Methylseleno-amino acid content of food materials by stable isotope dilution mass spectrometry

Wayne R. Wolf and Hanaa Zainal

Abstract

Selenium, an important dietary nutrient, is found in many foods. Selenium occurs in various chemical forms including in amino acids with methylselenium functional groups, such as selenomethionine (Semet) and Se-(methyl)selenocystine (Metsecys). We developed a procedure for determining methylselenium in foods such as wheat, a significant dietary source of selenium in the United States. This method is based upon the reaction of cyanogen bromide (CNBr) to cleave the CH_3Se-functional group of Semet and Metsecys to form the volatile compound, CH_3SeCN. Addition of stable isotope (^{74}Se) enriched selenomethionine to an analytical sample allows direct determination of naturally occurring protein bound Semet by gas chromatography/mass spectrometry (GC/MS), without a protein digestion step, using highly precise stable isotope dilution techniques. We found that a wheat gluten reference material (NIST RM 1818) contains 64% methylselenium of its assigned value of 2.58 μg Se_{total}/g. and that commercial selenium yeast tablets contained 73% of total selenium as methylselenium [147 ± 10 μg Se_{metse}/g (n = 9)]. These two materials would be good candidates for further study and characterization as reference materials for determining this important food component.

Key words: selenomethionine, methylselenocystine, selenium, food, isotope dilution mass spectrometry

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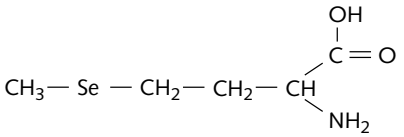
Introduction

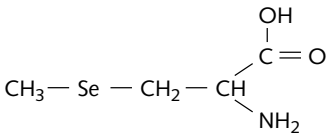
Selenium has recently been identified as an important nutrient in the diet due to its antioxidant capabilities [1] and is found in many foods, with grain products having a high concentration. The selenium concentrations in grains can vary widely from area to area, depending on the selenium concentration of the soil. Other sources of selenium are seafood (especially tuna), liver, and to a lesser extent, meats and eggs. While there is provisional information on the selenium content of foods [2] the selenium content of most food items is not well established. Selenium is found in various chemical forms, with some organic forms incorporated in protein bound amino acids such as selenomethionine (Semet), which is the selenium analog of the sulfur containing amino acid methionine.

Organically bound selenium, as Semet, is better adsorbed than inorganic selenium compounds [3]. Thus there is considerable interest in its metabolic and biological role. There is also increasing interest in the biological role of another selenoamino acid, Se-(methyl)selenocysteine (Mesecys) [4, 5]. Both Semet and Mesecys contain a functional methylselenium (metse) group (fig. 1)

The US Department of Agriculture encourages the populace to eat "plenty of grain products, vegetables and fruits" [6]. In addition to the fiber, macronutrients, and other components, grain products are considered to be a good source of selenium. With the current emphasis on antioxidants, it is easy to recognize the importance of being able to quantify the content of both total selenium and its organic forms in foods. We developed a procedure for accurately determining the Semet content of foods [7] based on the textbook reaction of cyanogen bromide (CNBr) with methionine, containing the CH_3S-functional group to form the volatile compound CH_3SCN. The chemistry for Semet is similar, with CH_3SeCN being formed (fig. 2). This volatile species can be detected by gas chromatography/mass spectrometry (GC/MS). We have also shown that this CNBr reaction does occur with the CH_3Se con-



Selenomethionine
MW = 196



Selenium (methyl)-selenocysteine
MW = 182

FIG. 1. Structure of selenomethionine and Se(methyl)-selenocystine.

taining species Mesecys, producing the same CH_3SeCN moiety [8].

When stable isotope (^{74}Se) enriched labeled Semet is added to an analytical sample and allowed to react with CNBr, an estimation of the protein bound Semet is possible via isotope dilution techniques. However, if this procedure is to be used by different labs, suitable reference materials (RMs) where the Semet concentration is known and verified are needed to determine the validity of the lab results. We have previously show that two high protein matrix-candidate RMs (NIST RM 8418 wheat gluten and a selenium enriched commercial yeast), contained significant amounts of their selenium content as Semet. These materials should be considered as candidates for further development as a suitable RM to determine the Semet content of foods [8].

Materials and methods

Wheat gluten (RM-8418) was obtained from the National Institute of Standards and Technology (Gaithersburg, Md., USA) and has an assigned value for total selenium content of 2.58 μg/g. A sample of a commercial product labeled to contain "selenium yeast" in tablet form was obtained at a local grocery store. The label claim was for 200 μg selenium per gram. During the course of our studies, we also obtained a sample of pure selenomethionine from the United States Pharmacopoeia (USP), which was a candidate standard sample under development by the USP. Commercial samples of selenomethionine were obtained from Sigma (Sigma, St. Louis, Mo., USA, No. S-3975) and Fluka, Biochemika (Buchs, Switzerland, No. 84925). ^{74}Se labeled selenomethionine stock solution (10^{-3} M) was obtained from C. Veillon, Nutrient Requirements and Functions Laboratory, USDA, ARS, BHNRC, Beltsville, Md., USA.

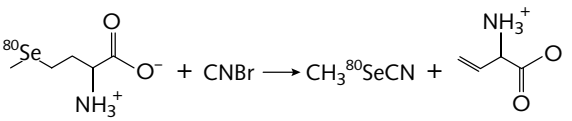


FIG. 2. Reaction of CNBr with selenomethionine to produce CH_3SeCN.

Preparation of solutions and samples

The weights of all additions for reagents, samples, solutions, etc. were determined gravimetrically to ensure accurate and precise quantification. Standard, wheat gluten or yeast samples were weighed into graduated 5 ml sample vials (Wheaton, Millville, NJ, USA, #986299). A two ml aliquot of the $^{74}Semet$ labeled acid solution (0.1M HCl) containing 2% stannous chloride (see below) was added directly to the test portions and vortexed to ensure complete incorporation of sample into the aqueous solution. Vials were held at 36 to 37°C overnight to ensure complete mixture of the protein material with the labeled spike. CNBr (200-μL) (Aldrich, Milwaukee, Wisc., USA, CAS# 506-68-3) was added, vortexed, then heated at 36–37°C for 4 hours. To extract the product, CH_3SeCN, 1 ml of chloroform ($CHCl_3$) was added vortexed, then allowed to stand at room temperature until phase separation was complete. The organic phase was separated and 1 μL aliquots injected into the GC/MS for analysis.

Labeled acid solution

In a 25 ml volumetric flask, $SnCl_2$-$2H_2O$ (~555 mg) (Aldrich, Milwaukee, Wisc., USA, No. 24,352-3) was dissolved in 12 ml of 0.1 M HCl (pH = 1). $^{74}Semet$ labeled stock solution (50 μL at 10^{-3} M) was weighed into the flask, mixed, brought up to volume with 0.1 M HCl and thoroughly mixed. Thus, the final concentration for the solution was 22.2 mg $SnCl_2$-2 H_2O (approximately 2% by weight) and 2 μL of $^{74}Semet$ label solution per gram of solution. The solution was held at 0°C. These quantities yielded enough spiked acid solution for 12 samples (2 ml per sample vial).

Instrumentation

The volatile CH_3SeCN was separated and detected using a Hewlett-Packard (HP, Agliant Technologies, Wilmington, Del., USA) 6890 Gas Chromatography System, with an HP 5973 Mass Selective Detector. The GC was fitted with an Agilent HP-5MS column, (cross-linked 5% phenyl-methyl diloxane column with an inside diameter of 0.25mm, film thickness of 0.25 mm, and length of 26 m). The injection port was operated in the splitless mode at a temperature of 250°C, pressure of 2.47 psi (X pa), with helium carrier gas.

The column was operated in constant flow rate of 0.4 ml/minute. The oven's initial temperature was 40°C, which was held for 4 minutes; ramped at 50°C/minute to a final temperature of 200°C, hold time 1 minute. Total run time was 8.20 minutes. The HP 5973 MSD was operated in negative chemical ionization (NCI) mode with a solvent delay of 3.25 minutes. The above parameters gave a retention time (RT) of 5.10 minutes for the CH_3SeCN reactant product from selenomethionine. Quantitative data were acquired in the selected ion mode (SIM); however, the scan mode was used to verify the presence of selenium by its characteristic selenium fingerprint of five isotopes. The methyl group is fragmented off of the CH_3SeCN molecule in the mass spectrometer, leaving SeCN (m/e 106) as the major ion of interest (m/e 100 for ^{74}Se labeled product). The CNBr reaction with Se(methyl)- selenocysteine gives the identical CH_3SeCN reactant molecule with identical chromatographic and mass spectrometric properties.

Results and discussion

Results obtained on solutions of commercial and USP standard Semet solutions are presented in table 1. Total selenium in these solutions was determined by gas chromatography/isotope dilution mass spectrometry (GC/IDMS) following acid digestion. Agreement of analyzed selenium as Semet (column 2) with analyzed total selenium (column 3) and/or expected selenium (column 1) was quantitative.

The enriched yeast tablets had a mean value of 146.8 ± 10.3 μg $selenium_{(as\ Semet)}$/g, as shown in table 2. These values show that 73% of the label claimed total selenium content is present as selenomethionine. As determined by IDMS, the commercial yeast tablets contained 210 mg Se_{total}/g (total selenium determined by IDMS by C. Veillon of the Nutrient Requirement and Functions Laboratory, BHNRC, USDA). The label claims are for an elevated level of "organic selenium"(200 mg/g), usually as Semet.

For the wheat gluten (RM8418) sample, repeatable results were also obtained (table 2) at a level almost two orders of magnitude lower than the yeast sample, which more closely reflect "natural" levels in most foods. These results showed that 64% of the assigned total selenium value of 2.58 μg/g in the wheat gluten RM was present as Semet.

The quantification of selenium in these two samples proved to be a bit of a challenge due to the matrix. The Semet, and previously reported Metsecys [8], stand-

TABLE 1. Standard selenomethionine solutions

Source of Semet	Selenium as semet Expected μg/ml	Selenium as metse Analyzed CNBr[a] μg/ml	Selenium total Analyzed IDMS[b] μg/ml
Commercial[c]	68	75	74.8
Commercial[c]	208.0	209.2	n.a.
USP[d]	0.235	0.232	0.217
USP[d]	260.7	258.9	262.0
Diet supplement study[e]	2.50	2.64	2.60

a. Commercial source of pure selenomethionine.
b. Candidate USP reference standard under preparation.
c. Tablets prepared commercially for a dietary supplement study at USDA.
d. Selenium as Semet determined by GC/IDMS following reaction with CNBr.
e. Total selenium determined by IDMS following acid digestion(C. Veillon, NRFL, USDA, Beltsville, MD.)

TABLE 2. Selenomethionine content of selenium yeast tablets and wheat gluten reference materials

Source	Total selenium expected	Selenium (as metse) (mean ± SD)	Metse/total selenium (%)
Enriched yeast tablet[a]	200 μg/g (label claim)	146.8 ± 10.3 μg/g (n = 9)	73
Wheat gluten[b] NIST RM 8418	2.58 μg/g (assigned value)	1.64 ± 0.15 μg/g (n = 11)	64

a. Commercial sample of selenium yeast.
b. RM 8418, National Institute of Standards and Technology (NIST), Gaithersburg, Md., USA.

ard solutions are liquids with no other components to interfere with the reaction. However, the samples of wheat gluten and the yeast tablets introduced a complex biological matrix. There were occasional problems in obtaining a complete suspension of the matrix in the liquid phase (0.1 M HCl). This was accomplished by ensuring that the yeast tablet was pulverized well with a mortar and pestle, and that the analytical test portions of both the yeast and wheat gluten were mixed well immediately upon addition to the aqueous phase. This prevented the formation of a semi-solid layer in the reaction vial. Another obstacle was occasional difficulty in obtaining clean phase separation upon extraction with chloroform. To correct for this, an additional amount of $CHCl_3$ (e.g. 0.5 ml) was added in these cases.

Conclusions

This is a quick precise method to determine selenomethionine in foods. There is a significant amount of Semet in both of the materials studies, and they would make good candidate RMs. The predominant form of methylselenium in these two protein-based candidate materials would be expected to be selenomethionine. Mesecys is expected to occur more often in other plant materials such as broccoli. This CNBr procedure would not distinguish between these two forms of organic selenium, i.e., selenomethionine and Se-(methyl)selenocysteine . Further studies are under way at the food composition laboratory to distinguish between these two forms and to evaluate distribution of Semet in various wheat-based food products.

References

1. Groff JL, Gropper SS. Advanced nutrition and human metabolism. 3rd ed., Belmont, Calif., USA: Wadsworth, 2000.
2. Gebhardt S, Holden J. Provisional table on the selenium content of foods. HNIS/PT-109 Washington, D.C.: United States Department of Agriculture, Human Nutrition Information Service, 1993.
3. Mangles AR, Moser-Veillon PB, Patterson KY, Veillon C. Selenium utilization during human lactation by use of stable isotope tracers. Am J Clin Nutr, 1990;52:621–7.
4. Sinha R, Kiley SC, Lu JX, Thompson HJ, Moraes R, Jaken S. Medina D. Effects of methylselenocystine on PKC activity, cdk2 phosphorylation and gadd gene expression in synchronized mouse mammary epithelial tumor cells. Cancer Lett. 1999;46(2):135–45.
5. Medina D, Thompson H, Ganther H, Ip C. Se-methylselenocystine: a new compound for chemoprevention of breast cancer. Nutr Cancer 2001;40(1):12–17.
6. U.S. Department of Agriculture, Department of Health and Human Services. Home and garden bulletin #232, Dietary guidelines for Americans, 4th ed. Washington, D.C.: Government Printing Office, 1995.
7. Wolf WR, Zainal H, Determination of selenomethionine contents of dietary supplements by SIDMS. Houston, Texax: AOAC International, September, 1999 (Abstract #411).
8. Wolf WR, Zainal H, Yager B. Selenomethionine content of candidate reference materials. Fresenius' J Anal Chem 2001;370: 286–90.

Dietary intake of essential minor and trace elements from Asian diets

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Abstract

In view of the limited data available from the Asian region on the daily intake of nutritionally essential trace elements, a study was taken up, as part of a coordinated research project of the International Atomic Energy Agency, to estimate the daily dietary intake and organ content of some selected trace elements of importance in radiation protection, and also in nutrition. Nine Asian countries—Bangladesh, China, India, Indonesia, Japan, South Korea, Pakistan, Philippines, and Vietnam—which represented more than 50% of the world's population, participated in this study. Analysis of about 700 diet samples was carried out for four minor (calcium, potassium, magnesium, and sodium) and eight trace (chromium, cobalt, copper, iron, iodine, manganese, selenium, and zinc) elements using nuclear and other sensitive analytical methods employing neutron activation analysis (NAA), inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma atomic emission spectrometry (ICP-AES), and atomic absorption spectrometry (AAS) techniques. These samples consisted of the total cooked diet, market basket, duplicate diets, and 225 staple foods. Emphasis was placed on the quality assurance and harmonization of the sampling techniques to ensure quality data. Significant inter- and intra-country variations in daily dietary intake of various trace elements were observed. The maximum inter-country variation was observed for iodine intake (factor of more than 45), being highest for Japan and lowest for Pakistan. For iron, an important trace element, the variation between the intakes was by a factor of four being lowest for Vietnam and highest for Pakistan.

Keywords: trace elements, dietary intakes, analytical methods, quality control, recommended dietary allowance

Introduction

A coordinated research project (CRP) on "Compilation of Physical, Anatomical, Physiological and Metabolic Characteristics for a Reference Asian Man (RAM-Phase I)" was carried out from 1989 to 1994 within the framework of a regional program in support of radiological safety needs [1]. The Phase I program was subsequently extended to phase II. The second CRP, "Ingestion and Organ Content of Trace Elements of Importance in Radiological Protection Reference Asian Man Project Phase II," was initiated in 1995 to estimate the daily dietary intake and organ content of the trace elements cesium, iodine, strontium, thorium, and uranium. These five elements are the stable counter-parts of the radionuclides encountered in various operations of the nuclear fuel cycle. This second phase also included the measurement of some essential elements, such as calcium, potassium, magnesium, chromium, cobalt, copper, iron, iodine, manganese, selenium, and zinc, in daily diets consumed by the populations of the Asian countries. A few of these elements (i.e., calcium and potassium) behave similar to some of the above stated five elements (strontium and cesium, respectively). Some of the nutritionally important trace elements are reported to have protective roles in radiation damage to the human system.

This program provided a unique opportunity to create a data base for the dietary intake of the above

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stated trace elements obtained from the diets consumed by the population in the Asian region, collected under carefully and meticulously developed protocols for representative sample collection and also the quality assurance in the analysis. Since the project involved a group of countries which had different levels of expertise in analysis as well as in the availability of instrumentation, emphasis was placed on establishing a reasonable mechanism to ensure accuracy and harmonization in the methodologies for generating analytical results. Both the internal as well as the external quality control steps were incorporated in the experimental design, including the appointment of a central reference laboratory (CRL) at the National Institute of Radiological Sciences (NIRS) in Japan to ensure adequate quality control in analysis. Highly sensitive nuclear and other related techniques were employed to perform chemical analysis.

Such geographic region specific data sets generated under harmonized conditions of sampling, sample preparation, and subsequent analysis are very valuable as they permit reliable comparison of daily dietary elemental intakes among countries from a selected region. The purpose of this paper is to summarize the findings for the nutritionally relevant elements in diets collected in the nine Asian countries listed above.

Approach

A protocol was developed on sampling and chemical analysis of diet and autopsy tissue samples to ensure representative samples from the population of each country, emphasizing the need for good quality data. In addition to the daily diet samples, some of the participants also collected and analyzed a number of individual food samples, as specified in the protocols for the CRP to assess the contributions of these foods to the total daily trace element intake. Another important task within the CRP was to normalize the data from different participating laboratories so as to allow a meaningful comparison of the analytical measurements between countries and their dietary practices. To achieve this objective, a number of certified reference materials were distributed among the participating laboratories (internal quality control) for the evaluation and assessment of the analytical capabilities of the individual laboratory in analyzing the similar kinds of samples containing trace elements in concentrations comparable to those found in actual diet samples. The investigating laboratories participated in both the internal and external quality control procedures through the analysis of the existing certified reference materials (CRM) as well as the Japanese total diet CRM which was certified during the course of this project. The responsibility of ensuring the external and internal quality control rested primarily with the CRL.

Experimental

Representative dietary intakes of the elements of interest were studied in the adult populations (20 to 50 years old) of the nine countries. The samples were collected from different geographical locations in the country as well as from the various socioeconimic groups representative of the population. Some of these details are discussed elsewhere [2]. The method of collection included market basket, duplicate diet, and in some cases, major staple foods. Nearly 700 food and diet samples were collected and analyzed for this project. The method chosen for the diet collection in a particular country depended on whether the population of the country was heterogeneous or homogenous, and this criteria was employed to ensure that the collected samples were truly representative of the dietary consumption of those countries. Whereas in a few countries the individual food components were cooked to reconstitute the total cooked diet of the population, in some countries, the samples of each individual food were analyzed separately to estimate their contribution to the total intake. Duplicate diet samples were analyzed to estimate the daily intake in those countries where the variations in the food habits between various population groups within the country were considered to be relatively small. Blenders with titanium-coated blades were distributed to the participating laboratories to avoid the mineral contamination of the samples during their homogenization and powdering.

The analytical methods employed to determine these 12 essential minor and trace elements, by both the CRL and the participating laboratories, included neutron activation analysis (NAA), inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma atomic emission spectrometry (ICP-AES), and atomic absorption spectrometry (AAS).

Role of central reference laboratory and quality assurance

The CRL contributed and ensured quality assurance in analysis by coordinating with the participant laboratories on both internal and external quality assurance (QA) requirements and in harmonizing the analytical steps, where practical. Briefly these included use of existing CRMs to validate the analytical methods for trace element analysis being employed in various country laboratories, generating reference values in selected reference materials where certified results were not available, preparing a new total diet CRM based on the Japanese total diet for use in the current project [3], and finally analyzing the 10% of all the samples collect and analyzed in various country laboratories to assess the performance of the individual analytical methods being used in analytical laborato-

ries. The samples received from various countries for analysis at CRL were in general stored at –20°C until analysis. The dry samples as well as the ash samples were stored at room temperature. Typical weights of the dried samples analyzed ranged between 0.2 to 1 g and 0.1 to 0.2 g of sample ash weight was taken for analysis. In all 260 samples were analyzed at the CRL, which included about 200 samples of diet (for quality control and back-up purpose) and 53 tissue samples. This quality control approach has been discussed in detail elsewhere [4].

In view of the difficulties faced by several participating country laboratories in the analysis of low concentrations of iodine in diet and tissue samples, IAEA arranged the analysis of iodine to be carried out by radiochemical neutron activation analysis at a highly reputed contract laboratory, that has established excellent analytical quality control using a range of certified reference materials for iodine. Sufficient emphasis was attached to ensuring the quality control exercised in the analysis of the low concentrations of iodine in the diet and other samples obtained from various countries.*

Results and discussion

Validation of the analytical methods

The analytical methods employed for the analysis of the food samples for the 12 elements at various country laboratories were tested for the reliability through the analysis of the CRMs. The role of the CRL in the development of the Japanese Diet CRM, certified for 14 elements and making available the reference values for 12 other elements underscores the analytical excellence brought to focus in the support of this project and the QA provided for the project as a whole. Duplicate analyses of the 10% of the total samples analyzed at the country laboratories were also carried out at the CRL and the results were compared. As a further development of this type of comparison, the methodology of proficiency testing process by IUAC was also followed [5]. This involved the calculation of Z scores for the results of the elemental concentration of each sample that has been analyzed at both laboratories (where Z is the difference between the participant's results and the CRL's results divided by the target value of the standard deviation). Experience to-date shows that most Z scores are acceptable even using 10% as the target relative standard deviation. This aspect has been discussed elsewhere.

For the Asian study, dietary trace element intake data were accepted only from participating country laboratories that had demonstrated proficiency in analysis through both the internal and external quality control. The results of the analysis of the CRMs obtained from NIST, which were distributed to the participating country laboratories for the trace elements reported by them, were in close agreement (within 10%) with the certified values for elemental concentrations.

The details of the dietary minor and trace element data reported from individual Asian countries are shown in table 1.

Calcium, magnesium, sodium, and potassium in Asian diets

The comparison of the range of daily dietary intake of 12 elements with the intake reported for International Commission for Radiological Protection (ICRP) Refer-

* J. Kucera. Nuclear Physics Institute, Prague, Czech Republic, Determination of iodine in Asian diet samples and reference materials by neutron activation analysis, personal communication, 2000.

TABLE 1. Details of the essential trace elements analyzed by the participating Asian countries

	Essential trace elements											
	Minor				Trace							
Country	Sodium	Potassium	Calcium	Magnesium	Chromium	Cobalt	Copper	Iron	Iodine	Manganese	Selenium	Zinc
Bangladesh	—	x	x	—	—	—	x	x	—	x	—	x
China	x	x	x	x	—	—	x	x	x	x	x	x
India	—	x	x	x	x	x	—	x	x	—	x	x
Indonesia	—	x	x	—	—	—	—	—	—	—	—	x
Japan	x	x	x	x	x	x	x	x	x	x	x	x
South Korea	—	x	x	—	—	—	—	—	x	—	—	x
Pakistan	—	x	x	x	x	—	—	x	x	x	x	x
Philippines	x	x	x	x	—	—	x	x	x	x	—	x
Vietnam	x	x	x	x	—	x	x	x	x	x	—	x

x, data reported. —, data not reported.

ence Man and the recommended dietary allowances for these elements are shown in table 2. Daily dietary intake for sodium was reported by four of the nine countries. The main source of sodium is generally common salt, which is added during cooking; and although sodium is known to be an essential element, a dietary deficiency of sodium is not common. The daily intake of sodium was found to be in the range of 1.96 to 3.9 g as compared to 4.4 g proposed for ICRP Reference Man [6] and is comparable with the recommended dietary allowance (RDA) of 1.3 to 3.3 g [7].

Potassium intake was reported by the nine participating countries. The daily intake ranged from 1.04 to 2.7 g, which was lower than 3.3 g proposed for ICRP Reference Man and also lower than RDA of 1.87 to 5.6 g.

Calcium is a structurally important element for the humans. Its daily intake, reported by all the countries, ranged from 0.22 to 0.72g; the lowest was for South Korea and the highest for China. The intake from all the Asian countries was lower than the ICRP Reference Man value of 1.1 g and the RDA of 0.8 g. The significantly lower intake of calcium could be attributed to the lower intake of calcium-rich milk and milk products in Asian countries, as compared to North America.

The intake of magnesium was studied by only five countries. The intake ranged from 0.14 to 0.46 g. The lowest intake was for Philippines and the highest for Pakistan. The intake of magnesium for Japan and Vietnam was also significantly lower, and close to the lower end of the range. Except for Pakistan, the magnesium intake reported for most countries was lower than the RDA.

