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The Role of Dietary Nucleotides in Neonatal and Infant Nutrition

V Y H Yu

ABSTRACT

Human milk has a higher concentration of nucleotides than bovine milk which is the source of most infant formulas. As the composition of human milk is considered the 'gold standard,' an increasing number of infant formulas are supplemented with nucleotides. This review summarises the biology of human milk nucleotides and evaluates the studies which investigated the clinical benefits of feeding infants with nucleotide-supplemented formulas. Although dietary nucleotides have been suggested to have beneficial gastrointestinal and immunological effects, nucleotide-supplemented formula feeding has not been shown to confer the same benefits as breast feeding, and randomised controlled trials have yet to prove that healthy term infants fed nucleotide-supplemented formulas compared to those fed nonsupplemented formulas, have accelerated physical growth and neurological development, better growth and development of their gastrointestinal tract resulting in improved digestive and absorptive functions, enhanced development of their immune system resulting in increased resistance to infection and lower bacterial and viral infection rates during infancy, and a more favourable intestinal microflora associated with a lower rate of infectious diarrhoea. However, a randomised controlled trial has reported that term infants with severe intrauterine growth retardation do have better catch-up growth with nucleotide supplementation. The hypothesis that nucleotides are semi-essential nutrients needs to be further studied, in particular in the presence of prematurity, fetal growth retardation, intestinal injury and limited nutrient intake. As no deleterious effects have been reported with the use of nucleotide-supplemented formulas, the first of which was introduced over 30 years ago, such products are considered safe when nucleotides are supplemented to an amount equivalent to the free nucleotide concentration of human milk. More basic and clinical research studies are awaited to further define the biology and role of human milk nucleotides, and to critically assess the potential benefits and appropriate level of nucleotide supplementation of infant formula.

Neonatal Intensive Care Unit
Monash Medical Centre
246 Clayton Road
Clayton,
Victoria 3168
Australia

V Y H Yu, MD MSc (Oxon), FRACP FRCP (Lond, Edin, Glasg), FRCPCH DCH
Professor of Neonatology and Director of Neonatal Intensive Care

Correspondence to:
Prof V Y H Yu

INTRODUCTION

Nucleotides are biologically active, non-protein, nitrogenous compounds present in the milk of human mothers and that of other species. As nucleotides are the structural units of nucleic acids, RNA and DNA, they and their related metabolic products are assumed to play a key role in many biological processes. In recent years, there has been an increasing interest in the role of dietary nucleotides in neonatal and infant nutrition. Traditionally, the composition of human milk has been assumed to be optimal for the growth and development of the infant, and serves as the 'gold standard' in the research and development of infant formulas. Human milk has been shown to contain considerable amounts of nucleotides, but nucleotides in the milk from ruminant species are present in a lower concentration and with a different composition. Because bovine milk is the source of the vast majority of infant formulas, an increasing number of infant formulas has been manufactured and marketed which are supplemented with nucleotides. The purpose of this review is to assess what is known about human milk nucleotides, and whether medical research has to date scientifically demonstrated the benefits of nucleotide supplementation of infant formulas according to the principles of evidence-based medicine.

Objective assessment of medical evidence

Biomedical research is continuing to improve our understanding of the biological process of early growth and development. The application of this new knowledge to the medical management of neonates and infants has resulted in the introduction of new and more effective medical interventions. Supplementation of infant formulas with nucleotides is potentially one such advance in infant nutritional management. However, the history of the development of neonatology has included medical strategies believed to be effective but which in retrospect were proven to be useless or even harmful. To avoid such mistakes of the past, it is essential that rigorous scientific standards for assessing efficacy and effectiveness be met before new medical interventions are to be endorsed. The principles behind the assessment of evidence concerning prevention and treatment of diseases of the newborn have been published[1]. The biomedical research in the development and assessment of medical interventions can be viewed under two categories: raising and testing of the hypothesis using laboratory and animal studies, and proving the hypothesis with medical evidence from clinical studies.

Raising and testing of the hypothesis using laboratory and animal studies

Is there a theoretical basis that the medical intervention can work, based on our understanding of biological processes? Has the physiology or biochemistry been elucidated in laboratory studies, for example, utilising cell cultures? Has the hypothesis been tested in animal models, in which the following questions have been asked: (1) What is the magnitude of the baseline risk, that is, what proportion of target group will experience an adverse outcome without the intervention? (2) Is there a real effect from the intervention, that is, an effect which is not due to chance? (3) What are the direction and magnitude of intervention effect? (4) Are there undesirable side-effects attributable to the intervention?

