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Role of Radicals in the Lipid Peroxidation Products of Commercial Infant Milk Formula

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Four commercial brands of infant milk formula were investigated, and the amount of shelf lipid peroxidation was determined by measuring malondialdehyde (MDA) or, more exactly, thiobarbituric acid reactive substance (TBARS) levels, which are the end products of lipid peroxidation. TBARS level, indicated by MDA concentration, was higher in the vegetarian brands. The vegetarian brands are, as expected, more prone to oxidation than dairy brands, as they contain more unsaturated fats. The introduction of formate anion diminishes the peroxide amounts initially present in the milk samples as well as those induced by radiation-induced oxidation.

KEYWORDS: Lipid peroxidation; infant formulas; ionizing radiation; scavengers

INTRODUCTION

Infant formulas on the market today should be aimed at providing the best alternative to breast milk for the babies of women who are unable to continue breast-feeding until 6 months of age, and it should form an adequate substitute for human milk, approaching the structural and functional effects observed in breast-fed infants after they reach 6 months of age. The aim is to mimic the functional outcome of the breast-fed infant (e.g., growth and development) and not to copy the composition of human milk. To that end, the following compounds have been added to formulas: long-chain polyunsaturated fatty acids (LCPUFA) for brain composition and neurodevelopment, probiotics and prebiotics for the fecal flora and local intestinal defense, and nucleotides for promoting immune response (*1*). Infant foods have a special place among food products mainly because of the unique nutritional aspects and preparation methods these foods require. It is predicted that the variety of available baby foods will increase markedly in the near future. The formulation, handling, and storage of baby foods are important to maintain both the nutritional quality and the physicochemical properties of these foods. During storage, some reactions and interactions occur that change the physicochemical and nutritional properties of baby foods. Lactose crystallization, the Maillard reaction, oxidation, and interactions between micronutrients and other components are the most important chemical reactions for which monitoring must be conducted in the preparation and storage of baby foods (*2*). In examining the effect of breast milk on plasma total antioxidant capacity (TAC), total peroxide (TP), and oxidative stress index (OSI), which are biomarkers of oxidative status, it was found that breast milk is a better antioxidant than formula milk (*3*). Preformed lipid peroxidation products present in the formula may contribute to the total reactive oxygen radical load on infants and may play a role in the pathogenesis of necrotizing enterocolitis and bronchopulmonary dysplasia (*4*, *5*). Exposure to light during tube feeding increases lipid peroxidation in infant formula but not in human milk. It has been observed that supplementing infant formulas with LCPUFA does not affect lipid peroxidation in the plasma of healthy, preterm infants (*6*). An investigation of the extent to which formula milk and stored breast milk, both commonly used in hospitals, could be pro-oxidant sources for newborn babies, found that there were notable differences in the oxidation parameters of several formula milk brands, particularly concerning the levels of lipid peroxides and total antioxidant capacity. No difference was found in the mean total antioxidant capacity between formula and breast milk (*7*). The oxidative degradation of polyunsaturated fatty acids contributes significantly to the reduced shelf life of many products (*8*). Lipid oxidation is a complicated process that leads to the formation of many compounds (*9*). Some of its effects include the development of off-flavors and -odors, changes in texture, and a loss of nutritive value (*10*). In addition, lipid peroxidation products seem to be directly involved in the development of atherosclerosis, cancer, and aging processes (*11*). Malondialdehyde (MDA) is one of the end products of lipid peroxidation (**Figure 1**) (*12*, *13*). MDA is in fact known to be a mutagenic species, a suspected carcinogen (*14*) that can react with DNA to generate mutagenic adducts.

The aim of this study was to compare MDA levels of soybean- and dairy-based baby milks, both commonly used in Israel, naturally and under conditions of oxidative stress.