TABLE 2. Comparison of the range of daily intake of 12 trace elements in Asian countries with that for the ICRP Reference Man

Elements	Range of intake in Asian countries[a]	Intake by ICRP reference man	RDA
Minor elements			
Sodium (g)	1.96–3.9	4.4	1.3–3.3
Potassium (g)	1.04–2.7	3.3	1.87–5.6
Calcium (g)	0.22–0.72	1.1	0.8
Magnesium (g)	0.14–0.46	0.34	0.35
Trace elements			
Chromium (μg)	59.9–224	300	25–160[b]
Cobalt (μg)	9.6–17.9	300	120 (?)
Copper (mg)	0.87–2.17	3.5	1.5–3.0
Iron (mg)	6.6–31.4	16.0	10.0
Iodine (μg)	60–2,990	200	150
Manganese (mg)	2.83–10.54	3.7	2.5
Selenium (μg)	52.6–141	150	70
Zinc (mg)	4.34–13.5	13	15

a. Bangladesh, China, India, Indonesia, Japan, South Korea, Pakistan, Philippines, and Vietnam.
b. Value based on the intake of chromium in 22 countries [8].

Trace elements in Asian diets

Iron, zinc, manganese, and cobalt

The intake of iron was reported by seven of the nine countries and ranged from 6.6 mg for Vietnam to 31.4 mg for Pakistan. Except for the Vietnam and the Philippines, the intake by the other countries was adequate when compared to the RDA for iron.

The intake for zinc was reported by all countries. The daily dietary intake ranged from 4.3 mg for South Korea to 13.5 mg for Pakistan. The intake of all the Asian countries was lower than the RDA value of 15 mg, however, with the exception of South Korea and Vietnam, the intake was only marginally lower.

Six of the nine countries reported the intake of manganese, which ranged from 2.8 for Philippines to 10.5 mg for Pakistan. In general, unlike the other elements, the dietary intake of manganese was more than the RDA and higher that proposed for the ICRP Reference Man.

Only three countries reported the intake of cobalt, which ranged from 9.6 μg for Vietnam to 17.9 μg for India. It is important to note that although all three intake values for cobalt agreed well with each other, they were more than 17 times lower than that proposed for ICRP Reference Man. The agreement between the intake of the three countries and the much higher value proposed for ICRP Reference Man indicates that the intake value for the ICRP Reference Man, may need revision. This new realistic intake value for cobalt was the outcome of strict quality control exercised while carrying out this study.

Copper, selenium, chromium, and iodine

The intake of copper was studied in five Asian countries and ranged from 0.87 mg for Vietnam to 2.17 mg for Bangladesh. These values are only marginally lower than the RDA range of 1.5 to 3.0 mg.

The dietary intake of selenium was studied in only four countries. The intake which ranged from 52.6 to 141 μg was not much different from the RDA value of 70 μg.

The intake of chromium was reported from three countries. The intake ranged from 59.9 μg for India to 224 μg for Japan. There are no recommended values for this element to compare with. However, these values

appear adequate when compared with the intake range of 25 to 160 μg of chromium by population groups in 22 countries, as reported by Iyengar et.al. [8].

Very large variations in dietary iodine intake were observed. The daily intake varied from about 60 μg for Pakistan to 2,990 μg for Japan. The inter-country variation of daily dietary iodine intake is almost 50 times. The higher intake of iodine in Japan could be due to the intake of iodine-rich seaweeds, which form an essential component of the Japanese diet. With the exception of Japan, China, and Vietnam, the intake of iodine in most of the other Asian countries appears to be low. The recently introduced policy of using iodated salt for cooking purposes in the Asian countries, will ensure that the intake of iodine in the Asian region is adequate.

Conclusions

Daily dietary intake of 12 essential elements: four minor (calcium, potassium, magnesium, and sodium) and eight trace elements (chromium, cobalt, copper, iron, iodine, manganese, selenium, and zinc) were estimated in nine Asian countries using nuclear and other sensitive analytical methods employing NAA, ICP-MS, ICP-AES, AAS techniques. The participating countries—Bangladesh, China, India, Indonesia, Japan, South Korea, Pakistan, Philippines, and Vietnam—represented more than 50% of the world's population.

The adequate quality control, both internal and external, exercised for the analysis of the diet samples and the harmonization of the sampling protocol ensured the generation of the reliable daily dietary intake data for the 12 elements.

A comparison of the daily intake of the above stated elements with the intake values proposed for the ICRP Reference Man as well as with the RDA, indicated that the intakes of calcium, magnesium, and potassium were lower and that for sodium and were comparable with the RDA values. With the exception of iron, zinc, and manganese, the intake of the other trace elements was generally lower. The Japanese diet contained very high quantity of iodine, but the intake of iodine for the other countries was in general lower than the RDA.

References

1. International Atomic Energy Agency. Compliation of anatomical, physiological and metabolic characteristics for a reference Asian man. Volume 1. Data summary and conclusions. IAEA-TECDOC-1005. Vienna: IAEA, 1998.
2. Kawamura H, Parr RM, Dang HS, Tien W, Barnes RM, Iyengar GV. Analytical quality assurance procedures developed for IAEA's reference Asian man. J Radioanal Nucl Chem 2000;245:123–6.
3. Yoshinaga J, Morita M. Certificate of Analysis. NIES/NIRS typical Japanese diet certified reference materials. Tsukuba, Japan: National Institute of Environment Sciences and National Institute of Radiological Sciences, April 2000.
4. Parr RM, Kawamura H, Iyengar GV. Acquisition of improved reference values for cesium, iodine, strontium, thorium, and uranium in selected reference materials. Biol Trace Element Res 1999;71/72:5–13.
5. Thompson M, Wood R. The international harmonized protocol for the proficiency testing of (chemical) analytical laboratories. Pure and Applied Chem 1993;65(9):2123–44.
6. International Commission on Radiological Protection. Report of the task group on reference man. Oxford: Pergamon Press, 1980.
7. National Academy of Sciences. Recommended dietary allowances. 10th edition. Washington, D.C.: National Academy of Sciences, 1989.
8. Iyengar GV, Wolf WR, Tanner J, Morris ER Content of minor and trace elements and organic nutrients in representative mixed total diet composites from the USA. The Science of the total Environment 2000;256:215–26.

Isotope ratios of trace elements in samples from human nutrition studies determined by TIMS and ICP-MS: precision and accuracy compared

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Abstract

Stable isotopes are used with increasing frequency to trace the metabolic fate of minerals in human nutrition studies. The precision of the analytical methods used must be sufficient to permit reliable measurement of low enrichments and the accuracy should permit comparisons between studies. Two methods most frequently used today are thermal ionization mass spectrometry (TIMS) and inductively coupled plasma mass spectrometry (ICP-MS). This study was conducted to compare the two methods. Multiple natural samples of copper, zinc, molybdenum, and magnesium were analyzed by both methods to compare their internal and external precision. Samples with a range of isotopic enrichments that were collected from human studies or prepared from standards were analyzed to compare their accuracy. TIMS was more precise and accurate than ICP-MS. However, the cost, ease, and speed of analysis were better for ICP-MS. Therefore, for most purposes, ICP-MS is the method of choice, but when the highest degrees of precision and accuracy are required and when enrichments are very low, TIMS is the method of choice.

Key words: TIMS, ICP-MS, isotope ratios, trace elements, copper, zinc, molybdenum, magnesium

Introduction

The use of stable isotopes for nutrition research in humans began in the late 1970s [1]. Early work used neutron activation analysis (NAA) and mass spectrometry of volatile metal chelates [2] for isotope measurements. These methods were generally not sufficiently accurate or precise for tracer studies and are rarely used now for isotope measurements.

In 1978 we began our stable isotope research using thermal ionization mass spectrometry (TIMS) because of its high precision and accuracy [3]. TIMS had been available for some time and was used primarily by geochemists and nuclear chemists. Nutritionists had not considered the approach. Commercial instruments were not yet available and we used MS6, a TIMS built at Lawrence Berkeley Laboratory (Berkeley, Calif., USA) by Maynard Michel. We could determine isotope ratios in 1 to 3 samples a day. Automated, commercial instruments became available shortly after we began using TIMS and these instruments improved the speed of analysis, but the approach still had drawbacks. An inductively coupled plasma mass spectrometer (ICP-MS) was developed for determination of trace elements in 1980 [4] and later began to be used for isotope ratio measurements. These two instruments are both available commercially and are the primary instruments used for isotope ratio measurements. We began using ICP-MS recently and this paper compares our results using TIMS and ICP-MS and discusses the advantages of each.

TIMS and ICP-MS compared

Purchase price

TIMS instruments are generally more expensive than ICP-MS instruments. We use a Finnigan MAT Model 261 TIMS (Thermo Finnigan, San Jose, Calif., USA) with multiple collector that cost US$335,000 in 1985. The current Thermo Finnigan MAT TIMS, the Triton TI, costs US$550,000. Our Perkin Elmer ELAN 6000 ICP-MS (Perkin Elmer, Shelton, Conn., USA) cost US$168,000 in 1996. The current model, the ELAN 6100 ICP-MS, costs about US$160,000. Other ICP-MSs with additional features cost more, up to US$700,000 for a multicollector, high resolution ICP-MS.

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Mention of the names of firms and commercial products does not imply endorsement by the United Nations University.

Operator training

TIMS are more complex instruments than ICP-MS and require more operator training. Approximately 2 to 3 months of training is needed for an individual to learn to operate a TIMS, while a person experienced with other mass spectrometers or an ICP can learn to use an ICP-MS in 2 to 3 weeks.

Sample purification and preparation for analysis

Samples must be free of all organic material and other minerals for analysis by TIMS. After eliminating organic material using a muffle furnace or by microwave oven digestion, we usually use ion exchange columns for separation and purification of the mineral of interest. A minimum of two column separations is required to eliminate impurities [5]. These separations are slow and time consuming. ICP-MS isotope ratio determinations can be done following elimination of organic material and a single column separation. We have been able to determine isotopic ratios of magnesium after eliminating organic material, without further purification [6]. Thus, sample purification and separation are considerably simpler and faster for ICP-MS.

The final preparation for analysis is considerably faster and easier for ICP-MS. Following sample purification, samples for TIMS analysis must be concentrated, applied to filaments, and dried. To increase sensitivity for some elements, such as copper, zinc, and molybdenum, samples are applied to filaments along with silica gel and phosphoric or boric acid [5]. This is a multi-step procedure and samples must be dried after each step. Slit plates are added to each filament on a 13 to 16 sample carousel, which is then placed in the ion source and the pressure must then be pumped down. ICP-MS samples, after column separations, are diluted and placed in an autosampler. The ICP-MS is generally left under vacuum, although the torch must be lit, stabilized, and optimized each day.

Sample throughput

ICP-MS analysis time is considerably faster than TIMS. Sample throughput (handling capacity) for our TIMS under automated conditions is limited to 13 samples (1 carousel) per day. When analysis cannot be automated, fewer samples can be analyzed per day. By ICP-MS we can analyze up to 70 samples per day (10 per hour plus 1.5 hours warm-up and optimization). A weekly full optimization of the ICP-MS requires 3 hours.

Consumables and gases

The cost of consumables, including gases, is lower on a weekly basis for TIMS than for ICP-MS. The liquid nitrogen used for TIMS costs US$48 per tank and lasts about six weeks. The rhenium filaments cost US$2 to $4 per sample, for a total cost of $150 per week at maximum operation. The liquid argon used for ICP-MS costs US$345 per tank that lasts about 3 to 4 weeks. Periodic replacement of peristaltic tubing, cones, torch, injector, coil, and detector is required. The total cost of consumables is about US$200 per week. However, since ICP-MS can analyze at least five times as many samples per week, the cost of consumables per sample is far less for ICP-MS.

Repair costs

Repair costs are highly variable, but TIMS generally has higher repair costs than ICP-MS. TIMS has more numerous and higher priced components and electronics damage sometimes occurs due to high voltage arcs with TIMS.

Interferences

TIMS has fewer interference problems than ICP-MS because of the high degree of purity required for analysis. After proper purification and preheating most elements have negligible interference. ICP-MS uses argon, which interferes with some calcium and iron isotopes, so they cannot be analyzed by conventional ICP-MS. Special methods have been developed with high resolution ICP-MS to allow analysis of these elements. ICP-MS is also vulnerable to other interference since less sample purity is required for analysis and ionization efficiency is very high.

Sample size

ICP-MS sample consumption is lower than TIMS by about a factor of 10. The sample sizes we usually use are shown in table 1. TIMS typically uses 1 to 5 μg of mineral per analysis, while ICP-MS usually uses 0.1 to 0.5 μg.

Precision and accuracy

The precision of TIMS and ICP-MS in our laboratory is shown in table 1. Typical TIMS precision is 0.01% to 0.1%, with best precision of 0.003%, considerably better than ICP-MS. Typical ICP-MS precision is 0.1% to 0.3%, with a best precision of 0.04%. With TIMS, internal precision is often better than external precision due to variations in fractionation between runs. With ICP-MS, external precision is usually better than internal precision due in part to the non-simultaneous scanning method.

The accuracy of TIMS is also much better than ICP-MS, but results of the two methods agree within 1%. Fractionation of the isotopes with TIMS analysis occurs due to an increased probability of lighter iso-

TABLE 1. Precision of isotope ratios determined by TIMS and ICP/MS for 10 replicates of unenriched standard solutions

Element	Isotope ratio	TIMS			ICP/MS		
		Sample size	Internal[a] RSD (%)	External[b] RSD (%)	Sample size	Internal[a] RSD (%)	External[b] RSD (%)
Magnesium[c]	25/24	5 mg	0.03	0.06	0.5 mg	0.20	0.11[d]
Copper	65/63	4 mg	0.01	0.08	0.15 mg	0.09	0.04
Zinc[c]	64/68	2 mg	0.02	0.06	0.15 mg	0.40	0.32
	67/68		0.04	0.08		0.42	0.15
	70/68		0.13	0.07		0.52	0.15
Molybdenum[c]	94/98	2 mg	0.10	0.05	0.25 mg	0.30	0.05
	97/98		0.06	0.08		0.10	0.07
	100/98		0.08	0.06		0.15	0.06

a. Internal RSD is the relative standard deviation of the ratio measurements within a single analysis.
b. External RSD is the relative standard deviation of ratio measurements of 10 replicates of the same sample.
c. Internal normalization was used for TIMS ratio measurements.
d. ICP/MS values for magnesium were based on five replicates.

topes evaporating from filaments than heavier isotopes. This produces a mass bias that is proportional to the square root of the ratio of the isotopic masses. The mass bias is in the range of 0.1% to 1% per mass unit difference. TIMS ratios can be corrected for fractionation when a ratio of two unenriched isotopes is available for normalization corrections [5, 7], such as for molybdenum and zinc.

With ICP-MS analyses the mass bias is more complex, since the argon torch efficiently ionizes all impurities in the sample. The complex mixture of ions is highly dependent on sample matrix and can vary over time. The bias is much larger than for TIMS, ranging from 1.5% to 15% per unit mass difference. Our ICP-MS software uses a mass bias correction procedure. A standard with natural isotopic abundance is analyzed and the measured isotopic ratios are divided by the accepted natural values to yield a ratio correction factor (RCF) for each ratio. In subsequent analyses each measured isotopic ratio is divided by its RCF to correct for mass bias. The resulting ratios are a function of the accepted natural values used and are relative values.

The isotopic ratios and enrichments measured using our TIMS and ICP-MS are compared in tables 2 to 4. For copper and zinc (tables 2 and 3), fecal samples were collected from subjects in human stable isotope studies. These samples were enriched with a range of enrichments in an isotope of each mineral. After organic material was destroyed, the minerals were separated and purified using two sequential anion exchange columns. Table 2 compares the measured 65/63 isotope ratios and enrichments for the samples enriched in ^{65}Cu, enrichments ranging from zero to 25%. Enrichment refers to the percentage change in isotopic ratio from the ratio of the natural sample. The average difference in copper isotopic ratios measured by ICP-MS versus those by TIMS was –0.8%. Table 3 compares the

TABLE 2. Isotope ratios of copper separated from ^{65}Cu-enriched fecal samples and measured by TIMS and ICP/MS

65/63 Ratio		Enrichment[a](%)	
TIMS	ICP/MS	TIMS	ICP/MS
0.4457	0.4463	0.00	0.00
0.4571	0.4569	2.54	2.38
0.4687	0.4669	5.16	4.62
0.4793	0.4747	7.54	6.36
0.4903	0.4791	10.0	7.35
0.5126	0.5089	15.0	14.0
0.5350	0.5359	20.0	20.1
0.5577	0.5469	25.1	22.5

a. Enrichment = [(measured ratio—natural ratio)/(natural ratio)] × 100%

TABLE 3. Isotope ratios of zinc separated from ^{67}Zn-enriched fecal samples and measured by TIMS and ICP/MS

67/68 Ratio		Enrichment[a]	
TIMS	ICP/MS	TIMS	ICP/MS
0.2172	0.2184	0.00%	0.00%
0.2285	0.2287	5.24%	4.72%
0.2349	0.2332	8.19%	6.78%
0.2470	0.2476	13.8%	13.4%
0.2604	0.2601	19.9%	19.1%
0.2853	0.2855	31.4%	30.7%

a. Enrichment = [(measured ratio—natural ratio)/(natural ratio)] × 100%

measured 67/68 isotopic ratios and enrichments for samples enriched in ^{67}Zn, with enrichments from 0 to 31%. The average difference in zinc ratios measured by ICP-MS versus those by TIMS was 0.02%. Natural and enriched molybdenum standard solutions were combined to create three sets of samples with a range of enrichments in one isotope. Table 4 compares the measured isotopic ratios and enrichments of three sets of molybdenum samples enriched either in ^{94}Mo, ^{97}Mo, or ^{100}Mo. The average difference in isotopic ratios measured by ICP-MS versus those by TIMS was 1.0% for 94/98, 0.6% for 97/98, and –0.3% for 100/98. The average difference in enrichments between TIMS and ICP/MS of samples with a range of enrichments for copper, zinc, and molybdenum was within 1% for all elements.

TABLE 4. Isotope ratios of molybdenum standard solutions enriched with stable isotopes and measured by TIMS and ICP/MS

^{94}Mo-enriched samples			
94/98 Ratio		Enrichment[a] (%)	
TIMS	ICP/MS	TIMS	ICP/MS
0.3806	0.3828	0.0	0.0
0.4145	0.4175	8.9	9.1
0.7250	0.7354	90.5	92.1
1.4129	1.4335	271	274
3.9604	3.9924	940	943
^{97}Mo-enriched samples			
97/98 Ratio		Enrichment[a](%)	
TIMS	ICP/MS	TIMS	ICP/MS
0.3945	0.3959	0.00	0.00
0.3986	0.4001	1.03	1.05
0.4054	0.4067	2.74	2.72
0.4304	0.4334	9.08	9.47
0.7569	0.7674	91.8	93.8
^{100}Mo-enriched samples			
100/98 Ratio		Enrichment[a](%)	
TIMS	ICP/MS	TIMS	ICP/MS
0.4016	0.3985	0.00	0.00
0.4052	0.4029	0.90	1.10
0.4123	0.4103	2.67	2.96
0.4379	0.4373	9.05	9.74
0.7632	0.7669	90.0	92.4

a. Enrichment = [(measured ratio—natural ratio)/(natural ratio)] × 100%

Conclusions

TIMS is considered the "gold standard" for isotope ratio measurements. Precision and accuracy are generally the best that can be obtained. Therefore, it is the method of choice when the highest degree of precision is required for the application. However, ICP-MS has numerous advantages, including lower cost, less sample preparation, much faster throughput, and ease of operation. For most applications, the precision and accuracy of ICP-MS suffice, so it is the method of choice in most situations.

References

1. Schwartz R, Spencer H, Wentworth RA. Measurement of magnesium absorption in man using stable ^{26}Mg as a tracer. Clin Chim Acta 1978;87:265–73.
2. Schwartz R, Giesecke CC. Mass spectrometry of a volatile Mg chelate in the measurement of stable ^{26}Mg when used as a tracer. Clin Chim Acta 1979;97:1–8.
3. Turnlund JR, Michel MC, Keyes WR, King JC, Margen S. Use of enriched stable isotopes to determine zinc and iron absorption in elderly men. Am J Clin Nutr 1982;35: 1033–40.
4. Houk RS, Fassel VA, Flesch GD, Svec HJ, Gray AL, Taylor CE. Inductively coupled argon plasma as an ion source for mass spectrometric determination of trace elements. Anal Chem 1980;52:2283–9.
5. Turnlund JR, Keyes WR. Automated analysis of stable isotopes of zinc, copper, iron, calcium, and magnesium by thermal ionization mass spectrometry using double isotope dilution for tracer studies in humans. J Micronutr Anal 1990;7:117–45.
6. Sabatier M, Keyes WR, Arnaud MJ, Turnlund JR. Preparation and ICP-MS measurements of magnesium stable isotopes in human samples. Trace Elements in Man and Animals 1999;10:208(Abstract).
7. Moore LJ, Machlan LA, Shields WR, Garner EL. Internal normalization techniques for high accuracy isotope dilution analyses—Application to molybdenum and nickel in standard reference materials. Anal Chem 1974;46:1082–9.

Bioavailability of iron from micro-encapsulated iron sprinkle supplement

Chandrani Liyanage and Stanley Zlotkin

Abstract

To improve the iron status of infants an effort was made to increase the iron content of complementary foods by adding 12.5 mg of elemental iron to the meal in the form of micro-encapsulated ferrous fumarate coated with a lipid. The contents of the packet were sprinkled directly on to infant foods. Relative absorption of iron from this supplement was determined in a prospective randomized study with 39 infants (mean age 33.6 ± 5.2 weeks) with initial hemoglobin values greater than 100 g/L. They were fed two complementary foods (rice-based and wheat-based) in which the supplement labeled with stable isotopes of iron ^{57}Fe and ^{58}Fe was incorporated. The erythrocyte iron incorporation was measured in the blood by inductively coupled plasma mass spectrophotometry. The incorporation of iron was significantly higher 11.9% $p < .001$ and 13.3% $p < .001$ and no difference was observed with the type of cereal in complementary foods. The use of ferrous fumarate sprinkles has proved to be efficacious in increasing the available iron intake of the infants.

Key words: iron absorption, supplement, stable isotopes, ^{57}Fe, ^{58}Fe, infants

Introduction

Iron-deficiency anemia is the most common of all the nutritional deficiencies affecting millions of infants and children in the developing world. It is prevalent in all the age groups of the Sri Lankan population in which 45% of preschool children are anemic [1]. Although a variety of infant foods that include pre-cooked cereals, cereal-fruit products, etc. are currently fortified with iron most infants and toddlers in the developing world have no access to these foods due to their prohibitive cost. Although iron supplementation is believed to be the most effective method of alleviating iron-deficiency anemia where the prevalence is high, it has not been shown to be an effective public health strategy. However, there are some potential problems with implementing an effective strategy of wide-scale iron supplement distribution to infants and young children. Iron supplements for infants and young children are presently given as a solution which has significant disadvantages as compared to tablets or pills including dispensing directions, shorter shelf-life, higher likelihood of dosage errors, possible staining of teeth, and a strong and unpleasant taste. As such, in an alternative delivery system for providing iron to infants and toddlers, a novel packaging method and a source of iron have been developed where iron is made available as micro-encapsulated ferrous fumarate in a packet as a single daily dose of 12 mg/per day.

The sprinkle-sized particles of ferrous fumarate are coated with a mono or diglyceride (hydrogenated soy lipid) and this thin coating protects the iron from the food (and food from the iron) and also masks the taste of the iron. The contents of the packet are sprinkled on the food that is served to the child. The iron will not react with the food altering its appearance or taste because it is encapsulated. The coating will dissolve in the stomach, releasing the iron salt, to be absorbed along with iron contained in the foods that constituted the meal. The availability of the added iron for absorption will be affected by inhibitors and enhancers of iron absorption that might be present in a meal fed to infants and toddlers. Knowledge of the bioavailability of iron supplements is therefore necessary for a sound approach to establishing strategies for meeting the needs of the absorbed iron in them. Of the

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several approaches that have been used to estimate the bioavailability of iron sources, the double stable isotope technique was selected to determine the absorption of iron from the erythrocyte iron incorporation.

Materials and methods

This was a randomized prospective study to determine the bioavailability of micro-encapsulated and non-encapsulated ferrous fumarate after mixing them with two different complementary foods.

Population

The study population consisted of 39 full-term healthy infants and toddlers between 7 and 12 months of age, who were of average birthweight and who attended the maternal and child health clinic at Godakanda, Galle. They had hemoglobin concentrations 100g/L or more and had already been introduced to complementary foods, that were similar to the test meals (porridge of rice or wheat), and clinically stable at the time of the initiation of the study. Consent was obtained from the parents to include their infants in the study after they were informed about the aims and procedures involved. The protocol was approved by the Ethical Committee of the Faculty of Medicine, University of Ruhuna, Sri Lanka.

The sample size was calculated to be large enough to detect a difference between the bioavailability of non-encapsulated versus encapsulated ferrous fumarate. As there had been no prior studies to estimate the variance in the outcome measure, a convenience sample of 40 infants were enrolled in the study. With an estimated dropout rate of 33% it was hoped to complete the study in about 30 subjects.