Proving the hypothesis with medical evidence from clinical studies

Scientific proof of a hypothesis can only be obtained from clinical evidence based on medical studies utilising human subjects, after preliminary data from animal studies have suggested efficacy and safety of the medical intervention. Medical studies can be classified into one of five categories listed in order of ascending methodological rigour: single case report, case series without controls, non-randomised study using historical controls, non-randomised study using concurrent controls, and randomised controlled trial (RCT). In contrast to observational studies, experimental studies such as RCTs offer maximum protection against selection bias which can invalidate comparisons between groups because of confounding variables. In addition to the four questions asked in the animal studies, three clinically relevant questions should also be included: (1) What are the economic costs and implications of the intervention? (2) Do the clinical benefits of the intervention outweigh the undesirable side effects and/or economic costs? (3) To whom are these results applicable, that is, are the findings derived from infants, rather than from older children or adults, from healthy term infants or from preterm or growth-retarded infants, and over what age period during infancy?

Concerning the choice of outcome measures selected for clinical studies, it is important to beware of what has been called the "substitution game", in which a risk factor (for example, blood cholesterol level in an adult, or intraventricular haemorrhage in an infant) is substituted for an event of prime clinical importance (that is, heart attack in an adult, or mental retardation in an infant). In these examples, it cannot be assumed that the medical interventions which result in a significant decrease in the blood cholesterol level in an adult or in the intraventricular haemorrhage rate in an infant are also effective in reducing respectively the rate of heart attack in the adult or mental retardation in the infant. Evidence of the latter effect can be obtained from a RCT which has as its primary outcome the rate of heart attack or mental retardation. How does this concept apply to nucleotide supplementation of infant formula? For infants fed a nucleotide-supplemented formula, this "substitution game" could potentially arise when, in attempting to determine whether nucleotide supplementation increases the infant's resistance to infection, an intermediate outcome (for example, the result of an immune function test performed at two months of age) was substituted for the relevant clinical outcome of the rate of bacterial or viral infection in the first year of life.

NUCLEOTIDES IN INFANT NUTRITION

Nucleotide biochemistry

Since nucleotides were first isolated from human milk in 1960, at least 13 acid-soluble nucleotides have been identified. They are ubiquitous, low molecular weight compounds consisting of a nitrogen containing base, a five-carbon sugar (ribose or deoxyribose) and one to three phosphate groups. The nitrogenous bases are derivatives of two parent heterocyclic compounds, purines (mainly adenine and guanine) and pyrimidines (mainly cytosine, thymine and uracil). The ribonucleotides and deoxyribonucleotides serve as the monomeric precursor units of RNA and DNA respectively. As nucleotides are also essential compounds in energy transfer systems (that is, in ATP and GTP), they are an integral part of carbohydrate, lipid, protein and nucleic acid metabolism and modulators of important neonatal physiological functions[2].

Nucleotide absorption and metabolism

Dietary nucleoproteins and nucleic acids are degraded by proteases and nucleases to nucleotides. The phosphate groups in nucleotides are cleaved by intestinal alkaline phosphatases and nucleotidases to form nucleosides which are the preferred form for absorption in the small intestine. Most of the absorbed nucleosides are degraded to uric acid and allantoin, and some are reconverted to nucleotides. Nucleotides can also be synthesised de novo using more elemental components such as amino acids and glucose, although this is a metabolically costly process. Some tissues such as the intestinal mucosa and bone marrow haematopoietic cells have a limited capacity for this de novo synthesis. These tissues depend more on the salvage pathway that produces nucleotides from either exogenous nucleosides originating from the diet or endogenous purine and pyrimidine bases released by the degradation of compounds like RNA and DNA.

The relative contribution of dietary nucleotides to the total pool in specific organs or in the entire body is unknown[3]. When the metabolic demand exceeds the capacity for de novo synthesis and the endogenous salvage pathway, dietary nucleotides may become an important source and thus be considered as a semi-essential or conditionally essential nutrient. By definition, a semi-essential nutrient is one which may become essential under certain conditions when the endogenous supply is insufficient for normal function, even though generally its absence from the diet does not normally lead to a clinical deficiency syndrome. Conditions under which a semi-essential nutrient might become essential include

periods of limited intake or rapid growth and disease states. Therefore theoretically, in the presence of prematurity, intrauterine growth retardation and diseases resulting in intestinal injury, an adequate intake of dietary nucleotides might spare infants the cost of de novo synthesis or salvage, and thus contribute to the optimisation of their physiological and metabolic function.