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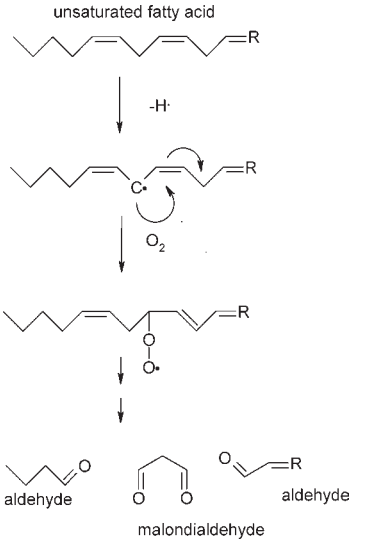

Figure 1. Scheme of lipid peroxidation and malondialdehyde end product. Adapted from ref *13*.

Table 1. Formula Composition

ingredients in 100 g of powder	A-d, dairy	A-s, soybean vegan milk	B-d, dairy	B-s, soybean vegan milk
total fats (g)	26.5	27.5	28.9	28.1
saturated fats (g)	14.5	12.4	18	9.13
unsaturated fats (g)	12.0	15.0	10.9	13.7
linoleic acid (mg)	5000	4400	5257	5.1
linolic acid (mg)	530	380	560	
ARA (mg)	115	144		
DHA (mg)	108	136		
vitamin E (mg)	10	10	16	12.9
vitamin C (mg)	60	65	47	53
vitamin A (IU)	1500	1500	1577	2000
iron (mg)	5.25	7.7	3.66	7.8

MATERIALS AND METHODS

Materials. All solutions were prepared from analytical grade chemicals and distilled water that was passed through a Millipore setup at a final resistance that was above 10 MΩ/cm.

All reagents and solvents were of analytical grade. Trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), and sodium formate were purchased from Sigma-Aldrich.

Milk Samples. Four commonly used newborn formulas were studied immediately after the milk powder container had been opened. Two of the formulas were cow milk (dairy) based and were from different companies (A-d and B-d), and two were based on soybean vegan milk from different companies (A-s and B-s). The lipid and antioxidant compositions of the four milk powders are listed in **Table 1** (according to the manufacturer's report on the package). Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are added to both cow and soybean milk powder of manufacturer A. The milk suspension was prepared by adding 5.0 g of powder to 50 mL of distilled water and then mixing well.

MDA Level Measurement. MDA levels were measured spectroscopically according to a procedure already published by François et al. (*15*) with minor modifications. The assay was based on the TBA reaction, a reaction between oxidized lipids and solution of 2-thiobarbituric acid under acidic conditions to yield a pink chromogen with a maximum absorbance at 532 nm (**Figure 2**) (*13*). Milk powder (0.5 g) was suspended in distilled water (10 mL) preheated at ~40 °C. There was no temperature effect (the study was made in the temperature range of 5−90 °C) on lipid peroxidation in the four brands of formulas. We added this important information to the text. An aliquot of this slurry (1 mL) was transferred to a 5 mL tube, followed by successive additions of TBA 1% in

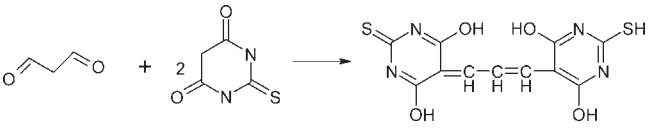

Figure 2. Chemical reaction between MDA and TBA to yield a pink chromogen. Adapted from ref *13*.

TCA 5% and BHT 0.8% in ethanol. The mixture was then homogenized and centrifuged at 2700*g* for 5 min and incubated in a 70 °C water bath for 20 min. Samples were subsequently cooled under tap water for 5 min and centrifuged for 5 min to separate flocculent material. The color produced by the chemical reaction was read at 532 nm against a blank reaction mixture, and the amount of MDA formed was determined by using the molar extinction coefficient $\varepsilon(530\ \text{nm}) = 1.56 \times 10^{-5}$ cm nmol^{-1} (*15*).

Spectrophotometric Measurements. All UV−vis measurements were performed using a Cary 100 Bio, UV−visible spectrophotometer.

Irradiations. γ-Irradiations were carried out at a ^{60}Co γ source, G-220 Gammacell, with a dose rate of 20 Gy/min, which was determined by means of Fricke dosimetry, using a G value of 15.6 (*16*, *17*)

All of the irradiations in this study were performed in N_2O-saturated solutions. The doses used were 300−1300 Gy.