Experimental design

The study subjects were selected during field visits by an investigator, and after obtaining written consent they were invited to the well-baby clinic on three days, two weeks apart, for the feeding trials. At each visit their body weight was measured and, a capillary blood sample was obtained before the test meal was given. A 10 µl sample for hemoglobin was drawn onto a microcuvette, followed by another sample of about 250 µl which was drawn into a heparinized plastic microtainer collection tube. The blood was transferred to the laboratory for subsequent ferritin and stable isotope analysis. Hemoglobin was determined immediately using the Heemocue photometer (AB Leo Diagnostics, Sweden).

Each infant was fed a test meal (rice-based) on the first visit with a packet of sprinkles added and, the mothers were instructed to return in 14 days. At the second visit the infants were fed the second test meal (wheat-based) with an iron packet added after measuring their body weight and obtaining a blood sample. The mothers were asked to bring their infants back in 14 days and the third blood sample was drawn and their weights were recorded.

The normal food intake of the infants was not controlled except that test meals were given four hours after the last meal to that ensure their stomach was empty enough for proper utilization of iron and, the mothers were asked not to give any other iron supplement during the day.

Test meals

The complementary foods tested were primarily composed of brown country rice (low extraction) or semolina (high extraction) 17.5g, lentils 5g, carrots 12.5g, and butter 2.5g. The ingredients were cooked in double-distilled water until soft. Butter and salt were added after mashing the mixture to a thick porridge. Each serving was weighted and a packet of iron sprinkles (ferrous fumarate, encapsulated or non-encapsulated) either unlabeled or labeled with ^{57}Fe of ^{58}Fe added and thoroughly mixed just before serving. The test meals were fed by the mother under the supervision of the investigators, and the weight of waste and leftover food was measured to obtain the infants' actual intake. To make sure that the infants consumed the given amount of sprinkles completely, they were first fed a small proportion of the test meal mixed with the sprinkles followed by the rest of the meal.

Iron supplies

Each packet contained 12.5mg of elemental iron, consisting of three forms of iron ^{58}Fe, ^{57}Fe, and unlabeled ferrous fumarate. The proportion of each labeled iron was 1.5mg of ^{58}Fe, 5.0 mg of ^{57}Fe, and 5.5mg of (remainder) unlabeled ferrous fumarate.

The enriched ^{57}Fe and ^{58}Fe were purchased as ferric oxide from Oak Ridge National Laboratory, USA and, converted to labeled ferrous fumarate. Micro-encapsulation was done only on ^{58}Fe fumarate by coating with hydrogenated soybean lipid. ^{57}Fe ferrous fumarate was not encapsulated and was added directly to the packet.

Ferrous fumarate microcapsules were prepared by small-scale rotational suspension separation encapsulation. Briefly, ^{58}Fe ferrous fumarate was dispersed in molten hydrogenated soybean lipid (Durkee Industrial Foods Corp.) at 60°C. The resulting mixture was then poured slowly into a container of silicone oil (DF

55–2000, Silchem Inc.) at an equivalent temperature. The mixture was stirred with an impeller at 72 rpm and then rapidly cooled with chilled silicone oil to a temperature of 35°C. The resulting particles were separated from the silicone oil matrix by centrifugation at 2000X g and rapidly washed (5 x) with chilled heptane.

Analysis of blood samples

From the aliquots of blood (about 250 µl) that were drawn on each of three visits, 100 ml was separated and digested using nitric acid and heated until the digest was clear. The digests were stored in sterile screw capped containers at room temperature, and taken to the Division of Paediatrics and Nutritional Sciences of the Hospital for Sick Kids in Toronto, for determination of the erythrocyte incorporation of stable isotopes. The isotope in the erythrocytes was determined using the method of Zlotkin et al.[2]. The percentage of iron that is absorbed is incorporated into newly formed erythrocytes and, the incorporation was measured from the iron intensities of the erythrocytes [3] in the digested blood samples (3 samples per infant) using an inductively coupled plasma mass spectrometer (ICP-MS Elan Model 6000, SCIEX, Inc. Thornhill, Ontario Canada) operated in the isotope ratio mode (appendix 1). The remaining blood from each sample was used for the ferritin assay by well-established IRMA method [4] using Coat-A-Count Ferritin IRMA kits, DPC, Los Angeles, Calif., USA.

Analysis of complementary foods (test meals)

Analysis of iron and calcium were made by atomic absorption spectrometry (Model 975; Varian, Techtron, Australia). Aliquots of the complementary foods were dried at 100°C and ashed for 24 hours at 600°c. The ash was dissolved in nitric acid to determine the iron concentration and measured by using a standard addition technique. For calcium, the ash was diluted with 1.5 mol hydrochloric acid per liter containing 0.5% lanthanium chloride. Duram wheat flour (reference material 8436, National Institute Standard Technology, Gaithersberg, Md., USA) was used as the reference. The phytate and tannin contents of the foods were determined using an Anion exchange method [5] and, modified FAS method [6], respectively.

Statistical analysis

The principle outcomes were the bioavailability of the encapsulated versus the non-encapsulated iron, and the effect of the type of diet on the bioavailability of the two forms of iron. These outcomes were analyzed using paired t-tests since each infant received both forms of iron in the same dosage and both diets.

Results

All the infants in the study were within the second six months of life at the time of study (table 1). This age is a period for the development of iron depletion and for the onset of iron-deficiency anemia. The subjects were non-anemic, with a mean hemoglobin of 114.14 ± 0.76g/L and without evidence of iron depletion (mean ferritin 67.38 ± 33.99 µg/L). The mean birthweight was 2.9kg with a mean body weight of 7.2kg at the time of study.

The mean weights for the test meals consumed were 73.7 ± 41.5g for the rice-based and 80.9 ± 40.7g for the wheat-based meal (table 2). Table 3 shows the mean isotopic ratio (MIR) of the stable isotopes of iron naturally occurring and, the baseline values for MIR in the study subjects. It is apparent that prior to study the subjects had the same isotopic ratio ($^{57}Fe/^{54}Fe$ and

TABLE 1. Characteristics of the infants included in the study

	N	Mean ± SD
Birthweight (kg)	39	2.988 ± 0.36
Baseline		
Weight (kg)	39	7.202 ± 0.80
Age (weeks)	39	33.71 ± 5.82
Hemoglobin (g/L)	39	114.14 ± 0.76
Ferritin (µg/L)	21	67.381 ± 33.99
End		
Weight (kg)	39	7.891 ± 0.29
Hemoglobin (g/L)	39	11.08 ± 0.56
Ferritin (µg/L)	25	66.734 ± 30.74

TABLE 2. Intake of the test meals and the contents of iron, calcium and inhibitory factors in a meal

	Rice-based Meal	Wheat-based Meal
Iron (mg)	1.3 ± 0.8	1.3 ± 0.6
Calcium (mg)	20.4 ±10.8	54.2 ± 26.8
Phytate (mg)	42.5 ± 2.4	18.4 ± 2.1
Tannins (mg)	0.0	0.0
Intake (g)	73.7 ± 41.5	80.9 ± 40.7

TABLE 3. Isotopic ratio report

Natural isotopic composition	$^{57}Fe/^{54}Fe = 0.379$
(Mean isotopic ratio, MIR)	$^{58}Fe/^{54}Fe = 0.0482$
Mean isotopic composition	$^{-57}Fe/^{54}Fe = 0.376 \pm 0.002$
(MIR) of the samples at baseline	$^{58}Fe/^{54}Fe = 0.046 \pm 0.001$

$^{58}Fe/^{54}Fe$) and, the MIR is quite close to the natural occurrence.

The effects of encapsulation and of the meal composition on erythrocyte incorporation are shown in table 4. It appears that the percentage of iron absorbed is directly related to the intake of the iron incorporated into the test meals. The higher the intake, the higher the absorption observed (11.9% and 13.3% absorption from the 5.07mg of ^{57}Fe and, 1.8% and 2.2% absorption from 1.57mg of ^{58}Fe). The iron content was the same in both types of meals, but the inhibitory factors (phytate and calcium) varies (table 2). However, for each form of iron (encapsulated or non-encapsulated) there was no effect of the type of cereal on the percentage incorporated. Absorption from non-encapsulated iron was significantly higher in both rice- and wheat-based meals.

In-vitro studies of release of iron from micro-encapsulated ferrous fumarate

In an attempt to further understand the potential release of iron after micro-encapsulation, two commercial sources of micro-encapsulated ferrous fumarate were used and differences in the release of iron were studied by an in vitro simulated "gastric digestion model" modified to mimic the gastric pH, temperature, fluid volume, and gastric emptying time of the infants between 6 to 12 months of age. The differences in the release of iron were striking between the two sources of encapsulated iron. Over a two-hour time span there was virtually no release of iron from one as compared to 70% release from the other. It is apparent, therefore, that the method of micro-encapsulation will have a significant effect on the release and bioavailability of iron and that one cannot necessarily assume equivalent bioavailability among the different forms of micro-encapsulated iron.

Discussion

The results of this study provide information on the absorption of iron from the two homemade complementary meals. Ferrous fumarate (non encapsulated) was reasonably well absorbed despite the phytate and calcium content of the complementary foods used in the study. There was no significant difference in absorption between the rice- and the wheat-based meals.

The encapsulated ferrous fumarate, however, was less well absorbed than the non-encapsulated ferrous fumarate. Theoretically, the micro-encapsulated iron will not react with the food it comes in contact with, yet the coating will readily dissolve in the low pH of the stomach. It is quite likely that the coating on the isotopically-labeled ferrous fumarate adversely affected the absorption of the iron in this study. The encapsulation of the isotopically-labeled ferrous fumarate was done in a university laboratory since a commercial form of isotopically-labeled iron was not available. The ratio of coating to iron was 10-fold higher with the laboratory prepared product, as compared to the commercially encapsulated non-isotopically-labeled iron. It is therefore most likely that because of the increased amount of coating, digestion of the coating was incomplete. Even with commercially micro-encapsulated iron, a significant difference in potential absorption and bioavailability would be expected depending on the process of micro-encapsulation. The results of the current study, therefore, cannot be directly extrapolated to estimate iron dosage for clinical use, since the release of iron appears to be highly dependent on the method of micro-encapsulation. However, the estimate of the absorption of isotopically-labeled ferrous fumarate will be useful in the determination of an appropriate dose of encapsulated iron to be included in a single dose packet.

One explanation of the high prevalence of iron-deficiency anemia in developing countries is the low iron content of typical complementary foods combined with the low bioavailability of the iron due to the relatively high fiber and phytate contents of the food [7]. In the industrialized world, the problem has been addressed by the fortification of commercial complementary foods with iron. Since the routine use of iron-fortified commercial complementary foods is not an option for most infants in the developing world, alternate strategies have to be employed. One strategy is to add iron in a soluble form to the home-prepared complementary

TABLE 4. The intake doses of labeled iron and percentage of erythrocyte incorporation of isotopes from micro-encapsulated (n = 32) versus non-encapsulated (n = 39) iron from rice-based and wheat-based meals

Iron isotope and intake doses	Percent absorption from meal					
	Rice-based		Wheat-based		Combined	
	% ± SD	p	% ± SD	p	% ± SD	p
^{57}Fe non-encapsulated	5.07 ± 0.11 mg	.001	13.3 ± 9.6	.001	12.5 ± 7.6	.001
^{58}Fe encapsulated	1.56 ± 0.03 mg		2.2 ± 2.5		2.0 ± 2.0	

food, without changing is taste, color, or appearance. In the present study the form of soluble iron (ferrous fumarate) tested caused no organoleptic changes and, the thin coating also masked the taste of the iron. As such, the use of a single dose supplement added directly to complementary foods has much appeal and this is especially true in developing countries because of the high prevalence of micronutrient deficiencies.

Acknowledgement

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References

1. Mudalige R, Nestel P. Combating iron deficiency. The Ceylon J Med Sci 1996;39(1):9–16.
2. Zlotkin SH, Lay D, Kjarsgaard J, Longley T. Determination of iron absorption in very low birthweight premature infants using two stable isotopes of iron (^{57}Fe and ^{58}Fe) J Pediatr Gastroenterol Nutr 1995;21:190–9.
3. Gray AL, Date AR. Inductively coupled plasma mass spectrometry using continuum flow iron extraction. Analyst 1983;108:1033–7.
4. Miles LE, Lipshitz DA, Bieber CP, Cook JD. Measurement of serum ferritin by a 2 site immunoradiometric method. Anal Biochem 1974;61:209–24.
5. Harland BF, Oberleas D. Anion exchange method for determination of phytate in foods; collaborative study. J AOAC 1986; 69:667–70.
6. Brune M. The inhibitory effect of phytate, calcium and phenolic compounds on non-heme iron absorption. Doctoral thesis. Gothenburg, Sweden: Department of Medicine II, University of Gothenburg, 1989.
7. Gibson RS, Ferguson EL, Lehrfeld E. Complementary foods for infant feeding in developing countries: their nutrition adequacy and improvement. Eur J Clin Nutr. 1998;52:764–70.

Appendix 1

Isotope ratio mode

Samples:	Digested whole blood (1:3 HNO_3) diluted with deionized water
Instrumentation:	PE Elan 6000
Analytical standard:	Iron high-purity standards (Cat. No. 100026-1)
Interference correction:	Isotopic-^{54}Fe ^{40}Ar ^{14}N Isobaric–^{58}Fe(^{58}Ni) ^{57}Fe ^{40}Ar ^{160}H ^{58}Fe ^{40}Ar 180
Mass-discrimination correction:	Used in all isotopic ratios

Use of Fourier transformed infrared spectrophotometer (FTIR) for determination of breastmilk output by the deuterium dilution method among Senegalese women

Aïta Sarr Cissé, Leslie Bluck, Babou Diaham, Nicole Dossou, Amadou Tidiane Guiro, and Salimata Wade

Abstract

Breastmilk output can be estimated from the mother's total body water and water turnover rates after oral administration of deuterium oxide. Usually the deuterium enrichments are determined using a isotope ratio mass spectrometer, which is expensive and requires a specialist for operation and maintenance. Such equipment is difficult to set up in developing countries. A less expensive method was developed which uses a Fourier transform infrared spectrophotometer (FTIR) for deuterium enrichment analysis. This study evaluated the constraints of using FTIR to study lactating women in Senegal. The deuterium isotope method was found to be adequate for free living subjects and presented few constraints except for the duration of the saliva sampling (14 days). The method offers the opportunity to determine simultaneously breastmilk output, mother's body composition, and breastfeeding practices. Deuterium sample enrichments measured with FTIR were fast and easy, but for spectrum quality some environmental control is required to optimize the results.

Key words: stable isotopes, deuterium, infrared spectrometer, breastmilk output

Introduction

Measurement of breastmilk intake is fundamental for infant nutrition in developing countries since it is well known that early introduction of weaning foods is one of the causes of child malnutrition [1]. In Africa, particularly in Senegal, while breastfeeding is widespread, only a small proportion of children are exclusively breastfed [2]. The conventional method for measuring breastmilk intake consists of weighing the baby before and after each feeding. This is time-consuming, inaccurate, and interferes with the mother's normal activities; also it cannot be used when the baby is fed on demand [3]. A more practical and accurate method is to measure breastmilk output by isotope dilution using stable isotope-labeled water. Deuterium oxide given to the mother or infant has been previously used [4-6]. Conventionally, sample enrichments were measured with an isotope ratio mass spectrometer, which is expensive, time-consuming, and requires a specialist for operation and maintenance. Such equipment is difficult to set up in developing countries. Hence, a fast, easy, and less expensive method was developed which uses a Fourier transformed infrared spectrophotometer (FTIR) to determine deuterium sample enrichments [7-10]. This study tested the deuterium dilution technique in field conditions in developing countries and evaluated the constraints of using the FTIR to measure breastmilk output in lactating Senegalese women.

Subjects and methods

Eleven lactating Senegalese women and their infants took part in the study. The study was approved by the ethics committee of the University and informed consent was obtained from the subjects before starting the study. All the babies were full term and averaged 3.7 months old. The mean age of the mothers was 24 ± 4 years and parity between one and five. Anthropometric measurements (weight and height) were done at the beginning and the end of the study. A dose (30 g) of deuterium oxide (99.8% purity, Cambridge Isotope Laboratories Inc., Andover, Mass., USA) was orally administrated to the mothers and saliva samples were collected from both the babies and the mothers before (for the determination of the natural deuterium abun-

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dance) and after administration of the dose, on days 1, 2, 3, 4, 13, and 14 (post-dose samples). Saliva from the mothers (~ 5 ml) was collected directly into small sterile vials. To obtain saliva from the babies, foam mouth swabs were cut and rolled around the baby's mouth until saturated. The foam was then placed in a 5-ml syringe, and the saliva squeezed into sterile tubes. This process was repeated until a sample of about 3-ml was collected. The samples were centrifuged for five minutes at 11,500 g and the supernatant kept at –20°C until analysis.

Enrichment of the saliva samples was measured using a Fourier transformed infrared spectrophotometer (Shimadzu 8300, Vienna, Austria) equipped with an automatic sample shuttle and a pair of matched calcium fluoride sample cells with 0.1mm path length. Before saliva measurement, the calibration procedure involved preparation of D_2O calibrator by dilution of D_2O with deionized water. The enrichment of this calibrator was confirmed by the isotope ratio mass spectrometer. For analysis, the pre- and post-dose samples were simultaneously loaded into the instrument and automatically positioned in the light beam. This minimizes any interfering effects due to the absorption of atmospheric carbon dioxide in the sample chamber. The infrared spectra were measured in the range 2,300 to 2,800 cm^{-1}. The magnitude of the response obtained from the FTIR is deducted from the deuterium absorption curve by an algorithm developed by the Medical Research Council-Human Nutrition Research (MRC-HNR). Breastmilk volume was determined from the FTIR spectra using Microsoft Excel software for the kinetic analysis. A simulation, analysis and modeling computer program was used for compartmental analysis [11].

A two-compartment model described by Coward et al. [12] was used to generate best-fit estimates for maternal and infant water fluxes. Flow is taken as unidirectional between mother and baby (fig. 1).

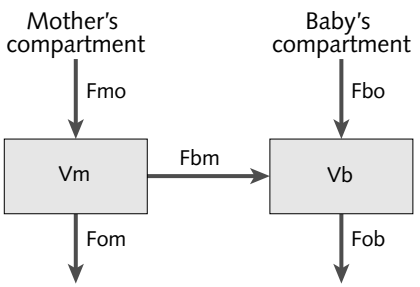

FIG.1. Two-compartment, steady-state model of water flows in a mother-baby pair. **F** indicates water flow. Subscript **m** refers to mother, **o** to outside and **b** to baby. **V** is the total body water. The combination of subscripts indicates the direction of fluxes (for example, Fmo indicates the flow to the mother from outside).

Results

The model predicts a monoexponential decay curve of deuterium in the mother's body water and a biexponential decay curve for deuterium appearance in the baby's saliva (fig. 2). The dotted points represent the experimental data of one mother-baby pair. The mean difference between the theoretical and the experimental data obtained from 11 pairs was 2 ppm and was less than the mean acceptable difference of 5 ppm.

Table 1 shows water flux indices including initial D_2 enrichment Cm (0), total body water (TBW, D_2 space), fractional rate constants (kmm, kbb, and kbm), and steady-state transports (Fom, Fmo, Fbm, Fob, Fbo) of one mother-baby pair.

Maternal total body water (TBW) was calculated from D_2 enrichment at time zero corrected by 1.04, which is the D_2 space. For the calculation of breastmilk output, TBW of the baby was derived from Friis-Hansen's formula [13]. Breastmilk volume was obtained as Fbm / 0.87, where 0.87 is the fraction of milk that is water, and Fbm the flow to the baby from the mother. The intakes of the baby other than milk (water and food supplements given to the baby) were estimated from Fbo.

The body composition of one mother is shown in table 1. The body composition was calculated from the TBW: TBW/0.73 for lean mass and body weight minus lean mass for body fat. The mean breastmilk intake and other metabolic water (food or water supplements) of the babies were 892 ± 108 g/day and 276 ± 185 ml/day, respectively.

Of the 11 lactating mothers in this study, only one was exclusively breastfeeding her baby: food or water supplement = 18 ml/day.

Discussion

Deuterium dilution is a non-invasive, simple, and safe method for measuring breastmilk output. The method

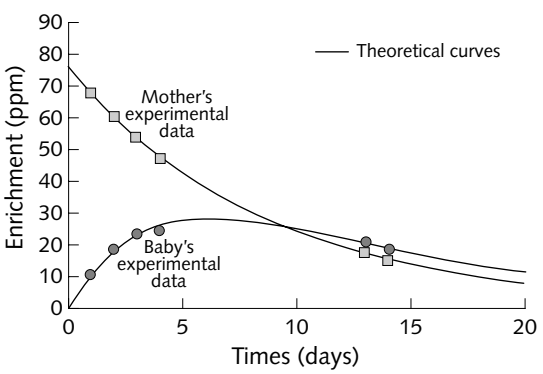

FIG. 2. Decay curves of D_2 enrichment

TABLE 1. Kinetic data baby intakes and body composition of one mother-baby pair

Kinetic data of one mother-baby pair	Baby intakes	Mother's body composition
Cm(0) = 762.91 ppm	Milk volume = 0.84 Kg/day^{-1}	Total body water = 34.07 Kg
Kmm = 0.12 day^{-1}	Fbo = 0.18 Kg/day^{-1}	Lean mass = 46.67 Kg
Kbb = 0.22 day^{-1}		Body fat = 14.59 Kg
Fbm = 0.73 Kg.day^{-1}		% of fat = 23.82
Kbm = 0.02 day^{-1}		

Cm(0), intercept of the curve for the disappearance of D from the mother's saliva.
Kmm, rate constant for irreversible loss of water for the mother.
Kbb, rate constant for irreversible transfer of water for the baby.
Fbm, water flux from the mother to the baby.
Kbm, rate constant for irreversible transfer of water from the mother to the baby.
Fbo, water flux from outside to the baby.

is especially useful for estimating the breastmilk contribution to nutrient intake in non-exclusively breastfed infants [12]. In many deuterium dilution studies, an isotope ratio mass spectrometer (IRMS) was used for deuterium analysis and isotope doses were determined on the basis of body weight [4–6, 12]. The expensive instrumentation and the need to measure each dose after weighing the mother have limited the widespread use of this method in the field, particularly in developing countries. IRMS requires a specialist for operation and maintenance, and is somewhat tedious because for isotopic measurements, the water is first transformed to hydrogen or equilibrated with hydrogen. Other methods have been proposed including infrared spectrophotometry (FTIR). For deuterium analysis, FTIR has been validated against IRMS and can be used to measure the enrichment of biological fluid samples as accurately as IRMS [7–10].

In this study a fixed isotope dose of 30 g was given to the mother. Conway et al. previously demonstrated that 30 g of deuterium oxide provide adequate enrichment in body fluids [10]. This dose is equivalent to 0.5 g/kg of body weight, as compared to the usual dose of 0.1 g/kg for IRMS analysis. The use of a larger deuterium dose did not pose any risk to the subjects [4, 14] and the cost is lower than that for the IRMS.

The results of breastmilk intake obtained in this study were comparable with those for well-nourished women from the industrialized countries and indicated that the breastmilk production of these Senegalese women was not impaired [15]. Despite a claim of exclusive breastfeeding by the mothers, the deuterium dilution method showed that only one baby was exclusively breastfed. In a recent national survey, 23.5% of the mothers stated that they were exclusively breastfeeding their infants [2]. The discrepancy between inquiries and measures makes the deuterium dilution method a good tool for assessing breastfeeding practices.

The deuterium dilution method using FTIR was easy to use, adequate for free living subjects, and presented few constraints except for the duration of the saliva sampling (14 days), which may cause some subjects to drop out. Mothers and babies accepted it without any resistance, the adequate quantity of saliva was easily obtained. The method was safe, simple, and accurate. The FTIR was fast and rapid but, for optimum spectrum quality, some environmental controls are required (temperature, humidity, dust, vibration, and smoke free atmosphere). In conclusion, the minimal time required for analysis and sample preparations for measurement of deuterium enrichment with FTIR make it suitable for community evaluation studies in developing countries.