Nucleotides in human milk

Up to 30% of the total nitrogen content of human milk is nonprotein nitrogen. Free nucleotides were reported to account for 2% – 5% of the nonprotein nitrogen[4]. The total free nucleotide content in human milk was found to range from 50 to 150 μmol/L or 2 – 6 mg/100kcal. This decreases with advancing lactation; at three months of lactation, the concentration falls to 75% of that in human colostrum[5]. More recent studies reported not only free nucleotides but also free nucleosides and pofymeric and cellular nucleotides derived from structural units of nucleic acids RNA and DNA. Expressed as 'total potential available nucleosides' (TPAN), the mean (± SD) concentration was reported to be 189 ± 70 μmol/L (range 82 – 402 μmol/L)[6]. Expressed as nucleotide equivalents, 68 ± 55 μmol/L were found to be present as nucleic acids, 84 ± 25 μmol/L as nucleotides, and 10 ± 2 μmol/L as nucleosides[7].

Nucleotides in formula milk

In contrast to human milk, nonprotein nitrogen accounts for only 2% – 5% of the total nitrogen in bovine milk. The nucleotide content of infant formulas derived from bovine milk is therefore considerably lower than that of human milk. Furthermore, cytidine and adenosine derivatives are present in relatively higher proportions in human milk than in milk from ruminant species. There have not been reports of clinical deficiency which might be attributable to the lower nucleotide content in infant formulas. However, as infant formulas are generally developed and manufactured to be as similar to human milk as possible, infant formulas supplemented with nucleotides have been marketed in Japan from 1965, in Spain from 1983, in the USA from 1989, and in some countries in South-East Asia from 1990. No deleterious effects have been reported to date, but the European Commission's Scientific Committee for Food has published guidelines in 1991 and 1996 on nucleotide supplementation of infant formulas[8,9]. The Committee limited its approval to five nucleotides and established their maximum limits as follows: cytidine 5' – monophosphate 2.5 mg/100kcal, uridine 5' – monophosphate 1.75 mg/100kcal, adenosine 5' – monophosphate 1.5 mg/100kcal, guanosine 5' – monophosphate 0.5 mg/100kcal, and inosine 5' – monophosphate 1 mg/100kcal. They authorised the use of the sodium salts of these nucleotides which are easily soluble in water and hydrolysed in the intestine and absorbed as nucleosides. The Committee also stated that the total nucleotide concentration should be in the same order of magnitude as the free nucleotides in human milk, that is, less than 5 kcal/100kcal. It rejected the proposal for a two- to-threefold increase in nucleotide supplementation which would be required in order to achieve a level equivalent to the TPAN content of human milk[9].

EVIDENCE FOR BENEFITS OF DIETARY NUCLEOTIDES

A large number of animal experiments together with several clinical studies on human infants has been conducted to investigate in particular the gastrointestinal and immunological effects of dietary nucleotides. A comprehensive review of the literature was published in 1995[2].

Intestinal growth and development

Dietary nucleotides were reported to be important in the growth and maturation of the developing animal gut. Frequently quoted to support this hypothesis is an animal study which was conducted in 21day old rats randomised to receive a nucleoside-supplemented diet (n = 10) or a nucleoside-free diet (n = 10)[10]. At the end of two weeks, the rats were compared for their body weight, intestinal size and histology, and intestinal diasaccharidase activities. No significant differences were found in their body weight gain or in their gut length, weight and mucosal weight. The gut protein and DNA content was significantly higher in the proximal segment in the supplemented group, but no significant differences were found in the middle and distal segments. Villus height was significantly higher in the proximal segment in the supplemented group, but no significant differences were found in the middle and distal segments. Crypt depth was significantly lower in the distal segment in the supplemented group, but no significant differences were found in the proximal and middle segments. Maltase activity was significantly higher in all segments. Sucrase and lactase activities were significantly lower in the proximal segment and significantly higher in the middle segment in the supplemented group, and not different in the distal segment.

