) or in a refrigerator (4°C) for varying intervals. Samples were then used for ROS analysis for in cell culture assays (see below). ANOVA statistical methods were applied, and pairwise comparisons were made using Fisher's least significant difference test, except for measurements of transmembrane electrical resistance (TEER), where repeated-measures ANOVA was applied. Data were considered statistically significant when p < 0.05.

Measurement of hydroperoxides and lipid peroxides

Total hydroperoxide content, including lipid peroxides, were determined in milk and cell culture samples by the FOX 2 or ferrous iron oxidation of the xylenol orange method.⁴ Essentially, lipid peroxides oxidize ferrous to ferric ions in dilute acid:

$$Fe^{++} + ROOH \rightarrow Fe^{+++} + RO' + OH^{--}$$

Resultant ferric ions can be determined with the ferric-sensitive dye xylenol orange at 560 nm. The aqueous phase of milk samples was obtained via centrifugation, extracted with hexane, and assayed in 96-well plates. Authenticity of the hydroperoxides was determined with glutathione peroxidase (10 μ m), and quantities titrated with H₂O₂.

The thiobarbituric acid reactive substance (TBARS) method was used to quantify secondary oxidation products of lipids in milk which form TBARS. Briefly, hydrogen peroxide standards were prepared at concentrations of 0.1 μ M to 1 μ M in deionized water. Pooled milk samples (2 mL) were combined in test tubes with 230 μ L of 40% trichloroacetic acid solution. Samples were vortexed then centrifuged at $1600 \times g$ for 5 minutes to precipitate proteins in samples. Supernatant (1.6 μ L) was removed and placed in glass test tubes, to which was added 1.6 μ L 2-thiobarbituric acid in 1 N HCl. Samples were again vortexed, then incubated at 75°C for 30 minutes. After cooling and centrifugation at $1600 \times g$ for 5 minutes, 300 μ L of supernatant was added to each well of a 96-well plate, and absorbance was determined at 532 nm in an Opsys® (Thermolab Systems, Uppsala, Sweden) microplate reader. Either superoxide dismutase (SOD) (Sigma Chemical Co., St. Louis, MO) or glutathione peroxidase (GPx) (Sigma Chemical Co.) were used as free radical scavengers where indicated to determine whether observed changes were, in fact, due to the generation of oxygen-free radicals.

Cell culture

Two in vitro models were used in different components of this study: Caco-2BBE and FHS 74 Int (both obtained from the American Type Culture Collection, Rockville, MD). The former is a subclone of the well-characterized Caco-2 human colon cancer cell line, which expresses characteristics of both polarized colonic and intestinal epithelial cells, and which forms confluent, tight barriers. The latter is a primary human fetal intestinal cell culture which does not form confluent, polarized monolayers in culture, yet is otherwise genotypically more similar to neonatal enterocytes. Caco-2BBE cells were used in transwell cultures chambers to determine the effects of ML supplementation on epithelial barrier function, as determined by TEER, and in apoptosis assays. FHS 74 Int cells were also used in assays measuring intracellular oxidative stress. Both cell lines were cul-

tured according to well-established protocols. Briefly, cells were grown in either 25 cm² polystyrene flasks or on Transwell[®] (Corning, Co., Ithaca, NY) culture chambers in DMEM (Dulbecco's Modified Eagle Medium; GIBCO-BRL, Grand Island, NY) containing 1% penicillin/ streptomycin, 10% fetal bovine serum, 200 mM L-glutamine, 1 mg/mL human transferrin, and 100 mM sodium pyruvate (Sigma-Aldrich Canada, Ltd., Oakville, ON) at 37°C at 5% CO₂:95% room air. Epidermal growth factor (EGF) was added to FHS 74 Int culture medium at a concentration of 10 μ M to increase the rate of cell proliferation. Cultures were either maintained for 14-21 days, after which Caco-2BBe cells are fully confluent; FHS 74 Int cells were maintained for 4 days in culture before experimental treatments.

Measurement of epithelial barrier integrity

Caco-2BBe cells were plated onto polycarbonate membranes with 0.4- μ m pore size in 12mm diameter Transwell® chambers (Corning Incorporated, Corning, NY). Cells were grown to confluency (14-21 days), as judged by appearance and by measurements of TEER made with an EVOMX hand-held electrometer (World Precision Instruments Inc., Sarasota, FL). After stable TEER measurements between 450 and 550 ohms were obtained, 0.5 ml of either whole milk or culture medium \pm ML (6 parts to 100; Mead Johnson Nutritionals), or milk + ML + SOD were added to the apical chamber (four wells per treatment group and incubated for an additional 4 hours at 37°C under cell culture conditions. In this fashion, epithelial cells were exposed as they would be *in* vivo. Media were then aspirated from the apical chamber and replaced with cell culture media, after which TEER measurements were made.