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References

1. Cohen RJ, Brown KH, Canahuati J, Rivera LL, Dewey KG. Effects of age introduction of complementatry foods on infant breast-milk intake, total energy intake and growth: a randomised intervention study in Honduras. Lancet 1994;344(8918):288–93.
2. UNICEF. Objectifs de la fin décennie du sommet mondial sur l'enfance. Rapport de l'enquête par grappe à indicateurs multiples (MICS II). Dakar, Senegal: UNICEF, 2000.
3. Coward AW. Measuring milk intake in breast-fed babies. Mini symposium. J Pediatr Gastroent Nutr 1984;3:275–9.
4. Coward AW, Whitehead RG, Sawyer MB, Prentice AM, Evans J. New method for measuring milk intake in breast-fed babies. Lancet 1979;2:13–4.
5. Infante C, Lara W, Vio F. Isotope dilution measurement of breast-milk production in chilean urban mothers. Hum Nutr Clin Nutr 1985;39C:379–86.
6. Orr-Ewing AK, Heywood PF, Coward AW. Longitudinal measurements of breast milk output by a $^{2}H_{2}0$ tracer technique in rural Papua New Guinea women. Hum Nutr Clin Nutr 1986;40C:451–67.
7. Jennings G, Bluck LJC, Chowings C, Podesta D, Elia M. Evaluation of an infrared method for the determination of total body water in a clinical context. Clin Nutr 1995;(suppl 2):53–4.
8. Jennings G, Bluck L, Wright A, Elia M. The use of infra red spectrophotometry for measuring body water spaces. Clin Chem 1999;45:1077–81.
9. Fusch Ch, Spririg N, Moeller H. Fourier transformed infrared spectroscopy. Measures $^{1}H/^{2}H$ ratios of native water with a precision comparable to that of isotope ratio mass spectrometry. Eur J Chem Clin Biochem 1993; 31:639–44.
10. Conway JM, Sadijimin T, Dibley MJ, Kjolhede CL, Caballero B. Infrared spectroscopy assay for deuterium in infant's urine after D_2O administration to the mother: comparaison with isotope ratio mass spectrometry. Clin Res 1992; 40 (suppl 2):A625.
11. Berman M, Weiss MF. SAAM manual. Washington, D.C.: US Department of Health, Education and Welfare 1978: 78–180.
12. Coward AW, Cole TJ, Prentice AM. Breast-milk intake measurements in mixed-fed babies by administration of deuterium oxide to their mothers. Hum Nutr Clin Nutr 1982;36C:141–8.
13. Friis-Hansen B. Changes in body water compartments during growth. Acta Paediatr 1957;46(suppl):1–10.
14. Lukaski CL, Johnson EP. A simple, inexpensive method of determining total body water using tracer dose of D_2O and infrared absorption of biological fluids. Am J Clin Nutr 1985;41:363–70.
15. WHO. Quantité et qualité du lait maternel ; rapport d'un comité d'experts. Geneva: World Health Organization, 1987.

Maternal smoking effects on infant growth

María del Rocio Berlanga, Gabriela Salazar, Carola Garcia, and Jimmy Hernandez

Abstract

The influence of maternal smoking the nutrient content of breastmilk and impact on infant longitudinal growth rate is unknown. From birth, 23 smoking (S), (7.1 ± 4.4 cigarettes/day) and 23 non-smoking (NS) mother-infant pairs were followed. The breastmilk volume by deuterium dilution, zinc (Zn), copper (Cu), and iron (Fe) in breastmilk and hair by atomic absorption (AAS) and cotinine levels by radio-immuno-analysis (RIA) were evaluated. Birthweight was similar in contrast to height, and infants grew normally. Height and height-for-age (ZHA) were significantly lower in S infants and weight-for-height (ZWH) was higher in S infants in the third month, caused by slower height growth. Cotinine was 19 times greater in the S mothers and six times higher in their infants, as compared to NS group. Breastmilk volume was 743 ± 119 g/day (S) and 742 ± 111 g/day (NS), with no difference in zinc, copper, iron contents, except for cadmium (Cd). In infant's hair, all minerals were higher in the S group. Smoking affected infant's height during breastfeeding, attributed to an eventual impaired bioavailability of essential nutrients.

Key words: smoking, infant growth, breastfeeding

Scientific background of the project

The effects of smoking on infant growth and maternal health, as well as other effects of smoking on later morbidity have been previously studied [1]. Smoking was found to alter body composition, as well as lowering the level of prolactine at the end of pregnancy, which has implications for lactation [2]. A recent paper showed the effect of smoking on growth until the third month in a smaller sample from this same study [3], while another study showed the effect of passive smoking in infants of non-smoking mothers, but with other smoking residents in their house [4]. Using the precise dose-to-mother deuterium dilution methodology, mothers who smoked produced significantly less milk at one month, which correlated with a slower rate of birth [5] at that age. This study investigated the concern that smoking could not only affect birthweight, but also that it could inhibit height growth due to the presence of cadmium in tobacco, which could impair zinc bioavailability.

Subjects and methods

Subjects

Mothers from low socioeconomic status were contacted after delivery in a hospital in the South-East health region of Santiago (Sotero del Rio). The mothers were invited to participate after verifying their maternal normal nutritional status and their infant's birthweight (3,000–4,000 g), that they were not taking any medication or had other factors thay affect normal lactation, their intention to breastfeed for at least three months. The study was thoroughly explained to the mother and written acceptance was required. Smoking mothers were selected with similar characteristics and a minimum of five cigarettes daily during pregnancy and lactation. After a careful screening of smoker mothers and infants, a sample of 23 pairs of smoking mothers and their infants (S) plus 23 non-smoking mothers (NS) and their infants were selected.

Methods

Infants were measured in their homes monthly until they were three months old. (A SECA balance was used

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to weight the infants and height was measured with an infant stadiometer, Precisión Hispana, Santiago, Chile). The mother's weight was checked at one-month postpartum.

When the infant was one month old, breastmilk production was measured using the dose-to-infant deuterium dilution methodology. A dose of 0.2g/kg of 99.9% of deuterium oxide was administered to the infants, after collecting a basal urine sample of 3 ml. At one month of age, urine samples were collected on days 1 to 8 after dosing, by a cotton layer over the diaper and careful extraction of the urine. Mothers were well-trained to collect urine samples, but to assure the validity of the milk measurement, the field worker personally collected samples during the first two days and the final two days of the sampling protocol. Mothers were interviewed on their infants' additional fluid intake. Samples were frozen at –20°C until measurement at the Institue of Nutrition and Food Technology (INTA), in a Europa Scientific HYDRA continuous flow IRMS (Crewe, Manchester, UK).

Hair from both the mother and infant were collected carefully at the first month, from the underneath hair in the back of the head, and stored immediately in sealed plastic bags at room temperature. The mother collected milk from 6 to 8 feedings during one day, 2 ml before and after each feed on one breast; and samples were pooled in one tube to perform micronutrient analysis (copper, zinc, and iron) by AAS (atomic absorption spectrophotometry). The first part was done at INTA, and cadmium was measured at the Department of Nutrition, Faculty of Medicine, University of Chile.

Results

A description of the subjects (mother/infant) at birth is shown in table 1. The characteristics of the subjects were similar except for the levels of smoking and cotinine as well as the infant's birth height

The infant growth and cotinine levels at one month of age are shown in table 2. Weight was higher in non-smokers, but it was not significantly different. Height was significantly different from birth (table 1). Cotinine levels were statistically different at the first month, and increased with time in smokers, ratifying a tendency of

TABLE 1. Description of subjects

Variables	Smokers (N = 23)	Non smokers (N = 23)
Mothers		
Age (yr)	26 ± 5	25 ± 5
Weight (kg)	70 ± 5	70 ± 6
Height (cm)	156 (153–160)[a]	156 (149–160)[a]
Parity	2 ± 1	2 ± 1
Cotinine (ng/ml)	1,026 (2,170–3,207)*[a]	54 (34–75)*[a]
Cigarettes (n)	7.1 ± 4.4	0
Infants		
Birthweight	3,300 ± 338	3,372 ± 252
Birth height	49.5 ± 1.2*	50.0 ± 1.3*
Gestational age	34.1 ± 1	31.5 ± 1

*$p < .05$.
a. Median and quartile.

TABLE 2. Growth and cotinine levels in infants one month of age

Variables	Non smokers (N = 23)	Smokers (N = 23)
	Mean ± SD	
Infant, one month		
Weight (g)	4,277 ± 351	4,087 ± 310
Height (cm)	53.6 ± 1.3*	52.3 ± 1.2*
Cotinine (ng/ml)	12 (1–15)*[a]	75 (12–122)*[a]

* $p < .05$.
a. Median and quartile.

TABLE 3. Zinc, copper, and cadmium levels in infants' hair at one month

One month	Zinc (μg/g)	Copper (μg/g)	Cadmium (μg/g)
	Median and quartile		
Non-smokers (N = 23)	135.8 (123.7–181.3)*	20.4 (11.7–25.3)*	0.13 (0.0–0.30)*[a]
Smokers (N = 23)	145.1 (119.2–172.3)*	12.4 (9.8–17.7)*	0.05 (0.02–0.23)*[a]

*$p < .05$.
a. Median and quartile.

TABLE 4. Zinc, copper, and cadmium levels in mothers' hair

One month	Zinc (μg/g)	Copper (μg/g)	Cadmium (μg/g)
	Median and quartile		
Non smokers (N = 23)	178.7 (158.6–193.2)*	13.5 (11.3–17.7)	0.0 (0.0–0.01)*
Smokers (N = 23)	167.8 (152.4–181.8)*	14.7 (9.6–18.9)	0.0 (0.0–0.03)*

*$p < .05$.

mothers to smoke more, as lactation progressed.

Levels of zinc, copper, and cadmium in infant's hair are shown in table 3. There were significant differences in zinc, copper, and cadmium levels between smokers and non-smokers, being higher in hair of infants of smokers. The fact that infants of non-smoker's have cadmium in their hair may be linked eventually to passive smoking.

The maternal levels of zinc, copper, and cadmium are shown in table 4. Maternal copper concentrations did not differ, which agreed with the normal nutritional status of the mothers. Levels of cadmium and zinc in hair differed between the two groups; low levels could be also attributable to passive smoking.

The accumulation of cadmium in the infant at the first month is probably due to *in utero* contamination and direct and passive ways [6]; the effect of passive smoking in the infants of non-smokers is also noticeable. After weaning, infants from smoking mothers ceased to receive cadmium from maternal milk.

Milk production was determined in part of the sample (13 non-smokers and 12 smokers), and showed no significant differences between smokers and non-smokers.

In figure 1 the Z scores for weight-for-age (WA), height-for-age (HA), and weight-for-height (WH) are shown. Height-for-age was significantly greater in infants of non-smokers from birth to three months of age (fig. 1b). Weight-for-height becomes significantly different in infants of smokers at the third month, mainly due to the deficit in height growth.

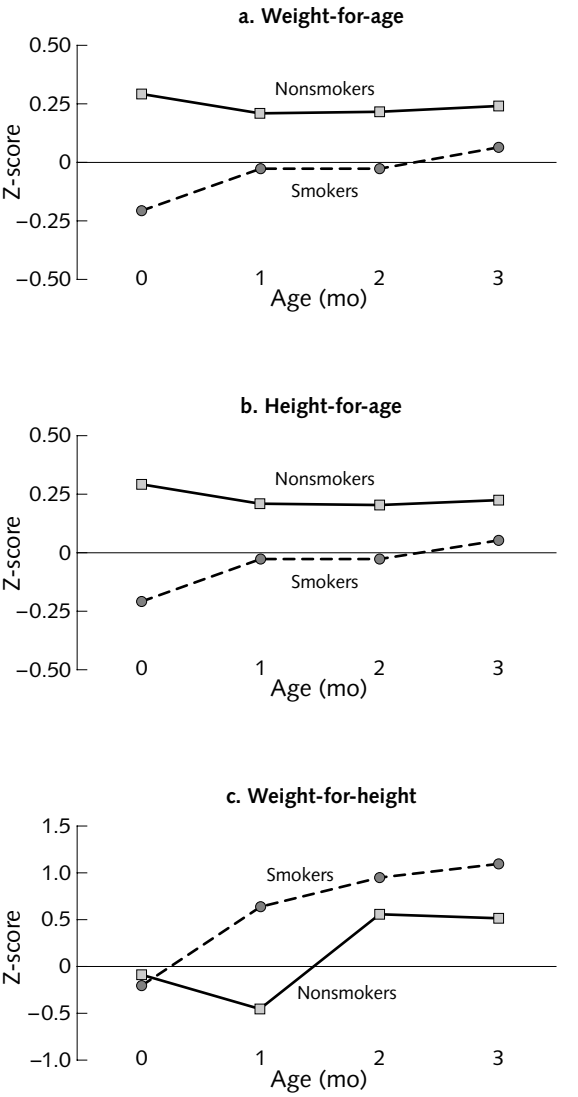

FIG.1. Z-scores for infants of smokers and non smokers

Discussion

Smoking affects the growth in the infant's height, due to the transfer of cadmium by tobacco smoke. Chronic contamination of cadmium is known to be caused by smoking or consumption of shellfish; although the placenta and breast present a barrier, cadmium has been shown to alter the cadmium:zinc and cadmium: copper ratios in fetal blood, as there is evidence that cadmium is preferably attached to α-lactalbumin or low molecular weight fractions in breastmilk [7, 8]. The most important interactions occur between cadmium and zinc and some results indicate disturbances of the metabolism of zinc, copper, and iron, especially when administered at low dose [6]. Accumulation of cadmium in the liver and kidney will also increase zinc levels in these organs due to binding to metallothionein. Our hypothesis is that this binding may alter the supply and bioavailability of zinc of infants of smokers during breastfeeding, altering growth in height significantly. Other studies have demonstrated widespread ossification retardation and marginal bone loss due to smoking in animal experiments and human adults [9, 10]. A possible interaction between calcium and cadmium may be a plausible cause. Both factors are linked to longitudinal growth during gestation and early growth.

Smoking has been shown to transiently affect growth in height preferentially during the period of breastfeeding. This may be due to eventual cadmium transfer by milk, produced by tobacco inhaled by the mother. In other studies cadmium altered zinc and copper metabolism, and zinc in particular, has an important role in longitudinal growth. The campaign carried out by the Ministry of Health should include the effects of the interference of toxic elements in infants' health and growth.

Future studies should look into the impact of cadmium in preferential binding of zinc by metallothionein, relating it to linear growth of infants of smokers' for longer periods. To further understand

these mechanisms, the placental transfer of micronutrient and toxic elements should also be studied, as well as other factors such as calcium availability, to assess the joint contribution from gestation and during lactation.

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References

1. Vio F, Salazar G, Yañez M, Pollastri A, Aguirre E, Albala C. Smoking effects on maternal body composition. Eur J Clin Nutr 1995;48:267–73.
2. Salazar G, Albala C, Yañez M, Seron-Ferre M, Vio F. Smoking effects on prolactin at the end of pregnancy. Nutr Res 1995;15:1599–1604.
3. Salazar G, Garcia C, Berlanga R, Ahumada M, Seron-Ferre M, Vio F. Maternal smoking effects on infant growth. Rev Med Chile 1998;126:1059–64.
4. Vio F, Salazar G. Passive smoking. Letter to the Editor. Rev Chil Pediatria 1997;68:139–40.
5. Vio F, Salazar G, Infante C. Smoking during pregnancy and lactation and its effects on breast milk volume. Am J Clin Nutr 1991;54:1011–6.
6. Goyer RA, Klaassen CD, Waalkes MP, eds. Metal Toxicology. San Diego, Calif, USA: Academic Press, Inc., 1995.
7. Kuhnert BR, Kuhnert PM, Debanne S, Williams MS.The relationship between cadmium, zinc and birth weight in pregnant women who smoke. Am J Obstet Gynecol 1987;157:1247–51.
8. Mata L, Perez MD, Puyol P, Calvo M. Distribution of added lead and cadmium in human and bovine milk. J Food Protec 1995; 59:46–50.
9. Nelson ED. Maternal passive smoking during pregnancy and fetal development toxicity. Part 1: growth morphological effects. Hum Exp Toxicol 1999;18: 252–6.
10. Lindquist LW, Carlsson GE. Jemt T. Association between marginal bone loss around osseointegrated mandibular implants and smoking habits: a 10 year follow up. J Dent Res 1997; 76:1667–74.

Body fat and cardiovascular risk factors in Indian men in three geographical locations

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Abstract

We studied cardiovascular risk factors in 149 rural, 142 slum dwellers, and 150 urban middle class Indian men (30 to 50 years, mean 40 years) in relation to their body fat. Mean body mass index (BMI) was 21.0, 22.3, and 24.3 kg/m^2 and mean body fat percent (bioimpedance) was 20.4, 22.5, and 30.4, respectively. A 75g oral glucose tolerance test showed no diabetes in rural subjects; 4% of urban slum dwellers and 10% of urban middle class men were diabetic. Hypertension (blood pressure ≥ 140/90 mm Hg) was present in 2% of the rural, 4% of the urban slum, and 10% of the urban middle class men. All cardiovascular risk factors were strongly related the percentage of body fat and waist to hip ratio. Two hour plasma glucose concentration and blood pressure were, in addition, independently related to geographical location (urban middle class were higher than slums who were higher than rural men). Our results suggest that urbanization increases the risk of hyperglycemia and hypertension independent of the percentage of body fat or its central distribution.

Key words: insulin resistance syndrome (IRS), coronary heart disease (CHD), body fat, oral glucose tolerance test, India

Introduction

India is experiencing an epidemic of type 2 diabetes, insulin resistance syndrome (IRS), and coronary heart disease (CHD) among its young adult and middle-aged population. It is projected that by the year 2020 India will have the highest number of diabetic patients anywhere in the world and that CHD will be the leading cause of premature death [1]. There is a striking excess in the prevalence of these conditions in urban compared to rural Indians. In a recent study of 38 year-old fathers of 8 year-old children in urban Pune, 8% were diabetic and 16% had impaired glucose tolerance (IGT) in the middle class men and 12% were diabetic and 18% IGT in slum dwellers. In another study in a nearby village (Pimpale Jagtap) only 4% of those over 40 years of age (mean 55 years) were diabetic and 4% had IGT [2, 3]. These findings suggest an increase in prevalence of insulin resistance syndrome in the near future with continuing rural to urban migration. The cause of this 'epidemic' is not clear but the major contributors to this are postulated to be the urban lifestyle as well as high genetic susceptibility of the population resulting in increased body fat percent and central adiposity, the main determinants of both diabetes and CHD.

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Research design and methods

We studied 150 men each (30 to 50 years of age) from three different geographical locations: rural, urban slums, and urban middle class who were selected by multi-stage random sampling.

Population sampling

The King Edward Memorial (KEM) Hospital and Research Centre is a tertiary care hospital with a rural outreach program in surrounding villages. We selected one geographical area in the vicinity of the Vadu Rural Health Centre. Of the six villages where we had conducted a community-based study of maternal nutrition and fetal growth, we selected randomly two villages. The villages were comprised of hamlets, which were listed, and five hamlets each were selected randomly from the two villages. A house-to-house survey was

done in these five hamlets to create a list of all men between the ages of 30 and 50 years from which the proposed number was selected.

The city of Pune is divided into 124 administrative wards. Four wards were selected randomly. Two wards were selected for the study of subjects living in the slums and two for studying middle class subjects. More than 1,000 houses were surveyed. A list of eligible men who were willing to participate in the study was created from which subjects were randomly selected.

Anthropometry

Height was measured using a stadiometer (CMS Instruments, London, UK), and weight using portable sohnell scales. Biceps, triceps, subscapular, and suprailiac skinfold thicknesses were measured on the left side of the body using Harpenden skinfold callipers (CMS Instruments). Head, mid-upper-arm circumference (MUAC), waist, and hip circumference were measured using a standard measuring tape. Fat mass was calculated from the sum of four skinfold thicknesses using Durnin's formula $((4.95/\text{density}) - 4.5) \times 100$, where density = $(1.1599 - (0.0717 \times \log_{10}$ sum of four skinfolds) [4]. The percentage of body fat was calculated using bioimpedance values and by the deuterated water method.

Laboratory methods

An 75 g oral glucose tolerance test (OGTT) [5] was done. Plasma glucose, cholesterol, triglycerides, and HDL-cholesterol were measured using standard enzymatic methods, and leptin concentrations using radioimmuno assay.

Statistical method

Data are represented by means and standard deviations unless or otherwise noted. Variables having skewed distributions have been log transformed to satisfy assumptions of normality. The comparisons across geographical locations have been made using ANOVA (analysis of variance).

Results and discussion

A study of the migration pattern across the geographical location revealed that 75% (table 1) of urban slum dwellers had migrated at least once in their lifetime as opposed to the rural and urban middle class who were more stable (40% and 31%, respectively).

Table 2 summarizes the lifestyles of the study population. Urban middle class men were more educated than their two counterparts while the percentage of those unemployed was similar in rural, urban slums, and urban middle class populations (3.1, 2.9, and 3.3, respectively). Alcohol consumption and smoking was higher in the urban slums (50.7 and 38.7, respectively).

The mean ages were 38 ± 5.9, 38 ± 5.9, and 41 ± 5.9 in rural, urban slum, and urban middle class men, respectively. The urban middle class men had the highest BMI (24.3 ± 3.9) followed by urban slum (22.3 ± 4.1) and rural (21.0 ± 2.8) men. The urban middle class men had greater subscapular and triceps skinfold thicknesses than the urban slum and rural populations (22.5 and 13.1, 15.9 and 9.0, 12.4, and 7.9, respectively)

Table 3 shows the anthropometric characteristics of the study groups. The urban middle class men had a higher percentage of body fat than those in the urban slums, while the rural men had the lowest percentage

TABLE 1. Migration history

	Rural (N = 149)	Urban Slums (N = 142)	Urban (N = 150)
Born and studied in the same geographical location (%)	75.8	42.2	76.0
Migrated at least once (%)	40.3	75.3	31.3
Years in the place of study	31	25	34

TABLE 2. Lifestyle

	Rural (N =149)	Urban Slums (N = 142)	Urban (N = 150)
Addicting habits (current)			
Smoking (%)	23.5	38.7	26.2
Tobacco chewing (%)	62.4	60.6	28.9
Mishri[a] (%)	55.7	26.1	12.1
Alcohol (%)	19.5	50.7	42.3
Education			
Mean years of education	7	7	14
No schooling (%)	23.1	18.9	0.6
Primary school (%)	34.7	34.5	2.7
Middle school (%)	12.6	23.7	17.3
Secondary school (%)	25.3	20.1	35.3
Intermediate (%)	—	0.7	4.7
Graduate (%)	3.2	0.7	26.7
Postgraduate (%)	1.1	1.4	12.7
Employment			
Unemployed (%)	3.1	2.9	3.3
Skilled (%)	9.5	12.9	28.0
Semi-skilled (%)	7.4	33.1	28.7
Unskilled (%)	80.0	51.1	40.0

a. Mishri is burned tobacco applied to teeth.

of fat. This holds true for the waist to hip ratio (WHR) as well as subscapular to triceps ratio (SSTR) (table 4). The total percentage of body fat was calculated by three different methods, and there was a good correlation between all of them (table 5).

Urban middle class men had higher glucose, cholesterol, triglyceride, and leptin concentrations than the other groups. There was no significant difference in the blood pressure between the three groups (table 6). Many of the insulin resistance syndrome outcome variables were higher in the urban middle class population and least in the rural population (table 7).

TABLE 3. Anthropometric characteristics

	Rural (N = 149)	Urban Slums (N = 142)	Urban (N = 150)
Age (yr)	38 (5.9)	38 (5.9)	41 (5.9)
Height (cm)	165.0 (5.6)	163.4 (6.5)	166.2 (6.8)
Weight (kg)	57.4 (8.4)	59.8 (12.5)	67.3 (12.1)
BMI (kg/m^2)	21.0 (2.8)	22.3 (4.1)	24.3 (3.9)
Head circumference (cm)	54.1 (2.2)	54.6 (1.7)	55.6 (1.6)
Mid arm circumference (cm)	26.3 (2.4)	27.4 (3.5)	28.9 (3.2)
Waist circumference (cm)	79.4 (9.1)	83.7 (14.1)	90.4 (10.2)
Hip circumference (cm)	88.1 (5.6)	90.9 (7.9)	96.0 (7.6)
Biceps (mm)[a]	4.1 (2.1–15.4)	5.1 (2.0–18.1)	6.9 (2.2–24.1)
Triceps (mm)[a]	7.9 (3.2-26.5)	9.0 (3.4-31.6)	13.1 (3.3-40.0)
Subscapular (mm)[a]	12.4 (5.1–40.0)	15.9 (4.8–48.6)	22.5 (8.0–46.4)
Suprailiac (mm)	15.8 (9.7)	18.7 (10.8)	26.6 (9.5)

Mean (SD)
a. Geometric mean, range.

TABLE 4. Body fat and its distribution

	Rural	Urban Slums	Urban
% Body fat			
Anthropometry	19.6 (5.8)	21.8 (6.6)	27.4 (5.3)
D2O	19.9 (6.2)	21.6 (6.9)	27.2 (7.1)
Bioimpedance	20.4 (10.2)	22.5 (10.9)	30.4 (8.1)
Weight:hip ratio	0.89 (0.06)	0.92 (0.09)	0.94 (0.09)
Subscapular:triceps ratio[a]	1.5 (0.6–3.7)	1.8 (0.9–3.2)	1.7 (0.6–4.2)
Leptin (ng/ml)[a]	1.8 (0.2–22.0)	3.9 (0.1–42.0)	7.6 (0.5–50.0)

Mean (SD).
a. Geometric mean, range.