The findings from this animal study are useful in helping to frame a scientific hypothesis but cannot be extrapolated to human infants. The study compared one diet which contained nucleosides with another which was completely nucleoside-free. This is a different comparison to one in which human infants fed milk with a higher nucleotide content (such as human breast milk or a cow's milk-based formula supplemented with nucleotides) is compared to those fed milk with a lower nucleotide content (such as a cow's milk-based formula). The lack of differences in overall weight gain and in most measurements of intestinal size and histology, and the higher protein and DNA content found only in the proximal intestinal segment in the supplemented group, put into question the hypothesis for a beneficial effect. Whether the few observed differences have functional implications in the rat or suggest clinically significant

effects in human infants must remain speculative. For the above reasons, this study has not provided definitive scientific evidence that, in normal healthy term infants, nucleotide supplementation of cow's milk-based formula above the quantity already present, results in significant benefits on their physical growth, growth and development of their gastrointestinal tract, or digestive and absorptive functions.

Intestinal microflora

Bifidobacteria, predominant in the stools of breast-fed infants, lower the pH of intestinal contents and impede the proliferation of pathogenic bacteria. Since bifidobacteria growth is enhanced in vitro when nucleic acids are added to a selective medium, a clinical study was conducted in healthy term infants to compare the effects of feeding human milk (HM group) and two versions of formula milk, one supplemented (NFM group) and one not supplemented (FM group) with nucleotides. The numbers of infants in the groups were 10, 11 and 12 respectively but they were not randomly assigned. Faecal samples were taken for bacterial culture at one and four weeks of age. Data from children who became ill during the study period (including diarrhoea or antibiotic therapy) were excluded from analysis. The study reported that the absolute bacterial counts (number of microorganisms per gram of dry faeces) for aerobes, anaerobes, lactobacilli, bifidobacteria, enterobacteria, enterococci, clostridia and staphylococci were not significantly different among the three groups at both one and four weeks of age. Only when the bacterial counts for lactobacilli, bifidobacteria and enterobacteria were expressed as percentages of the total bacterial count, were significant differences found. Compared to the HM group, a significantly higher percentage of lactobacilli was found in the FM group at one and four weeks and in the NFM group at four weeks. Compared to the HM group, a significantly lower percentage of bifidobacteria was found in the FM/NFM groups at one and four weeks. The percentage of bifidobacteria in the NFM group was significantly higher than that in the FM group at four weeks. Compared to the HM group, a significantly higher percentage of enterobacteria was found in the FM/NFM groups at one and four weeks. The percentage of enterobacteria in the NFM group was significantly lower than that in the FM group at four weeks.

The lack of random allocation, the small numbers in each group, and exclusion of data from ill children, increase the risk of selection biases in this study, which can invalidate comparisons because of confounding factors. The lack of significant differences among the bacterial counts of the eight bacteria groups obtained with the three feeding regimes at one and four weeks, indicated that infants fed a nucleotide-supplemented formula do not develop an intestinal microflora pattern which is quantitatively different from those fed a non-supplemented formula. Even when the data was expressed as a percentage of the sum of the bacterial counts, the data in NFM/FM groups were significantly different to that in the HM group, suggesting that the feeding of a nucleotide-supplemented formula does not result in an intestinal microflora similar to that from breastfeeding. The difference between the percentage of bifidobacteria and enterobacteria between the NFM/FM groups were statistically significant only at four weeks and only at the $p<0.05$ level, the magnitude of the difference being very much smaller than that reported between the HM group and NFM/FM groups. The small sample size together with the comparison of multiple variables within multiple groups increase the probability of finding a false-positive result due to a type I error. A higher level of statistical significance (for example, $p<0.01$) should be used to indicate an effect that is real rather than due to chance. For the above reasons, this study has not provided definitive scientific evidence that, in normal healthy term infants, nucleotide supplementation of cow's milk-based formula above the quantity already present, results in significant differences in the number and type of intestinal bacteria. Data from a more recent study also do not support the hypothesis that the intestinal microflora of infants fed nucleotide-supplemented formula are closer to that of breast-fed infants[12].