Apoptosis assay

Apoptosis in Caco-2BBE cells was determined by measurement of caspase 3. Caspase 3-mediated hydrolysis of the acetyl-Asp-Glu-Val *p*-nitroanalide peptide into *p*-nitroaniline was detected colorimetrically with a kit purchased from Sigma. The kit was used according to manufacture's directions, with modifications. Reactions were conducted in 96-well plates and optical density measurements made at 405 nm. Negative controls included homogenates to which were added 2 mM caspase 3 inhibitor Ac-DEVD-CHO (Acetyl-Asp-Glu-Val-al). The positive control included homogenates with 2 μ g/mL caspase 3. A sample of noninduced cells was used for the time control, and one cell culture was induced to undergo apoptosis by addition of 1 μ g/mL staurosporine. Each treatment group consisted of 10 replicates.

Intracellular oxidative stress

Determination of intracellular oxidative formation was based on the oxidation of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) to yield an intracellular-trapped fluorescent compound. FHS 74 Int cells grown in tissue culture-treated 96-well plates (Corning) were loaded for 30 minutes in 5 μ M CM-H2DCFDA, after which each well was washed with culture medium. Cells were then exposed to one of the following: culture medium, whole milk, milk plus ML, or milk + ML + 10 μ M SOD, or xanthine $(200 \ \mu mol/L)$ /xanthine oxidase (5 mU/mL). Cells were incubated under 5% CO₂:95% air at 37°C, and at 0, 1-, 2-, 3-, and 4-hour intervals, fluorescence was measured using a Fluoroskan Ascent fluorometer using (Thermolab Systems, Helsinki, Finland) equipped with a 485-nm excitation filter and a 527-nm emission filter. Each treatment group consisted of 10 replicates.

Measurement of interleukin-6 (IL-6)

Caco-2BBE and FHS 74 Int cultures were exposed to ML for 4 hours at 37°C, washed in three changes of culture medium, and incubated for 12 hours under standard culture conditions. Culture medium from these cells was then collected and the IL-6 titre determined by ELISA using a colorometric assay kit from R&D Sytems (Minneapolis, MN), with some modifications. Briefly, 200 μ L of 10 μ g/mL mouse antihuman capture antibody was adsorbed onto 96-well immulon microplates (Corning) by incubating overnight at room temperature. After washing each well three times with Trisbuffered saline (TBS) (pH 7.2), plates were blocked (1 hour, room temperature) with 1%

bovine serum albumin, 5% sucrose in phosphate-buffered saline to prevent nonspecific binding. Plates were again washed three times with TBS, then loaded with 100 μ L of either varying combinations of the standard (recombinant human IL-6) in culture medium or with culture supernatant from treated cells. Plates were incubated overnight at 4°C, followed by 1 hour incubation at room temperature.

After washing as described above, wells were incubated with 100 µL detection antibody (100 ng/mL biotinylated goat antihuman IL-6) for 2 hours at room temperature, then washed. This was followed by the addition of 100 μ L/well of Strepavidin-horse radish peroxidase, incubation at RT for 20 minutes, followed by aspiration and washing. Substrate solution (100 μ L) was added to each well, followed by the addition of 50 μ L/well of stop solution (2 N H₂SO₄). The optical density of each well was immediately determinered using an Opsys plate reader (Ithaca, NY) at 450 nm. Results are reported as the mean and standard deviation of triplicate measurements from three separate cell cultures.

RESULTS

Effects of microlipid supplementation on free radical production in milk

After 4 hours incubation with ML, significantly higher total hydroperoxide levels were observed in milk samples tested by means of the FOX 2 assay (Fig. 1). This increase was further evident after 8 hours of incubation at room



FIG. 1. ML-induced hydroperoxides in human breastmilk.