TABLE 5. Spearman correlations for body fat and leptin

	% Body fat			
	Anthropometry	D_2O	Bioimpedance	Leptin
% Body fat				
Anthropometry	1	0.80**	0.84**	0.72**
D_2O	0.80**	1	0.81**	0.64**
Bioimpedance	0.84**	0.81**	1	0.70**
Leptin (ng/ml)*	0.72**	0.64**	0.70**	1

* $p < .05$, ** $p < .01$, *** $p < .001$.

TABLE 6. Insulin resistance syndrome

Outcome (%)	Rural (N = 149)	Urban slums (N = 142)	Urban (N = 150)
Impaired glucose tolerance	9	12	20
Diabetes mellitus	0	4	10
Hypertension (≥ 140/90 mmHg)	2	4	10
Cholesterol			
Total ≥ 200 mg	3.4	8.7	14.8
HDL ≤ 35 mg	44.6	48.4	52.1
Triglycerides ≥ 150 mg	6.1	19.8	26.8

Conclusion

The percentage of body fat and central adiposity significantly increased from rural to the urban middle class men through the urban slum dwellers. An increased percentage of body fat predicted an increased insulin resistance and other cardiovascular risk factors. Central adiposity (waist:hip ratio and subscapular:triceps ratio) made a significant but relatively smaller contribution to this risk compared to the percentage of body fat.

Our results suggest that urbanization increases the percentage of body fat and central adiposity. Together these factors make a major contribution to the rise in the prevalence of insulin resistance syndrome. Body fat and its distribution do not account for all the geographic differences. Other environmental factors may also contribute to the rising prevalence of insulin resistance syndrome in India.

TABLE 7. Insulin resistance syndrome variables

	Rural (N = 149)	Urban Slums (N = 142)	Urban (N = 150)
Glucose (mg%)			
Fasting	91 (10.7)	94 (14.4)	99 (25.8)
30 minutes	152 (29.1)	156 (34.3)	163 (43.7)
120 minutes	102 (25.3)	117 (40.5)	136 (58.7)
Cholesterol (mg%)			
Total	148 (25.6)	153 (31.1)	164 (34.0)
HDL	38 (9.6)	39 (12.1)	36 (10.1)
Triglycerides[a]	82 (31–319)	95 (38–680)	108 (26–940)
Blood Pressure (mmHg)			
Systolic	113 (9.6)	115 (11.3)	118 (14.3)
Diastolic	66 (7.9)	70 (8.5)	74 (10.0)
Leptin (ng/ml)[a]	1.8 (0.2–22.0)	3.9 (0.1–42.0)	7.6 (0.5–50.0)

Mean (SD).
a. Geometric mean, range.
All blood measurements were on plasma samples.

TABLE 8. ANOVA (independent variable = geographical location)

	2 hour glucose	Systolic blood pressure	Cholesterol	Triglycerides
Location	9.1**	3.86 ns	4.7 ns	5.1 ns
Age (yr)	1.2 ns	0.08*	0.3 ns	0.2 ns
Body fat (%)	6.4***	7.70***	11.5***	13.5 ***
R^2 (%)	16.7	12.64	16.5	19.6
Location	9.1**	3.86 ns	4.7 ns	5.1 ns
Age (yr)	1.2 ns	0.01*	0.4 ns	0.2*
Body fat (%)	6.4***	8.77***	11.4***	13.9***
WHR	1.3*	0.56 ns	0.3 ns	2.2**
R^2 (%)	18.0	13.2	16.8	21.4
Location	9.1*	3.86 ns	4.7 ns	5.1 ns
Age(yr)	1.2 ns	0.01*	0.4 ns	0.2 ns
Body fat (%)	6.4***	8.77***	11.4***	13.9***
SSTR	1.9**	0.06 ns	0.0 ns	1.3**
R^2 (%)	18.5	12.7	16.5	20.5

Figures in table indicate percent contribution to R^2 values.
* $p < .0$, ** $p < .01$, *** $p < .001$, ns, not significant, SSTR, subscapular: tricepts ratio

References

1. Ramchandran A, Snehalatha C, Kapur A, Vijay V, Mohan V, Das AK, Rao PV, Yajnik CS, Prasanna Kumar KN, Nair JD. High prevalence of diabetes and impaired glucose tolerance in India. National urban diabetes survey. Diabetologia 2001;9:1094–1101.
2. Yajnik CS, Joglekar CV, Bavdekar A, Bhave SA, Pandit AN, Fall CHD. Parental risk of heavy birth weight child, 8 years after delivery. Paediatr Res 2001;50 (suppl):4A
3. Joglekar AA, Rolls S, Hirve S, Shelgikar KM, Joglekar CV, Yajnik CS. Glucose tolerance in the elderly (> 40 years) in rural India. Diabetologia 1997;40:suppl 1:A190
4. Durnin JVGA, Womersley J. Body fat assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 years. Br J Nutr 1974;32:77–97.
5. World Health Organization. Diabetes mellitus. Report of a WHO study group. Technical report series N.727. Geneva: WHO, 1985.

Isotopic tools for strengthening health and nutritional monitoring and their current applications in developing regions of the world

Mauro E. Valencia and Venkatesh Iyengar

Abstract

Nuclear and isotope methods in one form or another are regarded as essential tools for carrying out nutrition research besides enhancing sensitivity of nutrition monitoring techniques. They have been used extensively in industrialized countries to analyze human energy requirements, body composition including bone mineral density, to determine food composition, and to study metabolism of important nutrients such as protein, fat, vitamins, and minerals. The information acquired has led to many improvements in nutrition and health. Importantly, the developmental needs of countries in economic transition are being increasingly identified and resolved through field applications of isotopic tools, thus strengthening health and nutrition monitoring. Currently, several strategic applications of isotopic techniques are being introduced in developing countries where they can benefit millions through monitoring improvement in nutritional status, and serve as specific indicators of broader social and economic advances. These examples are highlighted in this report.

Key words: nutrition, health, stable isotopes, radioactive isotopes, developing regions

Introduction

Nuclear techniques have been used extensively to study animal and nutrient metabolism [1]. These techniques have used both radioactive and stable isotopes. They can be detected easily in physiological fluids; however, the use of radioisotopes can be a health concern because of radiation exposure or restricted on the basis of certain types of groups like pregnant women or infants. Also, radioisotopes can decay very rapidly and are of limited use in human studies.

On the other hand, extensive applications of stable isotopes in nutrition research have been documented [2, 3]. Stable isotopes can be used safely even in infants and pregnant women. They are innocuous in the amounts used, do not decay, can be handled with relative ease under laboratory or field conditions, and can be stored for long periods. However, they can be expensive and require high-technology analytical facilities, instrumentation, trained scientists, and technicians. Until recently, the use of nuclear techniques in human health in developing regions was used mainly for clinical diagnosis like radioimmuno assay (RIA) and very few applications of isotopic tracers were used to quantify metabolic events *in vivo*.

Stable isotope methodology

Human life is based mainly on a limited number of elements: hydrogen (H), carbon (C), nitrogen (N), and oxygen (O). These elements exist in nature in two or more forms that differ only in the number of neutrons in their nucleus. The major isotope (hydrogen-1, carbon-12, nitrogen-14, or oxygen-18) is accompanied by a constant proportion of minor heavier isotopes whose individual abundances range from 0.02% to 1.11%. An inventory of the human body shows that a 50 kg individual has an aggregate of 225 g of hydrogen-2, carbon-13, nitrogen-15, oxygen-17, and oxygen-18. Although there are variations in the proportions of ^{1}H to ^{2}H, ^{13}C to ^{12}C, ^{14}N to ^{15}N, and ^{16}O to ^{18}O, each has a characteristic baseline abundance to which tracer measurements are referred. The enriched form (e.g., $^{2}H_2O$ or $H_2{}^{18}O$) may be used directly, $^{13}CO_2$ may be incorporated into plants by biosynthetic procedures, or the isotope may be transformed through organic syntheses into labeled fats, carbohydrates, or amino acids.

Stable isotopes emit no externally measurable radiation and their presence in excess of natural levels is

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detectable only by changes in the ratio of minor isotope to major isotope. Such ratio is measured by an isotope ratio mass spectrometer in which heavy and light forms of the same molecule undergo separation and quantification. A purified sample of hydrogen gas, carbon dioxide, or nitrogen is admitted through a highly-restricted opening into an ion source under vacuum. The gas molecules are bombarded by a stream of electrons, whereby they acquire a positive charge and are accelerated into a magnetic field. Here the ionized gas molecules become segregated according to mass and strike individual collector plates. So ions generate currents that are proportional to their numbers and enable their quantification. Other assessing-methods have been developed recently (emission spectrometer and infrared absorption measurement).

These stable isotopes can be administered orally and the metabolic products into which they enter (e.g., body water, respiratory carbon dioxide, urea) can be detected in breath, saliva, milk, urine, and stool. The measurement of factors like breastmilk intake, energy expenditure, micronutrient status, macronutrient-utilization, body composition, and many more is important in assessing the nutritional status of infants, children, pregnant women, and nursing mothers, as well as that of individuals who subsist on marginal food supplies.

Another important use for stable isotopes is the assessment of trace element bioavailability and pools sizes, such as iron (^{57}Fe & ^{58}Fe) and zinc (^{67}Zn & ^{70}Zn). The uptake of these labeled micronutrients can be traced *in vivo*, which has been widely used for measuring the effectiveness of supplementation or fortification trials in several developing countries.

^{13}C-urea breath tests are used to examine bacterial colonization by *Helicobacter pylori.* The test measures the production rate of $^{13}CO_2$ in expired air, followed by oral ingestion of ^{13}C-labelled urea. Breath tests for *Helicobacter pylori* using stable isotopes are reliable and non-invasive tools that can be safely applied to children from developing areas where high rates of infection and malnutrition are observed.

Isotope dilution methods are used to assess vitamin A status. The principle relies on labeled carotenoid conversions to vitamin A, which can be traced with ^{13}C carotenoids. Vitamin A pool sizes are measured by the dilution of an oral ingested tracer into the different body pools. This technique has potential applications in measuring the effectiveness of vitamin A and carotenoid supplementation and fortification regimes in nutrition studies.

The role of nuclear and isotopic techniques in nutrition research

The International Atomic Energy Agency (IAEA) has played an important role in promoting the safe use of peaceful nuclear technology in many areas. One of its program areas is human nutrition and health-related studies. The IAEA's objective is to promote the use of nuclear techniques for development purposes. Nutrition is a top priority within the health sector, particularly in poorer countries, and a global cooperation priority. IAEA activities in human health as well as in technical cooperation, include a new emphasis on isotope techniques as tools to evaluate human nutritional status and the nutritional quality of foods within the context of national development programs. These techniques are now considered the best methods for measuring the up-take and bioavailability of many important nutrients. They are thus well-suited for determining the success of food supplementation programs and other interventions aimed at combating the many forms of malnutrition.

The IAEA's activities in nutritional evaluations were initiated to apply isotope techniques for assessments of human body composition, nutrient intake, and vitamin and mineral availability in developing countries. The application of stable and radioactive isotopes can have a major impact on socioeconomic development by providing added value to the evaluation of the interventions in shorter periods and by providing biological evidence that can result in improvement of the nutrition intervention program. This impact is in terms of both cost-savings and effectiveness.

IAEA human nutrition studies based on stable isotope methodology

Estimation of total energy expenditure

Caloric expenditure varies individually and is influenced by different biorhythm. Therefore, a field assessment is necessary. When doubly-labeled water ($^2H_2{}^{18}O$) is administered to a subject, both isotopes mix with body water and are eliminated in body fluids over a period of days. The turnover of body water can be estimated from the daily measurements of H-2 concentration in urine or saliva samples. When the samples are analyzed for O-18, the values will reflect a more rapid excretion rate than that for H-2 (deuterium) because the O-18 is also incorporated into exhaled carbon dioxide. The difference in excretion rates between O-18 and H-2 tracers thus reflects the volume of carbon dioxide produced over the period of observation. This parameter can be used to calculate the total energy expenditure of a subject.

Determination of lean body mass

A tracer dose of water labeled with H-2 or O-18 is administered and allowed to equilibrate for four to six

hours. The isotope concentration in saliva or urine will reflect the dilution undergone by the isotope. When the lean body mass is calculated, the difference in body weight is the amount of adipose (fatty) tissue.

A simple measure of overall nitrogen-flux

The nitrogen-flux balance stumbles in periods of stress, then the catabolic processes predominate over synthetic processes and a negative balance is the result. Whole body protein turnover is measured by administration of a single oral dose of an amino acid, or preferably a protein, labeled with N-15 [e.g., yeast grown in medium containing $(^{15}NH_4)SO_4$]. Urine is collected for 9 to 12 hours and the amounts of tracer nitrogen in urinary NH_3 and in the urea are determined. These two values provide a reliable estimation of whole-body protein turnover that is insensitive to changes in non-protein-nitrogen metabolism.

Nutrient absorption and utilization after diarrhea

Heliobacter pylori infection is likely to be the most common worldwide bacterial infection. It is estimated that approximately 50% of the general population is affected. The WHO has classified *H. pylori* as a Group 1 carcinogen.

Young children in developing countries that are the main targets of infection, with a substantial risk of developing gastric carcinoma during adulthood. High infection rates of *H. pylori* among newborns and young children in developing nations appear to be a major cause of chronic undernutrition and diarrhea syndrome with failure to thrive. The bacteria can survive in the acidic interior of the human stomach due to its capacity to secrete an enzyme called urease, which decomposes the urea contained in the stomach interior into ammonia and carbon dioxide increasing the pH underneath the protective mucous membrane in the stomach where it is protected from the caustic stomach acid. This transitory drop in stomach acidity explained by diminished gastric secretion and an increase in ammonia production during infection, promotes the transit of low bowel pathogens leading to repeated gastrointestinal infections, causing diarrhea and adverse consequences on nutrition and growth.

Weaning infants often have periods of infection leading to diarrhea. During this period nutrient intake is not sufficient to maintain growth and the incidence of infection can be diagnosed by labeled C-13 compounds. For example, when rice labeled with C-13 (exposed to $^{13}CO_2$, during periods of photosynthesis) is cooked (rice water for rehydration) and fed, digestion and absorption of the starch can be detected from the appearance of $^{13}CO_2$ in breath samples. The degree of malabsorption can be estimated from the recovery of tracer carbon in the total stool carbon.

Other applications

In *in vitro* studies to assess dialyzability the radioisotope ^{59}Fe is applied as a marker, this tracer along with ^{55}Fe and ^{65}Zn used for *in vivo* studies of micronutrient uptake and bioavailability. The dual energy x-ray absorptiomentry (DEXA) is widely used in laboratories to measure bone mineral density and body composition. For determining the trace element content in foods and human tissue a broad range of other nuclear analytical techniques are applied.

Developmental need of countries in economic transition

The application of isotope techniques in human nutrition addresses the need to ensure that food interventions are undertaken in an optimal way, using reliable biological indicators. Such interventions would normally comprise food supplementation, fortification, or dietary modification. It should be noted that, in many middle-income countries, the emphasis of national campaigns is currently moving from malnutrition to healthy aging and preventing obesity, coronary heart disease, and type 2 diabetes. The end users of the monitoring and evaluation of intervention programs would usually be public health agencies undertaking such interventions.

The Latin American context

Thanks to the existence of a strong regional research center in Chile, and a strong network of institutions in Latin America, an advisory panel to the IAEA, in a technical cooperation thematic planning meeting, strongly recommended that a regional program include Brazil, Chile, Cuba, Mexico, and Argentina. In Latin America, there was no established institutional network capable of evaluating nutritional interventions through nuclear techniques. On the other hand the region included several countries with large-scale intervention programs. Due to similar conditions it was recommended that the IAEA should investigate the possibility for similar activities in Asia.

In 1998 the IAEA together with the Institute for Nutrition and Food Technology (INTA) organized a planning meeting for a Latin American regional project at INTA in Santiago, Chile. This started the project RLA/7/008 on the use of stable isotopes to evaluate nutrition intervention programs in Latin American. In

the first meeting, scientists from the region and some officials in the health and nutrition sectors discussed the different nutrition intervention programs in their countries. Within this framework, preliminary proposals were formulated with the aim of having a Latin American regional project in which nuclear techniques could be applied to complement on-going evaluations of national programs in infants, preschool children, and pregnant and lactating women. The diversity of actions in the region is large, ranging from national supplementary feeding programs, distribution of milk, rice, and meals in day care centers, food assistance for malnourished children, programs for health education and nutrition for families in extreme poverty to problems related to energy expenditure, obesity, and physical activity.

Regional training course

Under technical cooperation actions a regional training course took place in Lima, Peru in 1996. The organization was in collaboration with the local nuclear authority and de Centro de Investigacion Nutricional. This course included Peru, Chile, Brazil, Argentina, Bolivia, Ecuador, Venezuela, Panama, Costa Rica, Cuba, and Mexico.

The objectives of the regional training course were to review human nutrition topics in the region related to the application of isotopic techniques for nutrition intervention and monitoring, to teach the ethical and practical principles of isotopic methods; and to teach the advantages of nuclear techniques in evaluating the impact of programs.

The course provided scientific and technical information regarding the uses and advantages of nuclear technology with both theoretical and practical demonstrations by international experts from industrialized countries and from experts in the region. However, the main success of the course was to initiate a network of research centers and scientists working in nutrition problems interested in using nuclear technology for improving evaluation of nutrition problems in their countries.

Further, in 1997 in Guatemala during the international congress of SLAN (Latin American Society of Nutrition), several meetings and symposia were held on the application of stable isotopes in nutrition studies in Latin America. A definite interest was seen in many of the participants countries who were invited to be part of this network for future actions and studies.

Technical and programmatic solutions

Stable and radioactive isotopes have allowed detailed evaluations of nutrient intake (breastmilk), micronutrient status, body composition, energy expenditure, and bioavailability of nutrients in food supplements and fortified foods. Nuclear techniques do not solve nutrition problems but they can provide important information for decision and policy makers and give added value to intervention programs.

The majority of the work is done through a unique IAEA mechanism called the coordinated research projects (CRP) which focus on method development and the introduction of isotopic techniques to developing countries. The resulting information and technological progress is transferred to improve human health through another mechanism called technical cooperation projects (TCP).

Coordinated research projects (CRP)

The coordinated research projects (CRP) in human nutrition started in the 1970s. Thematic areas were the development of the $^{55}Fe/^{59}Fe$ method for measurement of non-heme absorption in collaboration with the WHO, development of RIA for serum ferritin, and studies of trace elements in cardiovascular disease. In the 1980s, new CRPs began in the areas of trace elements in human milk and dietary intakes of trace elements and energy expenditure with the doubly-labeled water method. CRPs have concentrated on method development and the introduction of nuclear techniques to developing countries. CRPs have also been important in improving networking. In developing countries sharing common nutrition and health problems, CRPs have facilitated their work on their own problems as well as providing experience in the use of isotopic techniques. At the same time, researchers have been able to address and answer relevant scientific questions that were not possible with conventional methods used in nutrition research. In the beginning there were big (the industrialized country) to small (the developing country) partner type relationships. In these cases the big partner provided the analytical facilities and the field work was done by the small partner. However, at present many developing countries now have their own analytical facilities or are in the process of getting them.

The CRP mechanism is basically reflected in applied research to be carried out in a three to five year period with 10 to 20 country participants. A modest annual funding per institute (only the developing countries) is made available by the IAEA mainly for sample collection and analysis, minor equipment and supplies, temporary staff, and research coordination meetings (RCM). The RCMs are very important in that the different country protocols are discussed in detail. Investigators in the field from the industrialized and developing regions interact by contributing suggestions to improve the projects. Given the common thematic component, harmonized analytical information can

be obtained for different regions and countries using nuclear technology. The worldwide CRP mapping for research contract holders is shown in figure 1. A new type of CRP has been introduced recently that includes funds for supporting doctoral students under the concept of capacity building CRPs.

Technical cooperation projects (TCP)

There are various technical cooperation project (TCP) in which the IAEA assists developing country member states in solving technical problems and providing, at the same time, the necessary know-how. Actions include training courses, which are usually for two to three weeks, and can be attended by participants from developing countries. Fellowships provide technical training for people working in the subject area in which further training is requested. These actions can bring about human resource development for regional participants, improvement of the efficiency of nutrition program delivery, and evaluation and help to resolve challenges with respect to malnutrition and health of the population at large.

The purpose of TCPs is to contribute to socioeconomic development. They are programmed for two to four years, and include one to five participants. The annual funding per institute is approximately US$20,000.00 to $100,000. These funds can be used for training, expert visits, workshops, subcontracts, equipment, and supplies.

The implementation strategy for TCPs has to consider several criteria for approval. There has to be a nutrition intervention in need of evaluation. There must be direct links between technical cooperation counterparts and the public health agencies that can absorb the recommendations resulting from the research and use them to modify interventions if needed. One of the advantages of nuclear techniques over conventional methodology is the response time in the impact evaluation and the number of subjects required. Thus, the use of nuclear techniques can increase the efficiency of the evaluation of such programs. In nutrition interventions a multidisciplinary approach is required; a partnership with social authorities is important for a successful evaluation of the program.

Existing networks, involving technical cooperation counterpart institutes and public health agencies involved in the interventions are added advantages. Such a network exists in Latin America and another one is developing in Asia. TCPs should have a high probability of public acceptance, and simultaneously have an impact on development of member states through the use of nuclear technology. Finally, if the intervention is carried out through a multilateral or bilateral program, it should be established that sufficient Government ownership and commitment exists to ensure successful implementation and sustainability. Figure 2 show the worldwide map with technical cooperation contract holders.

Field applications of isotopic tools for strengthening health and nutrition monitoring

The IAEA through coordinated research projects and technical cooperation projects in the areas of health, nutrition, and environment are eminently positioned to provide the technical underpinnings to international efforts for improving the quality of life [4, 5]. To date,

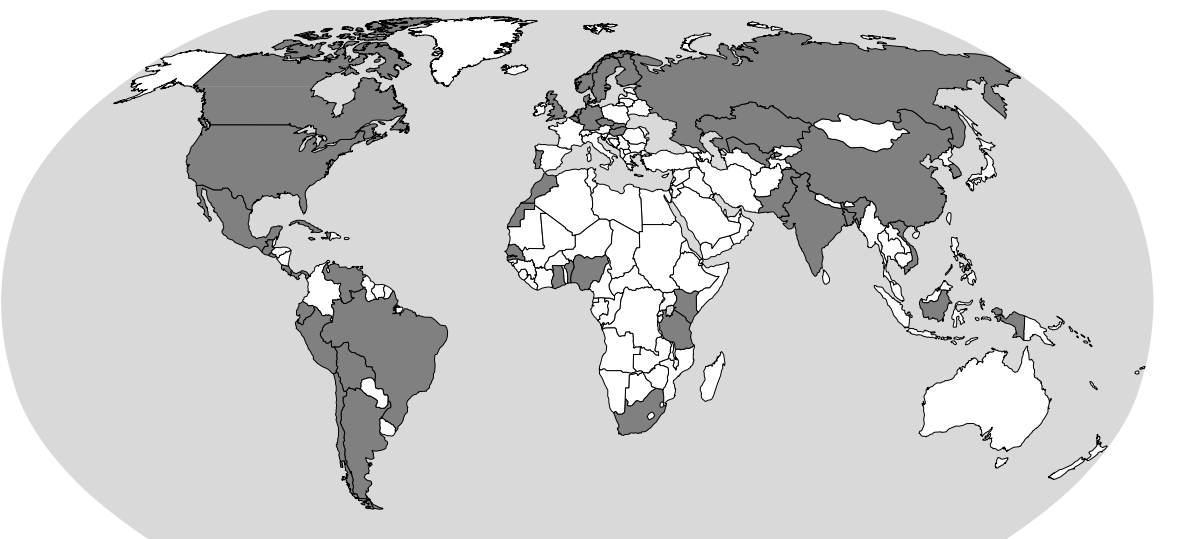

FIG. 1. Countries currently participating in CRPs on nutrition and environmental studies

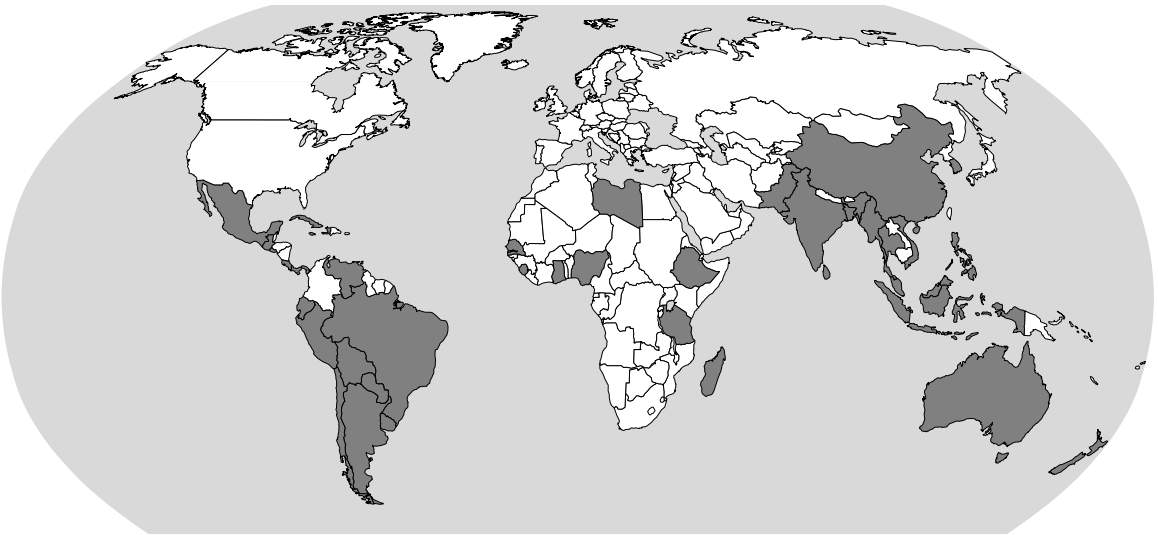

FIG. 2. Countries currently participating in TCPs on nutrition and environmental studies

isotopic strategies evolved through IAEA efforts to measure energy metabolism, resistance to insulin, rate of synthesis of fat, changes in protein synthesis, lactation performance, bone mineral density, food composition, efficacy of nutrient fortification, nutrient utilization, and prevalence of infection are implemented in more than 50 of its member states. A few examples are cited below.