Immune function and infection

Given the many in vivo animal studies and in vitro human studies suggesting significant effects of nucleotides on tests of cellular and humoral immunity, a study has been conducted to measure the effects of breast feeding (HM group) and feeding with two versions of formula milk, one supplemented (NFM group) and one not supplemented (FM group) with nucleotides, on the natural killer cell activity and interleukin-2 production in peripheral blood mononuclear cells at two and four months of age, and on the incidence and severity of infections in the four-month period. The numbers of healthy term infants in the groups were 9, 13 and 15 respectively, and the two formula-fed groups were randomly allocated. Physical growth, haematological indices and plasma biochemistry profiles among the three groups were not significantly different. Comparison between the NFM and FM groups showed that in the former group, the natural killer cell activity was significantly higher at two months of age (at the 50:1 and 25:1 effector-to-target cell ratios but not at the 12.5:1 ratio) but not significantly different at four months of age. Similarly, interleukin-2 activity was significantly higher at two months of age but not significantly different at four months of age. No significant differences were found between the HM group and the NFM/FM groups. No significant differences were found in either the incidence or the severity of infections among the infants in the three groups during the four-month study period.

As the study was conducted over the first four months after birth, the findings cannot be extrapolated to infants beyond the age of four months. The fact that this was a "study of limited sample size" according to the authors' own admission, together

with a study design in which a large number of variables were tested in three groups at two time intervals, increase the probability of finding one or more false-positive results, that is, an increased risk of a type I error (false claims of treatment benefit).

One out of twenty tests will show a treatment effect ($p<0.05$) even when there is actually no treatment difference. In such cases, any statistically 'significant' results should only be put forward as clues for future research rather than as conclusive findings. Given the biological complexity of immunological processes in infancy and childhood, it is questionable that differences in two tests performed on peripheral blood mononuclear cells between the NFM and FM groups at two months of age, even if they are considered as statistically significant, are likely to have any clinical relevance. Irrespective of how these findings are interpreted, the fact that there were no significant differences by four months of age shows that the effect even if real was a transient one. The lack of significant differences in the incidence or severity of infections among the infants in the three groups supports the opinion that the transient differences found in two peripheral blood mononuclear cell functions have no clinical significance. The issue of a "substitution game" arises here when, in an effort to support a particular point of view, an attempt was made to substitute an intermediate outcome (an immune function test) for a clinical relevant outcome of prime importance (the infection rate). For the above reasons, this study has not provided definitive scientific evidence that, in normal healthy term infants, nucleotide supplementation of cow's milk-based formula above the quantity already present, results in significant benefits on their physical growth, haematological and biochemical indices, immune function and infection risk in early infancy.

Diarrhoea and infection

Beneficial effects of dietary nucleotides on mucosal regeneration have been demonstrated in an experimental rat model of chronic diarrhoea[15]. A clinical study was therefore conducted to investigate the effects of feeding healthy infants two versions of formula milk, one supplemented (n = 194) and one not supplemented (n = 198) with nucleotides over a three-month period, on their physical growth, diarrhoeal episodes, presence of enteropathogens, incidence of infectious illnesses, and hospitalisation rate. The study population was from the periurban slums around Santiago in Chile. No significant differences were found in the body weight and body length at the end of the study period, nor in the total number of episodes of diarrhoea, total number of days with diarrhoea (all episodes), and duration of all episodes of diarrhoea. Only when the first episodes of diarrhoea were compared was the number significantly lower in the nucleotide-supplemented group compared to the non-supplemented group, but no significant difference was found in the duration of first episodes nor in the number of children who experienced more than one episode. No significant differences were found in the presence or type of enteropathogens, either with episodes of diarrhoea or from the asymptomatic infants. No significant differences were found in the incidence of upper and lower respiratory tract infection, skin infection, urinary tract infection, eye infection, other infectious diseases, and in the hospitalisation rate.

Because this study was conducted among infants who belonged to the low socioeconomic stratum and living in a contaminated environment, the applicability of the findings to an urban infant population within a developed country or an industrialised nation is questionable. No reference was made that this was a RCT. Twenty-six percent of the enrolled infants did not complete the study and were excluded for a variety of reasons, giving rise to the potential of biasing the results. Of the six different comparisons which described the incidence and severity of diarrhoea, five showed no significant differences between the two groups. It is difficult to justify the conclusion that nucleotide-supplemented formula decreases the incidence of diarrhoeal disease compared to non-supplemented formula when the data are viewed in their entirety. Similar to the two previous studies[11,12], this study reported no difference in intestinal microflora between the nucleotide supplemented and nonsupplemented groups. The hypothesis that nucleotide supplementation enhances immune function, increases resistance to infection and improves health is not supported by this study, as it did not demonstrate differences in the incidence of infectious illnesses and the need for hospitalisation between the two groups. For the above reasons, this study has not provided definitive scientific evidence that, in normal healthy term infants, nucleotide supplementation of cow's milk-based formula above the quantity already present, results in significant benefits on the intestinal microflora, physical growth, and the prevention of diarrhoea, infectious illnesses and hospitalisation.