Production of Radicals. The radicals were formed by irradiating the suspension with ionizing radiation. When ionizing radiation is absorbed by a dilute aqueous solution, the initial products are formed according to the reaction (*16*)

$$H_2O \xrightarrow{e^-,\gamma} {}^{\bullet}OH\ (2.65);\ {}^{\bullet}H(0.60);\ e^-_{aq}\ (2.65);\ H_2\ (0.45);\ H_2O_2\ (0.75);\ H_3O^+;\ OH^- \quad (1)$$

The G values are given in parentheses (G values are defined as the number of molecules of each product per 100 eV of radiation absorbed by the solution). The distribution of these products in the solution after 1×10^{-7} s is homogeneous (*16*).

When N_2O-saturated solutions, $[N_2O] = 2.2 \times 10^{-2}$ M (*17*), are irradiated, the hydrated electrons are transformed into ${}^{\bullet}OH$ radicals via (*18*)

$$e^-_{aq} + N_2O \xrightarrow{H_2O} N_2 + OH^- + {}^{\bullet}OH \quad k = 9.1 \times 10^9\ \text{M}^{-1}\ \text{s}^{-1} \quad (2)$$

Thus, at pH > 3, $G({}^{\bullet}OH) = 6.0$ is obtained (*18*). (The conversion of the irradiated dose to [OH] is as usually accepted in radiation chemistry; i.e., for 10 Gy of low LET radiation (γ radiation) adsorbed by a medium with a density of 1 g/cm^3 a product with $G = 1$ renders a concentration of 1 μM.)

RESULTS AND DISCUSSION

Lipids Peroxidation Measured in Shelf Products. The first aim of the study was to learn more about the oxidative status of dairy-based formula in comparison to soybean-based formula. For this purpose, levels of MDA were determined in A-d, A-s, B-d, and B-s, as mentioned above. The results are summarized in **Figure 3** and are discussed in terms of the formula composition shown in **Table 1**. All of the results are the average of at least six measurements. The study revealed notable differences in the levels of lipid peroxides in A-d and A-s compared to those levels in B-d and B-s. The levels of MDA in A-d and A-s exceeded those in B-d and B-s. The question is what conditions accelerate lipid peroxidation in milk powder? A high concentration of unsaturated fatty acids, especially LCPUFAs, in combination with vitamin C and iron, enables a well-known prooxidant process via a Fenton-like reaction (*19*) or the production of ferryl radicals (*20*−*22*). A low level of vitamin E, which is a lipid-soluble antioxidant, can also enable damage that is promoted by a lack of protection against oxidative stress. The results displayed in **Figure 3** seem to correlate well with the higher levels of unsaturated fats and the presence of LCPUFAs (ARA and DHA) in the milk powders from manufacturer A. The vitamin C concentration

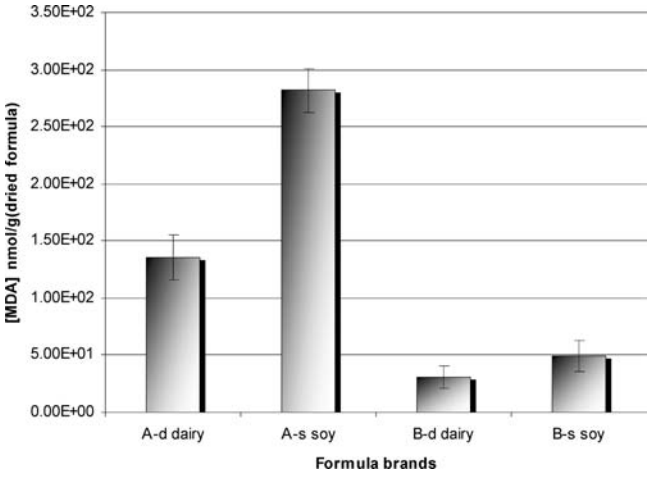

Figure 3. MDA levels for the four brands of formula milk, A-d, A-s, B-d, and B-s (data shown are average ± standard deviation) before irradiation and/or the addition of formate.