Latin America and Asia

The doubly-labeled water method ($^{2}H_{2}{}^{18}O$) is the only technique that can accurately determine the energy needs of people in their own environment. The results of investigations on energy expenditure of young children and adults in Cuba and Chile and Mexico based on doubly labeled water are being used by the FAO/WHO/UNU expert committee convened during 2001 to establish new energy recommendations. Prior to this regional project, data on energy expenditures were based on surveys in developed countries.

Methods based on isotope dilution using ^{2}H or ^{18}O are widely accepted for monitoring body composition especially in the context of onset of obesity. Over 2,000 subjects from Brazil, Chile, China, Cuba, India, Jamaica, Mexico, New Zealand, and Nigeria have been investigated to identify changes leading to obesity to formulate preventive care.

As a result of the existence of a strong regional research center in Chile, and a strong network of institutions in Latin America, an IAEA regional program (known as the Regional Latin America RLA/7/008) that includes Brazil, Chile, Cuba, Mexico, and Argentina using isotopes for evaluating nutrition intervention programs is being implemented. After three years, a measurement network in support of nutrition metrology has been established. Two major laboratories are operating in Chile and Mexico with internationally recognized expertise in applying stable isotope mass spectrometry for nutrition research. Other laboratories in the region are working with alternative techniques such as infrared spectroscopy (IRS and FTIR) for body composition and lactation studies, dual x-ray absorptiometry (DEXA) and RIA for determination of hormones and nutrients.

National nutrition interventions based on stable isotope technology have been introduced in Chile and Mexico. In Chile, 300 children participated in a study (designed to cover 1.3 million children) in a national nutritional intervention program (National Complementary Feeding Program). As a result, anemia was reduced from 30% to less than 5% after a year of providing iron-supplemented weaning foods, leading to increased use of foods fortified in iron and zinc. This is expected to impact on educational performance and decreased infections. Mexico is exploring the use of stable isotope technology in a national program (PROGRESA) to assess the effect of food supplements to a large number of pregnant and lactating mothers and preschool children currently being supplied with 20% of their energy requirements and 100% of iron, zinc, and vitamins A, C, E, B_2, $B_{12,}$ and folic acid. The effects on energy expenditure, physical activity, body composition, and breastmilk intake are being monitored by the doubly-labeled water technique.

Similarly, the first phase of the project in the Regional East Asia and Pacific (RAS/7/010 in China, Indonesia, Malaysia, Pakistan, Philippines, Thailand, and Vietnam), in which stable isotopic techniques were used to assess zinc and iron bioavailability to measure the effectiveness of multinutrient supplementation has

been successfully completed. For example, in Indonesia it is estimated that 35% of schoolchildren are underweight and 50% are micronutrient-deficient including iron and zinc. Technical and scientific input by IAEA to the national food fortification program has addressed the problem of iron and zinc through the wheat flour fortification initiative, which will benefit both children and adults.

Other CRPs addressing global health challenges

Persistent diarrhea accounts for over 60% of infant diarrheal deaths in Brazil, 47% in India, 36% in Senegal, and 26% in Bangladesh. Stable isotope techniques are the best and most cost effective modes of diagnosis of *Helicobacter pylori* (Hp) infection. A number of countries in Africa, Asia, and Latin America have joined a CRP on Hp infection and malnutrition addressing public health problems particularly in the young population. Through an IAEA project for facilitating diagnosis and preventive interventions, 1,300 children mainly from Bangladesh, India, Pakistan, and Benin have been investigated. Isotopic techniques using ^{13}C-labeled substrate breath tests for bacterial colonization and digestion and absorption of nutrients (lactose, amino acids, and triglycerides) that are sensitive tools to examine the significance of Hp and its consequences on poor nutrient assimilation in young children have been successfully used for breath sample analyses in these countries. It is estimated that by 2025 there will be 1.2 billion elderly people in the world. Techniques based on dual energy x-ray absorptiometry offer non-invasive methods for investigating the variation of bone mineral density. Under an IAEA initiative, over 6,000 subjects from Brazil, China, Russia, Turkey, and a few other countries have been investigated to assess bone mineral density (BMD) to evaluate measures for preventive health care. Highly significant differences in mean weight, height, and BMD between countries ($p < .001$) was found. Following adjustment for age, weight, and height, differences in BMD persisted between centers for both men and women. Significant differences existed in young adult bone mass which, if persisting into old age, may contribute to a two- to three-fold difference in fracture risk.

A CRP on the reference Asian man with the participation by several Asian countries (RAS project) generated reliable data sets for dietary intake for all participating countries (and in tissues by some) that will enhance their ability to resolve national problems of radiological protection, as well as to facilitate development of the characteristics of a reference Asian man, the primary goal of this project. Improved reference values have been derived for a number of additional elements and reference material matrices that will strengthen the capability to address also issues of nutritional interest.

Refined isotopic techniques resulting from a CRP on the isotopic evaluations of maternal and child nutrition to help prevent stunting have been used extensively in field studies in Latin America and Pakistan, and in an on-going CRP on isotopic evaluations on infant growth-monitoring, in collaboration with the WHO Growth Monitoring Programme.

Future prospects

There has been impressive progress in the instrumentation of isotope ratio mass spectrometry (IRMS) incorporating a gas-chromatographic interface. This improvement facilitates specific compounds to be converted to carbon dioxide, hydrogen, or nitrogen yielding compound specific isotope ratio measurements. This is expected to open new and exciting applications in nutritional sciences. New alternatives for stable isotope analysis, such as infrared techniques for $^{2}H_{2}$ and ^{13}C, offer the possibility of quantifying stable isotopes at cheaper costs. The IAEA has a system of research sub-contracts to pay for isotopic analysis, which usually go to the industrialized country laboratories. These technologies might help make developing regions more independent in their analytical facilities and hence in their capacity to address their own problems. However, these technologies must be properly validated.

Multiple rather then single micronutrient supplementation programs for bioavailability are very important because of potential nutrient and diet interactions in different populations. In agriculture the selection of species with a higher density of nutrients, like wheat higher in iron or genetically modified foods, will need to be addressed through more research.

In many developing countries, the changing dietary pattern, along with increased life expectancy and changing socioeconomic environment, has contributed to increased obesity and other diet-related chronic diseases that will have an enormous impact on the health care resources of these countries in the near future [6]. The problem of chronic disease, especially in reference to obesity and type 2 diabetes, will need to be explored not only with fasting and postprandial glucose levels, but will require a way to measure insulin resistance in different populations in the developing world were this epidemic is rampant. Body composition, in addition to body size, will have to be explored through dilution techniques and multi-compartment models to strengthen other field methods used to look at body composition. This will allow a study of the quality of growth by looking at changes in fat mass or fat-free mass in shorter periods, rather than only looking at body size changes. The DEXA methodology will be the most helpful and may be used also to look

at osteoporosis in the aging population. Milk intake measured by deuterium kinetics is important to evaluate the nutrient intake and health of lactating mothers. Nuclear techniques using deuterium analyzed by IR methods or dual x-ray absorptiometry are becoming more readily available to developing regions.

Poor countries of the world face old problems that have become new. Such is the case of the recent scurvy outbreak, probably complicated with other vitamin and mineral deficiencies in northern Afghanistan (before the conflict). This problem has affected about 10% of the population of the villages of Lafraye and Melgee [7]. These deserted and isolated areas require the distribution of flours, cereal/legume blends, and other foods fortified and/or enriched with vitamin C and other micronutients. These and other similar future emergency or long-term programs will have to be evaluated to be sustainable and effective.

References

1. Wolfe RR. Radioactive and stable isotope Tracers in biomedicine. New York: Wiley-Liss, 1992.
2. Hachey DL, Wong WW, Boutton TW, Klein PD. Isotope ratio measurements in nutrition and biomedical research. Mass Spec Rev 1987;6:289–328.
3. Mellon FA, Sandstrom B, eds. Stable isotopes in human nutrition London: Academic Press, London, 1996.
4. NAHRES, Nutritional and Health Related Environmental Studies. Targeting malnutrition, isotopic tools for evaluating nutrition worldwide. Vienna: IAEA, 2000.
5. IAEA. Roles of isotopic techniques in human nutrition evaluations, report of an IAEA consultants' meeting. Vienna: IAEA, 1996.
6. Popkin BA, Paeratakul S, Zahi F, Ge K. Obesity Res 1995; 3:145–53.
7. Fitsum A. Scurvy outbreak and erosion of livelihoods masked by low wasting levels in drought affected Afghanistan. Field article, field exchange. Emergency Nutrition Network. 2001;August:13–6.

Using stable isotopes to assess the bioavailability of minerals in food fortification programs

S. A. Abrams, I. J. Griffin, and S. Herman

Abstract

The fortification of various types of food with minerals is often undertaken without consideration of either their bioavailability or the potential nutrient-nutrient interactions resulting from their use. Stable isotopes provide a safe and accessible method of resolving these issues by providing the proper evidence in each case. They must be conducted according to strict safety and ethical guidelines and may be readily conducted in a field setting. Clinical studies in children enable researchers, policymakers, and food manufacturers to obtain the data necessary to determine the best way to fortify specific foods and beverages, in order to optimally enhance the nutritional health of growing children. We have shown the utility of this approach in studies in both developing countries and in the United States.

Key words: stable isotopes, minerals, bioavailability, food fortification

Introduction

Interventions to improve the micronutrient status of at-risk populations require specific evidence of their effectiveness. In particular, the rational provision of mineral-fortified foods to children requires accurate information regarding the bioavailability of the fortified nutrients in the target population. Currently, this information may best be determined using stable isotope techniques. Using stable isotopes allows for these assessments to be made in a completely safe and highly accurate fashion. Furthermore, stable isotope techniques are highly field-friendly, and are applicable to measurements made in both industrialized and developing countries.

In assessing bioavailability, it is crucial that the absorption of the stable isotope label closely matches that of the mineral it is tracing. This may require special dosing and batch preparation of the food to be consumed. A close collaboration among the isotope supplier, field team, and analytical team is necessary.

The benefits of fulfilling these challenging criteria are considerable. Currently, food fortification with minerals is undertaken as a national program in many developing and industrialized countries. Iron fortification of flour has become prevalent in many countries and the fortification of both beverage and flour products with calcium, zinc, and magnesium is on the rise. We will discuss two examples of the use of this technique in children. In one case, we will consider the use of stable isotopes to evaluate a potential zinc fortification program in Indonesia. In the other case, we will consider the use of stable isotopes to assess the effects of fortification of breakfast cereals with calcium.

Human use issues

An important aspect of mineral stable isotope studies is that they can be used in any subject population [1]. This includes children of all ages, as well as pregnant and lactating women. However, special considerations are involved when performing such studies in children. First, less blood generally is obtained from pediatric subjects, so special sample preparation techniques are required, as well as the use of fewer time points in kinetic analysis. Second, the rapid rate of bone turnover in adolescents mandates a greater demand for tracer, and in turn, administration of higher tracer doses to these young subjects than to adults of the same body weight [2]. Finally, analgesia during painful procedures, as well as consent and other ethical issues, must be carefully considered in performing pediatric studies.

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Mineral stable isotopes have been used in thousands of clinical studies with no reported complications related to their use. Complications would only be expected related to inappropriate isotope preparation or administration, especially of intravenous doses. Because of the widespread use of isotopes in pediatric studies, we have extensively evaluated approaches to ensure maximum safety in clinical protocols.

Our foremost concern is that the isotopes be obtained from sources which have thoroughly tested and demonstrated their purity, and which provide evidence, in the form of formal certification, documenting the origin of the isotopes, the isotopic content (enrichment), and the lack of excessive trace mineral contamination (table 1). Unfortunately, the open distribution of mineral stable isotopes from some sources has created the potential for isotopes to be sold on the open market without these safeguards. In some cases, very low prices may serve as a red flag indicating that those isotopes were not properly obtained or distributed. Typical market prices for the most commonly used stable isotopes are provided in table 2.

An important consideration is that the phlebotomy and infusion procedures be made as painless as possible. Children have a limited ability to tolerate uncomfortable procedures, a concern which should be kept in mind when interacting with those who volunteer to participate in research studies. It should be noted that often, pediatric subjects receive no direct benefit from the study. Clearly, on principle, any potential risks and discomforts that may be associated with a child's participation in an individual study should be very carefully scrutinized and minimized.

Prior to initiation of a study, children are interviewed, and often a screening blood sample is taken. This provides an opportunity to assess whether individual subjects are sufficiently comfortable with the process of venipuncture to allow this procedure to be carried out successfully throughout the entirety of the study time period. We may choose not to enroll a subject who wishes to participate in a study because of present or foreseeable difficulties with phlebotomy.

To minimize discomfort from venipuncture, we utilize an analgesic agent for all phlebotomies. Most commonly, we use a lidocaine/prilocaine cream [eutectic mixture of local anesthetics (EMLA)] cream which has proven effective in relieving pain associated with intravenous catheter placement [3]. The universal use of EMLA cream has made participation in research studies much more acceptable to our pediatric subjects. If children express interest in a particular study, they and their parents are given complete, detailed information. A child who is old enough to independently sign a consent form may be asked to do so, based on the guidelines of the individual institution's ethical review board. Ultimately, it must be assured that the children who participate in a study understand what that study will involve and what is expected from them, within the limits of their individual cognitive capabilities. It should be emphasized to the children that their enrollment in the study is optional, and that they can either refuse to participate or leave the study after enrollment without prejudice.

The feedback from our own pediatric subjects has been overwhelmingly positive, possibly because we place a strong emphasis on making their research experience an enjoyable one. In many cases, children have used their experience as a way of learning more

TABLE 1. Recommended guidelines for identifying the safe and legal purchase of mineral stable isotopes

It is recommended that purchasers obtain, or be able to readily obtain the following documents from any distributors:
» Original manufacturer's assay (must accompany the goods, with translation if needed)
» A certificate of origin from the country in which the material was produced.
» Third-party assay (with acceptable variation limits from the manufacturers to allow for deviation in measurement technology and quantification).
» A letter from the manufacturer that states the date of manufacture, the company to which the material was sold, when it was sold, and how it was transported out of the country.

Table 2. Costs and enrichments of commonly used stable isotopes

Isotope	Enrichment (%)	Price (US$)
Mg-24	99	1.50
Mg-25	98	8.00
Mg-26	98	8.00
Ca-42	90	52.80
Ca-43	52	145.00
Ca-44	97	25.00
Ca-46	6	140.00
Ca-48	92	145.00
Fe-54	99	10.00
Fe-56	99	1.25
Fe-57	94	10.00
Fe-58	90	70.00
Zn-64	98	3.00
Zn-66	98	3.00
Zn-67	80	37.50
Zn-68	98	3.50
Zn-70	88	100.00

Prices are approximate and vary according to quantity purchased, long-term contract orders, specific form required, etc.

Enrichments may vary according to the specific lot of material from which the isotopes are supplied.

Some higher and lower enrichments are available for the materials listed here. Prices will vary according to the specific enrichment ordered and its availability.

about nutrition and science through personal involvement. Many participants' families view the research studies as a special opportunity to teach the child about nutrition and health matters, as well as the scientific process. During the studies, the children meet many dietitians, physicians, and scientists. They learn about the importance of optimal nutrition during childhood and its impact on long-term health. For example, the girls who participate in a calcium intake study focusing on osteoporosis development have an excellent opportunity to learn about the importance of consuming foods containing sufficient calcium during childhood in order to lower their risk of osteoporosis in later life. Such direct experiences demystify the scientific process for children, who often find them entertaining as well as educational.

Methodological issues

Both single- and dual-tracer methods can be utilized to conduct mineral stable isotope studies involving calcium, zinc, and magnesium [1]. A detailed review of these issues is beyond the scope of this paper. However, in general, we prefer the dual-tracer method for these minerals because it obviates the need for fecal collections. That means greater acceptability by the children who participate, as well as the potential for improved accuracy of the results. In the dual-tracer method, one isotope is given orally and a second isotope is given intravenously. Urine and serum samples are collected after the tracer dosing to determine enrichment and calculate fractional absorption. In our isotope studies, each dose of intravenously administered isotope is prepared, labeled, and dispensed by a registered pharmacist using aseptic techniques and standard protocols. All isotope infusions are performed by a physician or registered nurse.

The method of oral administration depends somewhat on the specific research question being considered. For example, in calcium studies, it is common to give the tracer mixed with milk or the calcium-containing food that is being tested for calcium bioavailability. A fixed meal is usually used to maintain a constant level of the other nutrients being administered. The isotope is usually mixed into one or two servings of the food, depending on the amount of the isotope that is to be given.

The intravenous isotope may be administered in one of several ways. In studies in which frequent phlebotomy is planned related to kinetic measurements, it is necessary to place at least one intravenous access line in the subject prior to the study. The isotope may be infused either through that line or via a separate site. In most studies, especially those involving calcium or zinc, the isotope to be infused is diluted in a small amount of saline (1 to 10 ml) and given over a relatively rapid period of time, usually from 1 to 5 minutes. This approach is safe in that the amount of calcium given to children over two years of age is far below a level that would be likely to result in a measurable cardiovascular event. In infants, even smaller doses are usually given by using the least abundant calcium isotope, ^{46}Ca, for intravenous use.

The orally administered isotope tracer is absorbed into a central body pool, which for calcium is believed to represent serum, extracellular fluid, and some metabolically active bone. The oral tracer mixes with the intravenous (IV) tracer, which serves to "normalize" for variations in calcium distribution pool mass among subjects [4]. After administration of the tracers, a complete 24-hour urine collection is carried out. The relative fraction of the oral versus the IV tracer dose in this 24-hour urine pool is determined. This represents the fraction of the oral tracer dose that was absorbed. Because absorption is calculated from total urinary isotope recovery, it is not necessary to exactly sequence the time of administration of the oral and IV isotopes, as would be necessary if a single peak serum value were used [5].

Although iron absorption also can be measured using a dual oral and intravenous isotope method, we generally prefer not to give iron intravenously. As 80% to 90% of newly absorbed iron is rapidly incorporated into red blood cells, a standard value can be assumed to convert red blood cell incorporation into absorption [6]. This allows the second isotope to be used in a number of different ways. Two foods can be labeled with different isotopes and given on consecutive days. The ratio of the red blood cell incorporation of the two isotopes is equal to the ratio of their absorption. Using this method, direct comparison of iron absorption from two meals can be made in a single subject from a single blood sample.

Alternatively, the second isotope can be used as a reference dose. The reference dose is an aqueous solution of ferrous sulfate with an excess of ascorbic acid, given on an empty stomach. This form allows maximum iron absorption, and is an excellent measure of iron status. As iron status is regulated by changes in absorption, the reference dose absorption is highest in subjects with the lowest iron status. The reference dose absorption can therefore be used to statistically correct for differences in iron status between subjects and increase the statistical power of the study.

When designing a mineral absorption study, an early decision is required regarding the choice of a crossover or parallel design. In a crossover design, every subject receives all the different treatments (or labeled foods) in random order. Subjects act as their own controls and, if studies are conducted relatively close together in time, the mineral status of the subjects should not change over the course of the study. This helps to reduce variability, and increases the statistical power

of the study. Therefore, fewer subjects may need to be recruited.

There are, however, several problems with crossover designs. The first concern is that of an effect of the initial treatment on the results of the second study. It can often be very difficult to ascertain whether the effects of an intervention or treatment have been adequately eliminated. Use of washout periods may be beneficial, as may randomization to the initial treatment option.

In addition, there are considerable technical issues related to crossover design studies that use stable isotopes. For example, if a single measure of iron absorption is taken in a subject, then the isotopic ratio only needs to be measured at the end of the study. However, once an iron isotope is given, the red blood cells will remain enriched almost indefinitely. So if a second study is conducted, baseline enrichment must be measured. Therefore, to measure iron absorption once requires a single blood sample; to measure it twice requires three blood samples and isotope ratio measurements (two samples after the two isotope doses, and one sample before the second isotope); to measure it three times requires five samples. This procedure is particularly important for iron because its excretion is so low, and most body iron is in red blood cells. Once a calcium isotope is given, the bone (or bone and muscle, in the case of zinc) is likely to be enriched indefinitely; however, the enrichment in the plasma and urine compartment is much lower, and normally it is near baseline levels within 6 to 8 weeks. Frequently, studies can be repeated after 2 to 3 weeks if a baseline urine sample is collected.

In parallel study designs, each subject receives a single treatment. This makes the study much less intensive for the subject. However, it is possible that mineral status (or other baseline variables) may differ between groups. For this reason, larger numbers of subjects are needed for a parallel study than for a crossover study. Differences between groups can be minimized by stratified randomization. For example, in some studies of iron absorption to help ensure balance between the groups, we randomized subjects with low hemoglobin concentration separately from those with normal concentrations. Alternately, a statistical correction can be made after the study, to correct for between-group differences. In the case of iron absorption measurements, the serum ferritin can be used to correct for differences in iron status between individuals. The reference-dose iron absorption is probably even better; by using this to correct for intersubject differences in iron status, a parallel study can approach the statistical power of a crossover design.

Evaluation of food fortification in Indonesia

Food may be fortified with minerals to provide a greater supply of a nutrient, such as zinc or iron, when a dearth of that nutrient is perceived as limiting a child's growth or development. In that case, it is important to assess potential nutrient interactions, as well as the cost and optimal form of the nutrient to be delivered. These issues were evaluated in a study recently completed in collaboration with the Nutrition Research and Development Center in Bogor, Indonesia. One of the important questions weighed in this study was whether the bioavailability of zinc sulfate added as a fortificant to wheat flour would be the same as that of zinc oxide. That was a significant consideration because the oxide is potentially less expensive, but, cannot readily be used if it is not bioavailable. Furthermore, as flour in Indonesia is already iron fortified we wished to examine the effect of zinc co-fortification on iron absorption.

Recent studies have shown that zinc supplementation of high-risk populations in developing countries leads to significant decreases in mortality and morbidity from diarrhea and respiratory diseases, and may also improve growth [7–9]. This has led to the belief that sub-clinical zinc deficiency may be common in developing countries, and has sparked interest in fortifying food staples with zinc. Zinc fortification is common in industrialized countries, where the most common forms of zinc used are zinc oxide and zinc sulfate [10]. Wheat flour is generally a relatively poor source of iron and zinc (11.7 mg/kg and 7 mg/kg, respectively) [11]; it is commonly iron-fortified throughout much of the world. In Indonesia, consideration is being given to cofortifying iron-fortified flour with zinc. However, there is concern that zinc cofortification might reduce the absorption of iron from fortified flour [12].

Ninety healthy children (45 male and 45 female) were recruited from a rural outreach clinic in Situ Udik, a small village approximately 70 kilometers south of Jakarta, Java, Indonesia. Subjects were considered eligible for the study if they were between 4.0 and 8.0 years of age, had a height and weight greater than the third percentile for age, and had had no infectious diseases, respiratory tract infections, or diarrhea within the preceding 2 weeks. Subjects were not enrolled if they had any known chronic medical condition or were on any medications, including vitamin or mineral supplements. The nature of the study, and its potential risks and discomforts, were explained to the subject's parents by the study personnel; informed written consent was obtained from the subject's parents. The study received ethical approval from the Ethical Committee of the National Institute for Health Research and Development, Bogor, Indonesia; and from the Institutional Review Board of Baylor College of Medicine, Houston, Texas. To ensure that the results were not confounded by parasitic infections, the subjects received antihelminthic treatment with mebendazole (500 mg) as a single oral dose approximately two weeks prior to the start of the bioavailability study.