IN HUMAN DREASIMILK, EITHER WITH OR WITHOUT MICROLIPID ⁻ (ML)					
Milk (RT, LE)	Milk + ML (RT, LE)	Milk (RT, LP)	Milk + ML (RT, LP)	Milk (4°C, LP)	Milk + ML (4°C, LP)
3.61 ± 0.52	$4.69 \pm 0.57^{*}$	2.22 ± 0.31	4.11 ± 0.32*	2.20 ± 0.31	2.11 ± 0.23

TABLE 1. EFFECTS OF TEMPERATURE AND LIGHT ON HYDROPEROXIDE CONTENT IN HUMAN BREASTMILK, EITHER WITH OR WITHOUT MICROLIPID[®] (ML)

Samples were incubated for 4 hours at room temperature (RT) either exposed to light (LE) or light protected (LP), or were incubated at 4°C (LP). Values are presented as mean \pm SD. Asterisks indicate milk \pm ML hydroperoxide concentrations (M) which are significantly different from corresponding controls (nonsupplemented milk (p < 0.05; N = 5 replicates per sample).

temperature, an effect which was abolished by the addition of glutathione peroxidase. In contrast, ML added to milk did not generate significant changes in total hdroperoxides when sample were stored at 4°C (Table 1). In comparison with controls, photoprotection afforded a slight but nonsignificant decrease in hydroperoxides.

Lipid hydroperoxides were also evident after 4 hours incubation of milk with ML (Fig. 2). Milk supplemented with ML yielded significantly higher peroxide levels than the control (unsupplemented milk) after 4 hours, and this effect was abolished by the addition of exogenous SOD. Pasteurization of milk reduced but did not eliminate peroxide formation, and there was a trend to lower peroxide levels in pasteurized samples.

Intracellular oxidative stress

Milk supplemented and stored with ML for 4 hours generated slight, but statistically significant increases in fluorescence emitted by the reactive oxygen probe CM-H2DCFDA (Fig. 3). Both milk and ML-supplemented milk induced greater fluorescence signals in FHS 74 Int cells than in cells exposed to either cell culture medium or microlipid-supplemented medium. Interestingly, the effect of ML-supplemented milk was abolished by the addition of glutathione peroxidase (GPx), and fluorescence intensity was reduced below that of cells exposed to breastmilk alone.

Effects on TEER

In addition to generating significant hydroperoxides and lipid peroxides, ML also significantly reduced TEER across Caco-2BBE confluent monolayers (Fig. 4). Transwell cultures exposed via the adluminal surface to MLsupplemented milk that had been premixed and stored for 4 hours at room temperature had a nearly three-fold reduction in TEER; again, this effect was dependent on endogenous milk factors, as evidenced by a slight, but not significant, increase in TEER across monolayers exposed to ML in cell culture medium, and was abolished by a free radical scavenger (SOD). There were no significant differences in TEER values in cultures incubated with milk or cul-



FIG. 2. Effects of microlipid on lipid peroxide formation (TBARS assay).



FIG. 3. Effects of microlipid on intracellular oxidative stress.

ture media freshly supplemented with ML *IL-6 induction* (data not shown).

Caspase 3 activity

Milk samples supplemented and stored with ML for 4 hours at room temperature also induced significantly higher caspase 3 activity, a marker for apoptosis (Fig. 5). Compared to caspase 3 activity prior to supplementation, MLsupplemented breastmilk produced a greater than twofold increase, an effect comparable to that produced by staurosporine, an established pro-apoptotic agent. This effect was abolished by glutathione peroxidase. There was a slight, but nonsignificant increase in caspase 3 activity in cultures exposed to ML in cell culture medium, as was detected in cell cultures exposed to milk alone. As illustrated in Figure 6, exposure of cultured enterocytes to either 10 μ L peroxide or to ML significantly increased IL-6 expression. Coincubation of ML and SOD yielded IL-6 titres that were slightly but not significantly elevated above controls. Similar results were obtained with either cell culture assay system, although FHS 74 Int cell supernatants had significantly higher IL-6 titres when exposed to either peroxide or to ML.

DISCUSSION

We show herein that, after prolonged storage at room temperature, ML significantly evolves hydroperoxides and lipid peroxides in milk. Furthermore, such oxidative stress per-



FIG. 4. Effects of microlipid supplementation on TERR.



FIG. 5. Effects of microlipid supplementation on caspase 3.

turbs intestinal epithelial cells under *in vitro* culture conditions, causing increased apoptosis and decreased enterocyte barrier function. Finally, we demonstrate that oxidized ML induces expression of IL-6, a pro-inflammatory cytokine.