Catch-up growth in infants with severe intrauterine growth retardation

Although the clinical studies conducted to date on the possible beneficial effects of nucleotide-supplemented formula in healthy term infants have produced mainly negative results, a more recent study conducted in term infants with severe intrauterine growth retardation has demonstrated that its use was associated with better catch-up growth[16]. This RCT was conducted in infants whose birthweight was below the 5th percentile, who were fed nucleotide-supplemented (n = 39) and non-supplemented (n = 35) formulas over a six-month period. Unfortunately no comparison group who were breast-fed was included, and 17% of the enrolled infants failed to complete the study. The nucleotide-supplemented group has significantly higher mean rates of gain of weight, length and head circumference compared with the non-supplemented group. Since there was no difference in the pattern of illness between the two groups which might have influenced their growth rate, it was postulated that the improved growth in the nucleotide-supplemented group of is likely to have

been due to trophic effects of nucleotides on the intestinal mucosa previously damaged by intrauterine malnutrition. The findings in this study on infants with severe intrauterine growth retardation are in contrast to those from thirteen studies conducted on appropriately grown healthy term infants, all of which reported no effect of nucleotide supplementation on physical growth[2].

DISCUSSION

Nucleotides can be synthesised endogenously and thus are not essential nutrients. However, the hypothesis that they are semi-essential, that is, they may become essential under certain conditions, has lead to the suggestion that dietary nucleotides may spare the metabolic costs of de novo synthesis and salvage, and that they may confer beneficial effects upon intestinal growth and development, the immune system and physical growth. More research is needed to characterise the absorption and metabolism of nucleic acids, nucleotides, nucleosides, bases, and related metabolic products in humans, and the impact of conditions such as prematurity, fetal growth retardation, intestinal injury and limited nutrient intake. The effect of dietary nucleotides on hepatic function and lipid metabolism also require further investigation, as such data are even more limited. Additional studies are required which utilise improved technology for the collection and accurate analysis of the content of nucleic acid, nucleotides, nucleosides, bases, and their related metabolic products in human milk. Data on the specific biological effects of feeding individual nucleotides are also limited.

While further laboratory and animal experiments are being conducted to answer any of the basic questions on the biology of nucleotides, the hypothesis that dietary nucleotides are beneficial in infant nutritional management is already being tested with medical research which involves clinical trials conducted in newborn infants[11-14,16]. Properly designed and conducted RCTs have yet to be published which provide definitive scientific evidence which show that nucleotide-supplemented formulas confer the benefits of human breast milk and that healthy term infants fed nucleotide-supplemented formulas compared to those fed non-supplemented formulas, have accelerated physical growth and neurological development better growth and development of their gastrointestinal tract resulting in improved digestive and absorptive functions, enhanced development of their immune system resulting in increased resistance to infection and lower bacterial and viral infection rates during infancy, and a more favourable intestinal microflora associated with a lower rate of infectious diarrhoea. Until such benefits are proven it is advisable to refrain from making exaggerated or misleading nutritional performance claims especially in commercial promotional material associated with nucleotide-supplemented formulas.

A review article published recently in 1997 stated, "The role of human milk nucleotides for breast-fed infants is not known, and the issue of nucleotide supplementation of infant formula remains controversial". Nevertheless, available data have suggested that dietary nucleotides have important biological effects, and benefits of feeding nucleotide-supplemented cow's milk-based formulas are possible though yet essentially unproven. A variety of nucleotide-supplemented formulas have now been available in many countries in Asia, Europe and North America, in some for as long as 33 years. As no deleterious effects have been reported with their use in term infants, such products are currently considered safe, at least within the range of nucleotide concentrations approved by the Scientific Committee for Food of the European Commission[8,9]. However, it is prudent to await further research data before supplementation with a higher nucleotide concentration equivalent to the TPAN content of human milk is considered to be safe.

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