For oral administration, we prepared ^{67}Zn as both ^{67}Zn oxide and ^{67}Zn sulfate. These were mixed with the steamed dough balls that consisted of the food product to be fortified. Flour sufficient to make 36 portions of 25 g each was weighed out. This excess production (approximately 20%) was set up as a safeguard in case additional subjects needed to be recruited, to allow for the loss of subjects between the time the food was consumed and the time blood and urine samples were taken. The iron and zinc isotopes were added to water, which in turn was added to the dough. The isotopes were added in this manner in an attempt to ensure even distribution of the isotope throughout the dough. The mixture was seasoned with crushed garlic, salt, and pepper, and mixed by hand for 5 to 10 minutes. More water was added slowly, to produce the desired consistency. Once a smooth, pliable dough was produced, the dough mixture was re-weighed and divided into 36 equal portions by weighing the desired amount of dough on scales. The mass of dough in each portion (1/36 of the total mass) was weighed out to within 0.1g of the desired weight. Each portion (equivalent to 25 g flour, 1.5 mg iron isotope ± 1.5 mg zinc isotope) was divided into four balls and placed in an individual steaming bag, which was steamed for 5 to 10 minutes. The bags were frozen until required for use.

For intravenous use, ^{70}Zn, 90% enriched, was prepared as an aqueous solution of zinc chloride by the Investigational Drug Pharmacy of Texas Children's Hospital in Houston, and tested for sterility and pyrogenicity prior to intravenous administration. Isotopes were produced in Russia and purchased from Trace Sciences, Inc., Toronto, Canada.

After an overnight fast, subjects received an intravenous infusion of 0.2 mg ^{70}Zn. Afterward, the subjects received a meal consisting of the steamed dough balls that were reheated immediately before they were served by steaming for approximately 5 minutes. In addition, the subjects were fed a small amount (~2 tablespoons) of a seasoned tomato puree and 100 ml of water. Subjects fasted for an additional two hours before being discharged. Approximately 48 and 72 hours after discharge, subjects collected a urine sample for zinc isotope ratio analysis.

Urinary zinc isotope ratios were measured following acid digestion and anion exchange. Twelve-ml aliquots of urine were digested with 10 ml of 15 N nitric acid overnight on a hot plate. The dried sample was dissolved in 1 ml of 6 N hydrochloric acid and loaded onto an anion exchange resin column (AG 1-X8 resin, Bio-Rad Laboratories, Hercules, Calif., USA) that had been prewashed with 10 ml of double-distilled water and 5 ml of 6 N hydrochloric acid. The column was washed with serial 5 ml aliquots of 6 N, 3 N, 2 N, 1 N, and 0.5 N hydrochloric acid, and the samples eluted with 6 ml of double distilled water. Ten μL of 0.7 N phosphoric acid were added, and the sample dried on a hot plate overnight in a Teflon vial before being re-suspended in 0.5-ml double-distilled water. Ten to 20 μL of this solution, 2 μL of 0.7 N phosphoric acid and 6 μL of silica suspension were loaded onto rhenium filaments. Isotope enrichments were measured by magnetic sector thermal ionization mass spectrometry. Isotope ratios were expressed with respect to the non-administered isotope, ^{66}Zn, and corrected for differences in fractionation using the ^{64}Zn/^{66}Zn ratio. Replicate blocks of 10 scans were performed until the desired degree of precision was obtained [13]

Zinc absorption was calculated from the fractional excretion of the oral and intravenous isotopes in the 48- and 72-hour urine samples [1]. The two estimates of zinc absorption were averaged to give a final value. We found that there was no difference in zinc absorption between the children who received the zinc oxide (24.1 ± 8.2%) and those who received the zinc sulfate (23.7 ± 11.2%; p = .87). This result was somewhat unexpected. Because zinc oxide is less soluble than zinc sulfate, it has been generally expected that zinc absorption would be greater from zinc sulfate than from zinc oxide. However, there is little objective evidence to support a bioavailability difference. In fact, the small amount of preliminary evidence in humans suggests that zinc absorption is similar from zinc oxide and zinc sulfate [14]. Our data are consistent with this indication.

Iron absorption was 15.9 ± 6.8% in Group 1 (iron only), 14.0 ± 8.9% in Group 2 (iron and zinc oxide), and 11.5 ± 4.9% in Group 3 (iron and zinc sulfate), and tended to vary between the groups (ANOVA p-value = .068). Post-hoc testing (Fisher's PLSD—protected least square differences) revealed a significant difference between Group 1 (iron only) and Group 3 (iron +zinc sulfate; mean difference 4.36%, p-value = .021), but not between Group 1 (iron only) and Group 2 (iron and zinc oxide; mean difference 1.88%, p-value = .32). These data suggest that zinc sulfate, but not zinc oxide, may have an adverse effect on iron absorption. The possibility that iron can inhibit zinc absorption due to the two minerals competing for a shared absorptive pathway has been considered [15], and is felt to be likely, as these two minerals have similar physical properties [16]. There are several possible sites of inhibition, which have been reviewed elsewhere [17]. One intriguing, possible site of interaction between iron and zinc is at the duodenal transport protein DCT-1 (divalent cation transporter –1) also known as DMT-1 (duodenal metal transporter-1) [18]. This appears to be important in iron absorption, but can also transport many other metals, including zinc [18]. If iron and zinc can inhibit one other's absorption by competition for DCT-1, the effects would be expected to be most noticeable when one metal is in relative excess compared to the other. This is consistent with the observation that iron has little effect on zinc absorption when iron:zinc ratios are

1:1, but it has an inhibitory effect on zinc absorption when ratios are 2:1 or greater [20].

Although a number of studies have examined the effect of iron supplementation on zinc absorption, few have considered the effect of zinc supplementation on iron absorption. One study has examined the effect of zinc on radio-iron absorption from an aqueous solution [21]. Zinc had no effect on radio-iron absorption when the zinc:iron ratio was 0.36:1 (molar ratio 0.4:1), but a significant inhibition of radio-iron absorption was seen when the zinc:iron ratio was 1.14:1 (molar ratio 1:1). A second study showed that an iron:zinc ratio of 5:1 significantly reduced iron absorption from an aqueous solution, but did not affect heme iron absorption from a hamburger meal [22]. In our study, the zinc:iron ratio was 1:1, and a reduction in iron absorption was noted if zinc sulfate was the fortificant, but not if zinc oxide was. The reason for the difference between zinc oxide and zinc sulfate is not immediately clear. Zinc sulfate is, however, much more soluble in water than zinc oxide [23], and it is possible that not all the zinc oxide dissolved in the dough. If this were the case, the zinc:iron ratio in the aqueous phase might be less than 1:1 when zinc oxide was the fortificant. At this lower ratio, zinc may have no effect on iron absorption, or the effect may be sufficiently reduced to make it undetectable with a sample size of 30 per group. In our study, iron absorption fell from 15.9 % to 14.0% when zinc oxide was added, but this was not statistically significant. If this effect were true, a sample size of 274 per group would have been required to have an 80% chance of reaching statistical significance at $p < .05$.

Because zinc oxide is less soluble than zinc sulfate, it has been generally expected that absorption of zinc from zinc sulfate would be superior to that from zinc oxide. There is little objective evidence to support this, and the small amount of preliminary evidence in humans suggests that zinc absorption from zinc oxide and zinc sulfate is similar [14]. Indeed, the concept that *in vitro* solubility is directly related to mineral absorption is untested [24]. We found no difference between zinc absorption from zinc oxide and from zinc sulfate. An alternative hypothesis, therefore, is that the zinc oxide dissolved more slowly than the zinc sulfate. Perhaps this led to a higher zinc:iron ratio in the proximal gastrointestinal tract when zinc sulfate was used as the fortificant than when zinc sulfate was used as such, leading to a significant interaction between iron and zinc sulfate, but not between iron and zinc oxide.

Because of the low levels of iron and zinc in flour, cofortification with iron and zinc oxide or zinc sulfate would lead to significantly increased absorption of both iron and zinc. However, our study was carried out in the context of existing iron fortification of flour, and the question we addressed was whether adding zinc oxide or zinc sulfate might reduce iron absorption. The results of our study suggest that co-fortification with zinc sulfate *might* reduce iron absorption. As zinc absorption was similar from zinc sulfate and zinc oxide, we suggest that zinc oxide might be a preferable choice, especially as it is cheaper than zinc sulfate (a significant consideration).

Iron and zinc deficiencies are huge public health issues in developing countries, and it is important that the results of a single, relatively small, study are not over-interpreted. Further studies are urgently needed to confirm or refute our preliminary findings. These studies should not only evaluate iron and zinc absorption from fortified foods, but examine the effects of prolonged consumption of fortified foods on iron and zinc status, and on mortality and morbidity. Clearly, we have much to learn about the optimum ways to fortify food staples with iron and zinc in developing countries, and the potential interactions that may result between minerals.

Calcium fortification of breakfast cereals

Fortification of commonly eaten food products is increasing worldwide, even in those regions in which malnutrition is not widespread. This may be attributed to the dual goals of preventing acute nutrient deficiencies and decreasing long-term morbidity related to micronutrient inadequacy. For example, flour is now commonly fortified with folate and iron, and salt is typically fortified with iodine. At this time, the very low dietary calcium intake of children and adolescents in the United States is a serious public health concern [25]. Dietary calcium from dairy products and other natural food sources is generally not ingested in amounts sufficient to meet current dietary recommendations. Specifically, over 80% of adolescent females do not meet the 1300 mg/day calcium intake currently recommended for 9 to 18-year-olds [26, 27]. Therefore, the fortification of food and beverages, especially breakfast cereals and fruit juice, has been widely undertaken by food manufacturers. Food labeling laws in the United States currently allow foods to be described as "good sources" of calcium if they contain 100 mg of calcium per serving. However, there is no requirement that the bioavailability of this calcium, or any other nutrient, be evaluated.

We sought to determine whether the addition of calcium to cereal would have a net positive effect on calcium absorption without decreasing iron absorption [28]. Twenty-seven US children, 6 to 9 years of age, were provided two servings per day (30 g of cereal per serving) of either a low (39 mg per serving) or fortified (156 mg per serving) calcium-containing cereal product for 14 days. Calcium absorption was measured using stable isotopes added to milk (44 Ca, extrinsically labeled) and to the calcium-fortified cereal (48 Ca, intrinsically labeled. The cereal used in this study

was Kix®, a corn-puffed cereal (General Mills, Inc., Minneapolis, Minn., USA). The manufacturer supplied special batches of the cereal for use during the study, and measured servings were provided to the families during the two-week adaptation period. The cereal was calcium-fortified by adding calcium carbonate to the dry mix of the cereal prior to cooking and puffing.

The 27 children who completed the study had a mean age of 7.8 ± 1.0 years (range 6.1–9.0 years). Thirteen children were Caucasian, two were African-American, nine were Hispanic, and three were multiethnic (meaning that these three children had one Caucasian and one Hispanic parent). All of the children were prepubertal. Their mean weight was 27.7 ± 7.4 kg (range 18.0 to 43.7 kg).

The fortified cereal contained 156 mg of calcium, while the unfortified cereal contained 39 mg of calcium per 30 g serving. Thus, two servings of fortified cereal provided an additional 234 mg calcium per day compared to the unfortified product. When calculated as part of the entire diet, the calcium intake of the subjects averaged 699 ± 58 mg per day when they received unfortified cereal, and 912 ± 55 mg per day when they received fortified cereal.

The study was designed to compare the absorption of calcium from cereal with that from milk. To determine whether addition of the fortified cereal lowered milk calcium absorption, subjects were also studied when fed a low-calcium-containing cereal.

Calcium absorption from the fortified cereal was assessed using a special batch of cereal that had been labeled with ^{48}Ca during production. This was consumed along with ^{44}Ca-labeled milk with breakfast. A dose of ^{46}Ca was given intravenously afterwards. A second serving of ^{48}Ca-labeled cereal was given with lunch.

Fractional absorption of calcium from milk when given with fortified cereal was 28.9 ± 6.6% and when given with noncalcium-fortified cereal, it was 30.8 ± 6.6 ($p = .17$). Calcium absorption from the labeled cereal was 30.6 ± 7.8%. This value was not significantly different than the absorption of calcium from milk when given with either type of cereal ($p > .2$ for each comparison). Total calcium absorption (269 ± 45 vs. 215 ± 45 mg/day, $p < .001$) was greater in the children with a higher calcium intake.

This study demonstrated a marked nutritional benefit to prepubertal children by increasing their calcium intake via fortification of a breakfast cereal that they consumed with milk, and also as a snack. The net effect of fortifying two servings each day with a modest amount of calcium was an increase in calcium retention (balance) of 56 mg per day. This benefit, if maintained over one year, would lead to a relative increase in retention of 20 g of calcium per year, which would represent approximately 4% of a typical 500 g calcium skeleton in a child.

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References

1. Abrams SA. Using stable isotopes to assess mineral absorption and utilization by children. Am J Clin Nutr 1999;70:955–64.
2. O'Brien KO, Abrams SA. Effects of development on techniques for calcium stable isotope studies in children. Biol Mass Spectromet 1994;23:357–61.
3. Cordoni A, Cordoni LE. Eutectic mixture of local anesthetics reduces pain during intravenous catheter insertion in the pediatric patient. Clin J Pain 2001 17:115–8.
4. Abrams SA, Vieira NE, Yergey AL. Interpretation of stable isotope studies of calcium absorption and kinetics. In: Wastney ME, Siva Subramaniam KN, eds. Kinetic models of trace element and mineral metabolism. Boca Raton, Fla, USA: CRC Press, 1995:283–90.
5. Yergey AL, Abrams SA, Vieira NE, Aldroubi A, Marini J, Sidbury JB. Determination of fractional absorption of dietary calcium in humans. J Nutr 1994;124:674–82.
6. Abrams SA, Wen J, O'Brien KO, Stuff JE, Liang LK. Application of magnetic sector thermal ionization mass spectrometry to studies of erythrocyte iron incorporation in small children. Biol Mass Spectromet 1994;23:771–5.
7. Bhutta ZA, Bird SM, Black RE, Brown KH, Gardner JM, Hidayat A, Khatun F, Martorell R, Ninh NX, Penny ME, Rosado JL, Roy SK, Ruel M, Sazawal S, Shankar A. Therapeutic effects of oral zinc in acute and persistent diarrhea in children in developing countries: pooled analysis of randomized controlled trials. Am J Clin Nutr 2000;72:1516–22.
8. Bhutta ZA, Black RE, Brown KH, Gardner JM, Gore S, Hidayat A, Khatun F, Martorell R, Ninh NX, Penny ME, Rosado JL, Roy SK, Ruel M, Sazawal S, Shankar A. Prevention of diarrhea and pneumonia by zinc supplementation in children in developing countries: pooled

analysis of randomized controlled trials. Zinc Investigators' Collaborative Group. J Pediatr 1999;135:689–97.
9. Umeta M, West CE, Haidar J, Deurenberg P, Hautvast JG. Zinc supplementation and stunted infants in Ethiopia: a randomised controlled trial. Lancet 2000;355:2021–6.
10. Whittaker P. Iron and zinc fortification in humans. Am J Clin Nutr 1998;68(suppl):442S-6S.
11. USDA, ARS. USDA nutrient database for standard reference. Nutrient Data Laboratory Home Page. Release 13 ed: Washington, D. C.: U.S. Department of Agriculture, Agricultural Research Service, 1999.
12. Solomons NW. Competitive interaction of iron and zinc in the diet: consequences for human nutrition. J Nutr 1986;116:927–35.
13. Griffin IJ, King JC, Abrams SA. Body weight specific zinc compartmental masses in girls significantly exceed those reported in adults: a stable isotope study using a kinetic model. J Nutr 2000;130:2607–12.
14. Diaz M, Rosado JL, Munuz EC, Westcott JE. Bioavailability of zinc sulfate and zinc oxide added to corn tortilla: a study using stable isotope-not "isotopes" FASEB J 2001;15:A732.
15. Solomons NW. Competitive interaction of iron and zinc in the diet: consequences for human nutrition. J Nutr 1986;116:927–35.
16. Hill CH, Matrone G. Chemical parameters in the study of in vivo and in vitro interactions of transition elements. Fed Proc 1970;29:1474–81.
17. Fairweather-Tait SJ. Iron-zinc and calcium-iron interactions in relation to zinc and iron absorption. Proc Nutr Soc 1995;54:465–73.
18. McMahon RJ, Cousins RJ. Mammalian zinc transporters. J Nutr 1998;128:667–70.
19. Conrad ME, Umbreit JN. Iron absorption and transport-An update. Am J Hematol 2000;64:287–98.
20. Solomons NW, Jacob RA. Studies on the bioavailability of zinc in humans: Effects of heme and non-heme iron on the absorption of zinc. Am J Clin Nutr 1981;34:475–82.
21. Crofton RW, Gvozdanovic S, Kin CC, Brunt PW, Mowat NAG, Agget PJ. Inorganic zinc and the intestinal absorption of ferrous iron. Am J Clin Nutr 1989;50:141–4.
22. Rossander-Hulthen L, Brune M, Sandstrom B, Lonnerdal B, Hallberg L. Competitive inhibition of iron absorption by manganese and zinc in humans. Am J Clin Nutr 1991;54:152–6.
23. Budavari S, O'Neil MJ, Smith A, Hecklemna PE, eds. The Merck index: an encyclopedia of chemicals, drugs, and biologicals. Rahway, N.J. USA, Merck and Co. Inc., 1989.
24. Heaney RP. Factors influencing the measurement of bioavailability, taking calcium as a model. J Nutr 2001;131: 1344S–48S.
25. Heaney RP, Abrams SA, Dawson-Hughes B, Looker A, Marcus R, Matkovic V, Weaver C. Peak bone mass. Osteoporosis Int 2000;11:985–1009.
26. Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Washington, D.C.: National Academy Press, 1997.
27. Committee on Nutrition, American Academy of Pediatrics. Calcium requirements of infants, children, and adolescents. Pediatrics 1999;104:1152–7.
28. Abrams SA, Griffin IJ, Davila P, Liang L. Calcium fortification of breakfast cereal enhances calcium absorption in children without affecting iron absorption. J Pediatr 2001;139:522–6.

Stable isotopes and $^{13}CO_2$ breath tests for investigating gastrointestinal functions

Yvo Ghoos, Benny Geypens, and Paul Rutgeerts

Abstract

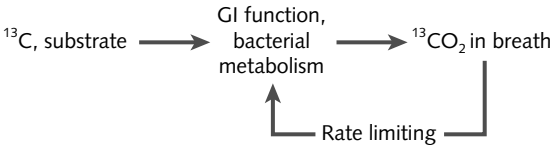
In medical investigation there is a need for non-invasive methods. Moreover, patients ask for easy methods that are simple to perform and medical doctors demand reliable techniques. With the advent of stable isotopes a new area of tracer technology became available. In gastroenterology, $^{13}CO_2$ breath tests are used which fullfill all the conditions needed in modern clinical research and investigation.

Key words: Gastrointestinal function, breath test, digestion, absorption, fermentation, transit, stable isotopes

Introduction to breath tests

The $^{13}CO_2$ breath test (BT) is a non-invasive and reliable method to study the main gastrointestinal (GI) functions including the assimilation of food ingredients. Stable isotopes offer the possibility to monitor various metabolic events, including the fermentation processes in the colon.

Breath tests have the common characteristic that a substrate, that bears the functional group in which a normally present ^{12}C atom has been replaced by the stable isotope ^{13}C, is administered to an individual. This functional group is cleaved enzymatically under specific circumstances, either during the transit through the gastrointestinal tract, during absorption or during further metabolism of the absorbed substrate. After cleavage the marked subgroups undergo a metabolic process that ends with expiration of the labeled CO_2. It is necessary that the speed determining (*rate limiting*) factor of the whole physiologic process is directly related to the genesis of $^{13}CO_2$. The $^{13}CO_2$ mixes with the body pool of CO_2-HCO_3^- and is breathed out. In this way the exhalation of $^{13}CO_2$ reflects the function to be investigated, as indicated by the following scheme:



The ^{13}C, substrate has to be chosen in such a way that the enzyme/function/bacteria is the rate-limiting step in $^{13}CO_2$ evolution to demonstrate by $^{13}CO_2$ measurement either enzyme activity, a well-defined GI function, or bacterial metabolism. When the excretion of the tracer in breath is expressed as % dose per hour and/or as cumulative % dose excreted over a defined time period, a dynamic analysis of the examined parameter of the gastrointestinal tract is obtained in the course of time.

$^{13}CO_2$ breath tests may be considered excellent investigation methods, as their scientific bases are sound and well-conceived, the results have been validated in an unequivocal way, and their applications are accepted by an increasing number of scientists. $^{13}CO_2$ breath tests can be combined with the ^{14}C,tracer or/and the H_2 measurement in breath. Additional information on the fermentation processes in the colon is obtained by labeling bioactive molecules with nitrogen-15 (^{15}N)and deuterium (^2H).

There is a need for non-invasive methods, which parallel the information of the classical methods, that are simple to perform and that can be executed at lower costs. $^{13}CO_2$ breath tests may meet this need. They have great advantages over conventional methods as the gastrointestinal function can be displayed in course of time. Furthermore they are not invasive for the patient, and can even be performed at home. The medical doctors ask for tests that can be done in a repetitive way without major discomfort or radiation hazard for the patient and without special equipment and personnel. In no way, however, it is claimed that

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$^{13}CO_2$ breath tests are exclusive tests. The breath test has to be considered as an important clinical investigation tool. Therefore, the interpretation of the test result should be done in close discussion with the medical doctor in the clinical unit.

Special attention should be paid to the execution of $^{13}CO_2$ breath tests. These tests seem simple to perform, and some investigators even try to render the test more simple by reducing the sample numbers or by changing test conditions (meal, sampling time, calculations of results…). These modifications could lead to false interpretation of the results, and make them unsuitable for inter-laboratory comparison. The best way to safeguard uniformity in test design is to do breath tests in a specialized clinical unit.

Breath tests

At the digestion and absorption laboratory at the University Hospital Gasthuisberg, Leuven, the breath tests used in clinical practice are shown in figure 1.

Substrates used in $^{13}CO_2$ breath tests

Hepatic functions:
» demethylating and oxidative capacity: [^{13}C]aminopyrine [1]
» hepatic mass: [^{13}C]galactose [2]
» mitochondrial activity: [^{13}C]keto isocaproic acid [3]
Transit measurement:
» gastric emptying: [^{13}C]octanoic acid and [^{13}C]glycine [4, 5]
» orocecal transit: lactose-[^{13}C]ureide [6, 7]
» small intestinal transit: by mathematical deduction [8]
Helicobacter pylori in stomach
» [^{13}C]urea [9]
Digestive, absorptive, fermentative functions
» carbohydrates: [^{13}C]naturally enriched compounds, starch, lactose [10–12]
» lipids: [^{13}C]mixed triglyceride [13, 14]
» proteins: [^{13}C],[^{15}N]egg-white proteins [15]
» fermentation process: lactose-[^{15}N]ureid [16] ; [^{15}N], [^{2}H]proteins [17]
Bacterial overgrowth–bile acid malabsorption
» the only [^{14}C]substrate in use, i.e,. glycocholic acid + 3 days fecal collection + [^{3}H] PEG transit marker correction [18–20].
Note: mathematical expression of the functions
» an elegant method has been developed to express the meaning of gastrointestinal events by a mathematical formula. [21, 22].

Further developments are being studied to express different gastrointestinal functions by mathematical analysis when several markers are used simultaneously. In the near future we will try to make it possible to measure, by a single meal, gastric emptying ([^{14}C]octanoic acid), protein digestion ([^{13}C]leucine-protein), orocecal transit time and small intestine half emptying time (hydrogen marker+mathematical convolu-tion/deconvolution method) and fermentation pattern ([ring,^{2}H]-phenylalanine-protein, lactose-[^{15}N]ureid), plus fecal protein loss ([^{15}N]leucine-protein). Apart from the ^{14}C,tracer (estimated dose 0.2mSv) these methods can be applied in children also, and they are suitable for use in nutritional and pharmacological research.

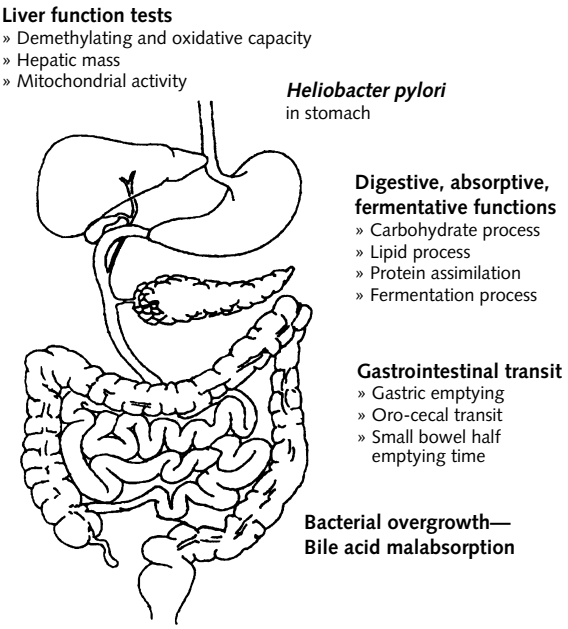

FIG. 1. Gastrointestinal functions that can be investigated by $^{13}CO_2$ breath tests, the dynamics of which can be evaluated by mathematical expressions

These studies have been made possible only by close collaboration with the medical doctor in the hospital. Even when the tests are executed routinely for diagnostic purposes, it is a constant reflection on how helpful these tests can be for the individual patient.