More specifically, our data suggest that, after 4 hours incubation at room temperature, ML can generate significant hydroperoxides and lipid peroxides in breastmilk. The same conditions generate intracellular oxidative stress, as measured by the oxidation-sensitive intracellular probe, H₂DCFDA. The latter implies that, at least in vitro, extracellular free radicals can perturb the balance intracellular oxidants and reductants. Consistent with this notion, cultured intestinal epithelial cells manifest such stress via changes in at least three different physiological endpoints: barrier function, apoptosis, and IL-6 secretion. Compromise in barrier function is of particular concern in neonates, given the role of the intestinal mucosa in regulating fluid and electrolyte balance and in preventing the translocation of infectious agents across the gut.^{6,7}

It is generally recognized that lipoxygenase (LOX) products, which include hydroperoxides and lipid peroxides, are implicated as regulators of cell proliferation and apoptosis.^{8,9} Our results are consistent with this notion, and suggest that one mechanism by which lipid-derived free radicals affect epithelial barrier integrity is by stimulating apoptosis. However, there may be other free radical-mediated effects that contribute to the observed degradation of barrier function: altered transepithelial transport, disrupted cytoskeletal or cell-cell adhesion proteins, and/or disregulation of genes or gene products associated with epithelial cell maintenance.¹⁰ Certainly, apoptosis is a normal part of mucosal growth and differentiation;¹¹ however, it is conceivable that overactivation of apoptotic pathways can disrupt mucosal integrity in the gut. This may be of particular con-



FIG. 6. Effects of ML on IL-6 secretion.

cern in the neonate, in whom the intestine is a relatively "leaky" barrier in the first few days *ex utero*.⁶ Our results suggest that enteral factors, such as oxidized lipids, can increase apoptosis, thereby degrading transpithelial integrity. There are many possible pathways by which apoptosis may be activated; these include upregulation of pro-apoptotic genes such as *p53* or *bax*, or downregulation of antiapoptotic genes such as *bcl*,^{12,13} or by less selective mechanisms involving lipid peroxide-mediated activation of nonspecific esterases.¹⁴ Our data show that the latter method of activating apoptosis is most plausible.

The findings that ML induced IL-6 production in both cell culture assay systems is interesting in that it suggests that both the cell line (Caco-2BBE) and primary human small intestinal cells behave in a similar manner when exposed to oxidative stress. We can only speculate as to why the response is higher in FHS 74 Int cells; it may be that fetal cells exhibit an enhanced inflammatory response. That both assay systems responded in a similar manner argues for the validity of using the Caco-2BBE cell line as an appropriate indicator of how fetal enterocytes respond to ML or other sources of oxidative stress. The clinical significance of these findings is clear: IL-6 is one of the key inflammatory cytokines associated with inflammatory gut disorders, including necrotizing enterocolitis.¹⁵ In that oxidized ML appears to induce IL-6 production in vitro suggests that oxidized ML may act in a similar manner in vivo.

The period for which ML was incubated with milk for the cell-based assays was chosen on the basis the time required for measurable increases in oxidation products (4 hours). This represents an outer limit for the storage of these two components together in the special care or neonatal intensive care nursery, based on the experience of one of the authors (W.D.-J.), although storage practices can vary widely from unit to unit. Thus, it is conceivable that such storage times and conditions may be used in the nursery, especially when one considers the need to conserve milk supplies. The results of our study indicate that storage of human breastmilk supplemented with ML can induce the formation of lipid peroxides. The clinical implications are that such storage conditions (room temperature for 4 hours) have the potential to cause enterocyte dysfunction, and that 4 hours is likely an unsafe interval for these components to remain as an admixture.

Photoprotection did not confer significant protection from hydroperoxide generation over the interval tested, although refrigeration appears to significantly reduce this effect. While the need for shielding ML-fortified enteral feeds has also yet to be established, and we suggest that, based on the well-known effects of light on peroxidation of lipids in parenteral feeds given to neonates,⁵ this may be an important area of investigation, particularly under conditions of strong ultraviolet exposure such as phototherapy. Ultimately, we hope that such information may lead to the development of safer, better-informed use of human breastmilk.

ACKNOWLEDGMENTS

We thank Mr. Ben Wiebe and Mr. Vhaibav Shirwardkar for technical assistance, and we acknowledge the Association of Women's Health, Obstetrical and Neonatal Nurses (W.D.-J.) and the Natural Sciences and Engineering Foundation (Canada) (J.K.F.) for funding support.

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