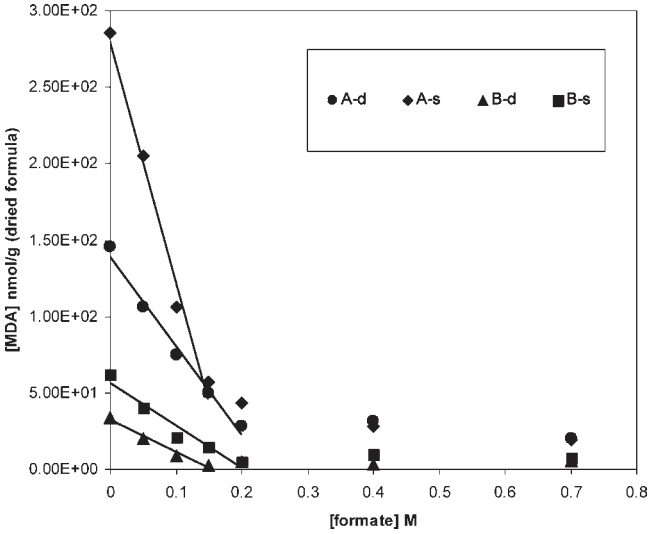

Figure 4. Influence of sodium formate on lipid peroxidation (MDA level) in the four brands of milk powder, A-d, A-s, B-d, and B-s.

is also higher in A milk powders. The lower amounts of MDA in B-d and B-s correlate with the higher vitamin E content, known to be a strong reducing agent (antioxidant) and, as such, to protect against oxidative stress in the latter. The elevated levels of MDA in the soy formula (A-s and B-s) versus the dairy formula (A-d and B-d) support the hypothesis that soy milk is more sensitive to oxidative stress than cow milk, as the vegetarian brands contain more unsaturated fats and iron than the dairy powder.

To distinguish between the amounts of lipid peroxides and free MDA contained by the powder prior to its dissolution in water and those produced with the addition of water, a process that may increase the diffusion of the existing radicals, the formate anion, NaCOOH, a radical scavenger, was added to the water before preparation of the emulsions. Formate ions react with oxidizing radicals, producing $CO_2^{\bullet -}$ radicals, which are strong reducing reagents (−1.7 V vs NHE).

When the four brands of formula were dissolved in aqueous solutions containing different concentrations of formate, a pronounced decrease in MDA concentrations was observed (**Figure 4**). It is well-known that the formate anion, HCO_2^-, reacts quickly (usually diffusion-controlled reactions) with

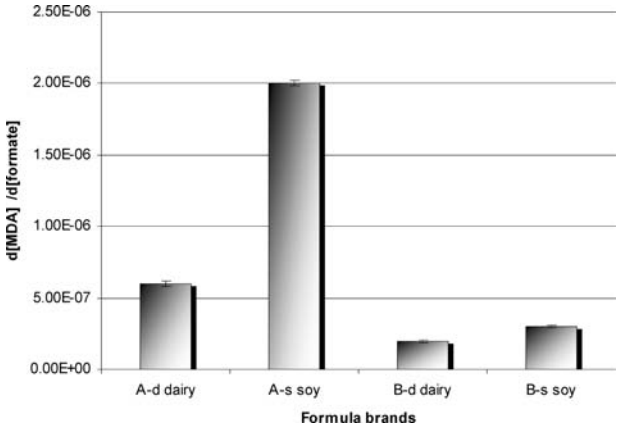

Figure 5. Slope determined from the linear dependence region of curves shown in **Figure 4**.

oxidizing radicals, rendering a powerful reducing agent, the formate radical anion (*15*):

$$R^{\bullet} + HCO_2^- \longrightarrow HR + CO_2^{\bullet -}; \quad R^{\bullet} = \text{oxidizing radical} \quad (3)$$

The results show unequivocally the effect of radical scavenging by the formate anion. Although in all cases the reactions are linear until they each plateau around 0.2 M NaCOOH, a different slope (in the case of A-s, the difference approaches 1 order of magnitude) was obtained for each infant milk powder (summary of slopes, **Figure 5**). The results seem to confirm that the observed change in the amount of peroxide radicals is a function of the starting amounts, as expected, but the relatively large amount of formate anion required is intriguing. This last observation may be explained by the heterogeneity of the medium; the work is performed in conditions at which an emulsion, rather than a clear solution, is formed. Formate anion diffusion to the radicals or vice versa appears difficult as the mixed formulas are characterized by biphasic conditions, conglomerates, or other forms of solids that tend to markedly reduce the rates. Therefore, a large excess of scavenger is needed.

Lipids Peroxidation Induced by Ionizing Radiation. When solutions containing the four brands of formula were subjected to oxidizing media produced by ionizing radiation (hydroxyl radicals), lipid peroxidation increased as a function of the irradiation dose, as expected (**Figure 6**). In addition, it was also expected that the vegetarian brands, which contained more unsaturated fats, would be more prone to oxidation. The amount of MDA detected increased linearly with the irradiation dose. The differences in slopes correlate to the relative amounts of unsaturated fats in the infant formulas: A-s > B-s > A-d > B-d (**Table 1**). The larger amounts of iron and vitamin C, which are known to induce oxidation (via Fenton or Fenton-type reactions), in the vegetarian brands (A-s and B-s) also help explain the greater amounts of MDA formed in B-s. There is also the possibility that a second type of radical was formed in A-s relative to that formed in B-s, as the exact nature of the initial unsaturated fats is unknown and may differ between brands.

In all cases, 0.2 M formate was added prior to irradiation, and the amounts of MDA measured after irradiation (even at the highest doses) were similar to those detected in the plateau region (**Figures 4**−**6**). These experiments show conclusively that formate is an excellent radical scavenger for preventing lipid peroxidation.

Conclusions. Commercial milk powder for babies that is based on soybean fats is more susceptible to oxidative damage due to the relatively high levels of unsaturated fatty acids and a combination of high concentrations of vitamin C and iron. The results

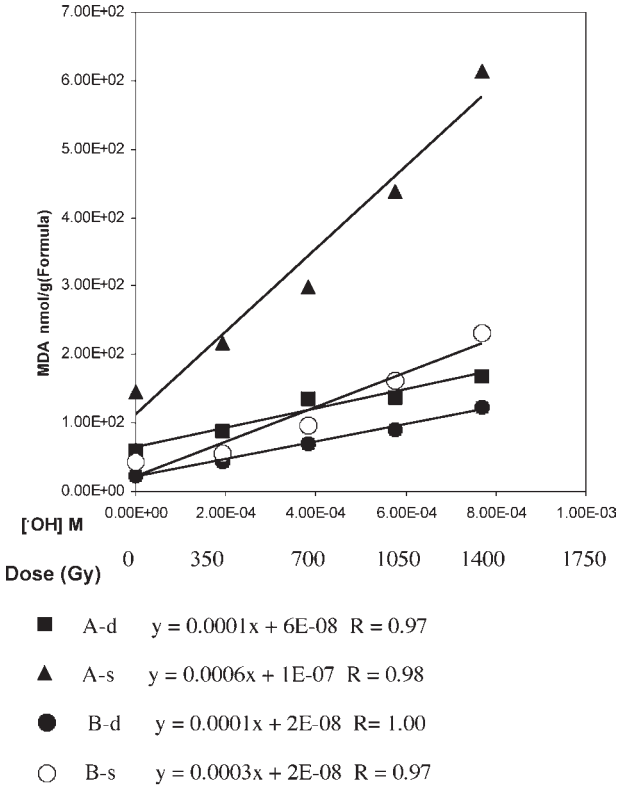

Figure 6. Amount of MDA formed as a function of [•OH].

also suggest that milk formulas containing added ARA and DHA (A-d and A-s) are even more susceptible to oxidation. The extraordinary protection against oxidative damage provided by the formate anion, even at the highest concentrations of hydroxyl radicals (ionizing radiation), suggests that adding a biological lipid-soluble antioxidant such as lycopene to the milk powder may also prevent oxidative damage to the lipids, thus benefitting the infant.

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