How attractive the test designs might be, standardization of test execution, and harmonization of test protocols are needed for collaboration and for inter-laboratory comparison of results. The first attempt was made by the European concerted action BIOMED PL93-1239 [23]. The on-going evolution in mathematical analysis of test results will contribute markedly to the better understanding of basic gastrointestinal processes in the individual. The mathematical expression of their results represents a new area for forthcoming application and further development. Mathematics is

a welcome exposition of physiologists view of how the process of science can lead to reliable results.

Although these breath tests are under current evolution at the laboratory, they can be applied successfully in less privileged countries, as in their simple form they are very reliable to investigate gastrointestinal functions. No radioactive tracer or radioactive waste is involved. The tests, which can be presented in a test kit, can be used in all members of the population. The breath samples can be kept unaltered in exetainers at least for six months before being sent to a centralized analytical laboratory.

References

1. Mion F, Queneau PE, Rousseau M, Brazier JL, Paliard P, Minaire Y. Aminopyrine breath test: development of a ^{13}C,breath test for quantitative assessment of liver function in humans. Hepato-gastroenterol 1995;42:931–8.
2. Berry GT, Nissim I, Mazur AT, Elsas LJ, Singh RH, Klein PD, Gibson JB, Lin Z, Segal S. In vivo oxidation of ^{13}C galactose in patients with galactose-1-phosphate uridyltransferase deficiency. Biochem Mol Med 1995;56: 158–65.
3. Lauterburg BH, Grattagliano I, Gmur R, Stalder M, Hildebrand P. Noninvasive assessment of the effect of xenobiotics on mitochondrial function in human beings: studies with acetylsalicylic acid and ethanol with the use of the carbon 13-labeled ketoisocaproate breath test. J Lab Clin Med 1995;125:378–83.
4. Maes BD, Ghoos YF, Geypens BJ, Mys G, Hiele MI, Rutgeerts PJ, Vantrappen G. Combined carbon 13-glycine/carbon-14-octanoic acid breath test to monitor gastric emptying rates of liquids and solids. J Nucl Med 1994;35:824–31.
5. Veereman-Wauters G, Ghoos Y, van der Schoor S, Maes B, Hebbalkar N, Devlieger H, Eggermont E. The ^{13}C-octanoic acid breath test: a noninvasive technique to assess gastric emptying in preterm infants. J Pediatr Gastroenterol Nutr 1996;23:111–7.
6. Heine W, Berthold H, Klein P. A novel stable isotope breath test: ^{13}C-labeled glycosyl ureides used as noninvasive markers of intestinal transit time. Am J Gastroenterol. 1995;90:93–8.
7. Geypens B, Bennink R, Peeters M, Evenepoel P, Mortelmans L, Maes B, Ghoos Y, Rutgeerts P. Validation of the lactose-(^{13}C)ureide breath test for determination of orocecal transit time. J Nucl Med 1999;40:52–7.
8. Geypens B, Maes B, Ghoos Y, Luypaerts A, Rutgeerts P. Correlation of ileal emptying and gastric emptying calculated from simultanuous application of two breath tests. Gastroenterology 1998;115:A755.
9. Perri F, Ghoos YF, Maes BD, Geypens BJ, Ectors N, Geboes K, Hiele MI, Rutgeerts PJ. Gastric emptying and Helicobacter pylori infection in duodenal ulcer disease. Dig Dis Sci 1996;41:462–8.
10. Ghoos Y, Hiele M, Rutgeerts P, Vantrappen G. Use of naturally ^{13}C-enriched substrates for the study of carbohydrates and protein assimilation by means of $^{13}CO_2$-breath tests. In: Baillie TA, Jones JR, eds. Synthesis and application of isotopically labelled compounds. Amsterdam: Elsevier Science Publishers B.V, 1986; 693–8.
11. Hiele M, Ghoos Y, Rutgeerts P, Vantrappen G, Carchon H, Eggermont E. $^{13}CO_2$ breath test using naturally ^{13}C-enriched lactose for detection of lactase deficiency in patients with gastrointestinal symptoms. J Lab Clin Med 1988;112:193–200.
12. Hiele M, Ghoos Y, Rutgeerts P, Vantrappen G. Starch digestion in normal subjects and patients with pancreatic disease, using a $^{13}CO_2$ breath test. Gastroenterology 1989;96:50 3–9.
13. Ghoos YF, Vantrappen GR, Rutgeerts PJ, Schurmans PC. A mixed-triglyceride breath test for intraluminal fat digestive activity. Digestion 1981;22:239–47.
14. Vantrappen GR, Rutgeerts PJ, Ghoos YF, Hiele MI. Mixed triglyceride breath test: a noninvasive test of pancreatic lipase activity in the duodenum. Gastroenterology 1989;96:1126–34.
15. Evenepoel P, Hiele M, Geypens B, Geboes KP, Rutgeerts P, Ghoos Y. ^{13}C-egg white breath test: a non-invasive test of pancreatic trypsin activity in the small intestine. GUT 2000;46:52–7.
16. Geypens B, Evenepoel P, Peeters M, Luypaerts A, Rutgeerts P, Ghoos Y. Direct demonstration of the effect of lactulose on colonic bacterial metabolism by application of lactose-[^{15}N]-ureide breath test. Gastroenterology 1998;114:A1246.
17. Evenepoel P, Claus D, Geypens B, Geboes KP, Hiele M, Rutgeerts P, Ghoos Y. Amount and fate of egg protein escaping assimilation in the small intestine of humans. Am J Physiol 1999;277:G935–43.
18. Vantrappen G, Janssens J, Hellemans J, Ghoos Y. The interdigestive motor complex of normal subjects and patients with bacterial overgrowth of the small intestine. J Clin Invest 1977;59:1158–61.
19. Rutgeerts P, Ghoos Y, Vantrappen G, Eyssen H. Ileal dysfunction and bacterial overgrowth in patients with Crohn's disease. Eur J Clin Invest 1981;11:199–206.
20. Hellemans J, Joosten E, Ghoos Y, Carchon H, Vantrappen G, Pelemans W, Rutgeerts P. Positive $^{14}CO_2$ bile acid breath test in the elderly people. Age and Ageing 1984;13:138–43.
21. Maes BD, Mys G, Geypens BJ, Evenepoel P, Ghoos YF, Rutgeerts PJ. Gastric emptying flow curves separated from carbon-labeled octanoic acid breath test results. Am J Physiol 1998;275:G169–75.
22. Geypens B, Maes B, Luypaerts A, Ghoos Y, Rutgeerts P. Double deconvolution of breath tests to assess the pharmacological modulation by cisapride of proximal gastrointestinal tract. Gastroenterology 1999;116:A997.
23. Ghoos Y, Coward A. European concerted action. GUT 1998;Suppl 3:1–24.

Stable isotope aided evaluation of Community Nutrition Program: effect of food supplementation schemes on maternal and infant nutritional status

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Abstract

The supplementation program of the community nutrition project (PNC) launched by the Senegalese Government in order to protect the most vulnerable groups (children and women) was evaluated. Using a stable isotope (deuterium), we assessed the effect of the PNC on breastmilk output, mother's body composition, and baby's growth at three months of lactation. Breastmilk triglycerides, lactose, protein, and zinc were also determined. Mothers who were supplemented more than 60 days during pregnancy showed a significant increase in fat-free mass as compared to those who were supplemented for less than 30 days ($p = .03$*). Breastmilk output was not influenced by the supplementation, but breastmilk lactose, total protein, and zinc contents increased significantly (*$p < .01$*) in the supplemented mothers. Growth of the babies of the supplemented mothers was better than that of those whose mothers were not supplemented. It was concluded that the food supplementation had beneficial effects on both mothers' and babies' nutritional status depending on the onset of the supplementation.*

Key words: stable isotopes, breastmilk output, food supplementation, Senegal, West Africa

Introduction

From 1986 to 1993, the prevalence of malnutrition in Senegal has risen from 6% to 12% for children 6 to 36 months old and to 20% for children under five years old [1]. National authorities undertook a series of measures to protect the most vulnerable groups (women and children) and an externally funded high priority community nutrition project (PNC) was launched by the Senegalese Government under the supervision of the Presidency of the Republic [2]. The PNC consisted of three parts:food supplementation for pregnant and lactating women and moderately malnourished children, nutrition education and a follow-up study of child growth, and provision of drinking water. The PNC was launched in 1994, with the support of the World Bank, the World Food Programme, and the German "Kreditanstalt Für Wiederaufbau." The Public Works and Employment Agency (AGETIP) executed the program. A National Commission for Combating Malnutrition was created to monitor the PNC. Community nutrition centers involved in the project use local contractors to carry out certain tasks, such as monitoring malnutrition and educating beneficiaries on issues involving food and sanitation. A supplementary food, based on local foodstuffs (millet and maize) was provided for over six months to pregnant women during their last trimester and to lactating women. The supplementation was carried out through network operational centers in poor urban areas.

A stable isotope, deuterium, was used to assess the effectiveness and quality of the supplementation on breastmilk output, maternal body composition, and babies' growth at three months of lactation. The evaluation aimed to determine the optimal timing of supplementation during pregnancy as well as the composition of breastmilk and nutrient transfer from the mother and from outside to the baby.

Subjects and methods

Subjects

There were 76 supplemented women and 57 age-

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matched controls in the study. All pregnant supplemented women from four sites of the PNC centers: Arafat, Wakhinane,

Hann-Yarakh, and Grand-Yoff were recruited. The control group was recruited from lactating mothers with a baby less than one month old in Wakhinane and Hann-Yarakh. Both groups were comparable in term of socioeconomic conditions, and lived in poor suburban areas of Dakar. Among the beneficiaries 41 received the supplement based on millet and 35 the supplement based on maize. The composition of food supplements is presented in table 1.

Approval of the ethics committee of the University of Dakar as well as consent of the subjects and the community was obtained before starting the study.

Methods

Anthropometric measurements (weight and height) of the mothers and babies were carried out at one, two and three months after delivery. Body mass index (BMI) was calculated as weight/height^2. The babies' anthropometric indices, weight-for age (WA), height-for-age (HA) and weight-for-height (WH), were calculated using the NCHS standards [3].

At three months of lactation, the deuterium dilution method (D_2O) dose-to-mother was used for the determination of breastmilk intake of the babies [4]. The method was tested previously in the field [5, 6]. Briefly, 30 g of deuterium oxide (99.8% purity, Cambridge Isotope Laboratories Inc., Andover Mass., USA) were orally administrated to the mother. Saliva samples were collected from both the babies and the mothers before (pre-dose samples) and after administration of the dose on days 1, 2, 3, 4, 13, and 14 (post-dose samples). Enrichments of the saliva samples were measured using a Fourier transformed infrared spectrophotometer (Shimadzu 8300, Vienna, Austria). Body composition of the mothers (lean body mass and fat mass) was calculated from the total body water component.

TABLE 1. Composition of the food supplement

	Millet	Maize
Grain (%)	55	60.6
Beans (%)	23.6	18
Peanut (%)	11	9
Sugar (%)	10	12
Vitamin-mineral complex[a] (%)	0.34	0.4
Energy (kcal/100 g of dry matter)	422	400

a. The vitamin minerals complex (%) included calcium (100–123 mg), zinc (8–6.15 mg), iron (6–9.84 mg), vitamin A (1300–1600 IU), vitamin C (30–36.9 mg), niacin (5–6.64 mg), vitamin D_1 (100 µg), vitamin B_1 (123 µg), vitamin B_2 (400–492 µg), vitamin B_{12} (1–1.23 µg), and folic acid (50–61.5 µg).

Total body water (TBW) was assumed to be equal to D_2 space divided by 1.04. Fat-free mass was calculated as TBW / 0.73. Body fat was computed as body weight minus fat-free mass.

On day 14, breastmilk samples were manually collected for macro- and micronutrient analysis. Mothers expressed a volume of milk from each breast into a sterile vial. Samples were collected two times during the day, mid-morning and mid-afternoon, with a minimal interval of four hours according to the method of Roquelin et al. [7]. Both samples were pooled for the determination of the breastmilk composition. Lactose was determined by an enzymatic method using a kit for lactose/D-galactose measurement [8]. Triglycerides were measured by an enzymatic method after clarification of breastmilk according to Lucas and al. [9] using a kit for serum determination. Total nitrogen (N) was determined by the Kjeldhal method and protein expressed as N X 6.38. No correction has been made for non-protein nitrogen. Energy was calculated by adding the values for protein, lactose, and fat concentrations using the conversion factors 5.65, 3.95, and 9.25 kcal/g, respectively [10]. Breastmilk zinc content was determined by an atomic absorption spectrophotometer (Analyst 300 Perkin Elmer, Courtaboeuf, France). Skim milk powder supplied by the National Institute of Standards and Technology (NIST, Canada) and the Agricultural Research Center (ARC, Finland) were used as reference materials. Results are expressed as means ± SD and statistical analysis was performed using Epi Info 6.04 (CDC, Atlanta, Ga. USA) and Systat 8.0 (SPSS Inc., Chicago, Ill.,USA).

Results

Table 2 shows the mean age, BMI, and body composition of the mothers at three months postpartum. There were no significant differences between the supplemented and the non-supplemented groups. Body composition of the mothers, according to the duration of the supplementation, is presented in table 3. There was a significant increase in total body water and fat-free mass in mothers who were supplemented more

TABLE 2. Characteristics of the subjects at three months postpartum (mean ± SD)

	Supplemented		Non-supplemented (n = 57)
	Millet (n = 41)	Maize (n = 35)	
Age (yr)	27 ± 6	27 ± 6	27 ± 6
BMI (kg/m^2)	23 ± 3	23 ± 4	22 ± 4
TBW (kg)	28 ± 3	29 ± 4	28 ± 3
Fat-free mass (kg)	39 ± 4	40 ± 5	37 ± 4
Fat mass (kg)	19 ± 6	19 ± 7	19 ± 7

than 60 days ($p < .05$). In contrast, the duration of the supplementation had no effect on their fat mass.

Breastmilk output and metabolic water from outside (water and supplements given to the baby) are showed in table 4. Breastmilk intakes as well as supplements given to the baby were comparable in both groups. However the composition of breastmilk was significantly ($p < .01$) influenced by the supplementation. In the supplemented group, concentrations of lactose, total nitrogen, and zinc increased particularly when the food supplement was based on millet (table 5).

Anthropometric indices of the babies at three months are presented in table 6. WA and HA indices were better ($p < .05$) in the babies of the supplemented mothers than in those of non-supplemented mothers. The WH index was not influenced by the supplementation.

Discussion

The nutritional intervention of the PNC involved pregnant women during their last trimester of pregnancy. The trial was designed to improve nutrient availability during pregnancy and lactation for nutritionally at risk women. The effects of maternal nutrition supplementation on breastmilk output and infants' growth were assessed, and the deuterium dilution method was used to measure the breastmilk intake of the offspring and the body composition of the mothers. The deuterium dilution method has been found to be more accurate than the test weighing technique [11] and has been used in both industrialized and developing countries [4, 12–16]. An isotope ratio mass spectrometer (IRMS) has been used to measure the deuterium enrichment of the samples [17], but recently, a modified version of the technique, based on the analysis of the sample enrichment with a Fourier transformed infrared spectrophotometer (FTIR) was developed. It was tested against IRMS, and found to be accurate and suitable for use in field studies [18]. Using a FTIR and the deuterium dilution method, breastmilk output was not impaired in three-month Senegalese lactating women [5].

The lactation capacity of underprivileged women living in developing countries has been questioned for a long time. Despite its importance for public health, the question of whether poorly nourished women can improve their lactation performance when increasing their dietary intake was conflicting [19]. Several studies in developing countries have reported a positive or no relationship [19–27].

A comparison between the supplemented and the non-supplemented women indicated that breastmilk output was not impaired. In both groups, the breastmilk intake of the babies was within the normal range expected at three months of lactation [20, 23, 27, 28]. Milk-output of these underprivileged mothers was not influenced by the supplement or the duration of the supplementation. These results are in accordance with previous studies conducted in the Gambia [26, 27], Indonesia [25], and Kenya [22], supporting a lack of association between supplementation and breastmilk output.

In contrast, the food intervention had a positive impact on breastmilk quality. Although the composi-

TABLE 3. Body composition of the subjects according to the duration of the supplementation (mean ± SD)

	Duration of the supplementation		
	≤ 30 days ($n = 14$)	30–60 days ($n = 35$)	> 60 days ($n = 28$)
TBW (kg)	27 ± 3	29 ± 3	30 ± 3*
Fat-free mass (kg)	37 ± 3	40 ± 4	41 ± 5*
Fat mass (kg)	19 ± 7	18 ± 6	21 ± 7

* $p < .05$.

TABLE 4. Breastmilk and other intakes of the babies (mean ± SD)

	Supplemented		Non-supplemented ($n = 57$)
	Millet ($n = 41$)	Maize ($n = 35$)	
Breastmilk intakes (g/day)	918 ± 186	992 ± 205	943 ± 207
Other intakes[a] (ml/day)	234 ± 144	150 ± 100	158 ± 145

a. Water or food supplements given to the baby.

TABLE 5. Breastmilk composition (mean ± SD)

	Supplemented		Non-supplemented ($n = 57$)
	Millet ($n = 41$)	Maize ($n = 35$)	
Lactose (g/L)	62 ± 8	60 ± 8	55 ± 8**
Triglycerides (g/L)	29 ± 7	33 ± 10	31 ± 10
Protein (g/L)	15 ± 3	15 ± 2	12 ± 3**
Zinc (mg/L)	1.7 ± 0.7	1.5 ± 0.6	1.3 ± 0.5**

** $p < .01$.

TABLE 6. Anthropometric indices (Z scores) of the babies at three months of age (mean ± SD)

	Supplemented		Non-supplemented ($n = 57$)
	Millet ($n = 41$)	Maize ($n = 35$)	
Weight-for-height	0.33 ± 0.85	0.02 ± 0.76	0.25 ± 1.04
Weight-for-age	0.32 ± 0.85	–0.07 ± 0.86	–0.06 ± 1.06*
Height-for-age	0.01 ± 0.78	–0.18 ± 0.94	–0.40 ± 1.02*

* $p < .05$.

tion of the breastmilk produced by the subjects was within limits observed among well-nourished women for protein, lactose, fat, and total energy [28], the average protein and lactose contents were significantly increased in the supplemented mothers, indicating that the food supplement was beneficial to them. Similar findings have been reported for Gambian women during the farming season [26].

The increase of zinc concentration in the breastmilk of the supplemented women might be related to the enrichment of the food supplement with zinc: 8 mg/100 g in the food based on millet and 6 mg/100 g in the food based on maize.

Until three months of age, the rate of babies' growth in both groups was comparable to NCHS standard curves. However, the rate of growth of the babies from the supplemented mothers exceeded that of the non-supplemented ones. In the non-supplemented group, 10% of the babies were stunted at one month of age (HA < –2 Z scores) compared to 2% in the supplemented group (not shown). Mean stature growth, weight-for-age and height-for-age were higher in the babies of supplemented mothers suggesting growth-limiting nutrients in the breastmilk of these underprivileged women.

The nutritional status of the women at three months of lactation, assessed with BMI, was not influenced by the food supplementation, but the duration of the supplementation during pregnancy had an impact on the body composition of the mothers, resulting in an increment in fat-free mass at three months postpartum. The results suggest that the length of dietary supplementation during pregnancy should be at least 60 days before delivery in order to have an impact on the mothers' body composition.

In conclusion, the PNC nutrition intervention was beneficial to the community. Although the supplementation did not influence the quantity of breastmilk, it significantly changed its composition for the benefit of the babies' growth, particularly when the food supplement was based on millet.

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References

1. Direction de la Statistique et de la Prévision. Enquête démographique et de santé II (EDS II)> Dakar: Ministère de l'Economie, des Finances et du Plan du Sénégal, 1992/1993.
2. World Bank / Agence d'Exécution des Travaux d'Intérêt Public (AGETIP). Staff appraised report: Community nutrition project, Republic of Senegal. Washington, D.C.: World Bank, 1995.
3. World Health Organization. Physical status. The use and interpretation of anthropometry. Report of a WHO expert committee. Technical Report Series No. 854.Geneva: WHO, 1995.
4. Coward AW, Cole TJ, Sawyer MB, Prentice AM. Breastmilk intake measurement in mixed-fed infants by administration of deuterium oxide to their mothers. Hum Nutr Clin Nutr 1982;36C:141–8.
5. Cissé AS. Utilisation de la spectrophotométrie infrarouge à rransformée de Fourier (FTIR) dans l'estimation de la production lactée de femmes dakaroises. Dakar: Université Cheikh Anta Diop, Mémoire de Diplôme d'Etudes Approfondies, 1999;5:27.
6. Cissé AS, Bluck L, Diaham B, Dossou N, Guiro AT, Wade S. Use of Fourier transform infrared spectrophotometer (FTIR) for determination of breast-milk output by deuterium dilution method among Senegalese women. Food Nutr Bull 2002;23(3Suppl): PAGES THIS ISSUE.
7. Roquelin G, Tapsoba S, Mbemba F, Traissac P, Prevel YM. Lipid content and essential fatty acid (EFA) composition of mature Congolese breast-milk are influenced by mothers' Nutritional status: Impact on infants' EFA supply. Eur J Clin Nut 1998;52:164–71.
8. Cunuff P, ed.. Official methods of analysis. Arlington, Va., USA: AOAC International, 1995.
9. Lucas A, Hudson GJ, Simpson P, Cole TJ, Baker BA. An automated enzymatic micromethod for the measurement of fat in human milk. J Dairy Res 1987;54:487–92.
10. Garza C, Butte NF, Dewey KG. Determination of the energy content of human milk. In: Jensen RG, Neville MC, eds. Human lactation 1: milk components and methodologies. New York: Plenum Press, 1985:121–5.
11. Butte NF, Garza C, Smith EO, Nochols BL. Evaluation of deuterium dilution technique against the test-weighing procedure for the determination of breast-milk intake. Am J Clin Nutr 1983;37:996–1003.
12. Butte NF, Wong WW, Patterson BW, Garza C, Peter DK. Human milk intake measured by administration of deuterium oxide to the mother: a comparison with the test-weighing technique. Am J Clin Nutr 1988;47: 815–21.
13. Infante C, Lara W, Vio F. Isotope dilution measurement of breast-milk production in chilean urban mothers. Hum Nutr Clin Nutr 1985;39C:379–86.
14. Orr-Ewing AK, Heywood PF, Coward AW. Longitudinal measurements of breast milk output by a $^2H_20$ tracer technique in rural Papua New Guinea women. Hum Nutr Clin Nutr 1986;40C:451–67.
15. Lucas A, Ewing G, Roberts SB, Coward WA. Measurement of milk intake by deuterium dilution. Arch Dis Child 1987;62:796–800.

16. Butte NF, Garza C, Smith EO, Nichols BL. Human milk intake and growth in exclusively breast-fed infants. J of Pediatr 1984;104:187–95.
17. Coward AW, Whitehead RG, Sawyer MB, Prentice AM, Evans J. New method for measuring milk intakes in breast-fed babies. Lancet 1979;2:13–4.
18. Conway JM, Sadijimin T, Dibley MJ, Kjolhede CL, Caballero B. Infrared spectroscopy assay for deuterium in infant's urine after D_2O administration to the mother: comparison with isotope ratio mass spectrometry. Clin Res 1992;40 (suppl 2):A625.
19. Brown KH, Dewey KG. Relationships between maternal nutritional status and milk energy output of women in developing countries. In: Picciano MF, Lönnerdal B eds. Mechanisms regulating lactation and infant nutrient utilisation. New York: Wiley-Liss, 1992:77–95.
20. Brown KH, Roberston AD, Akhtar NA, Ahmed MG. Lactational capacity of marginal nourished mothers: relationships between maternal nutritional status and quantity and proximate composition of milk. Pediatrics 1986;78:909–19.
21. Naing KM, Oo TT. Effect of a dietary supplement on lactation performance of undernourished Burmese mothers. Food Nutr Bull 1987;9(3):59–61.
22. Van Steenberger WM, Kusin JA, De With C, Lacko E, Jansen AAJ. Lactation performance of mothers with contrasting nutritional status in rural Kenya. Acta Paediatr Scand 1983;72:805–10.
23. Gonzalez-Cossio T, Habitch JP, Rasmussen KM, Delgado HL. Impact of food supplementation during lactation on infant breast-milk intake and on the proportion of infants exclusively breast-fed. J Nutr 1998;128:1692–702.
24. Girija A, Geervani P, Nageswara Rao G. Influence of dietary supplementation during lactation on lactation performances. J Tropic Paediatr 1984;30:140–4.
25. Van Steenberger WM, Kusin JA, Kardjati S, De With C. Energy supplementation in the last trimester of pregnancy in East Java, Indonesia: effect on breast-milk output. Am J Clin Nutr 1989;50:274–9.
26. Prentice AM, Roberts SB, Prentice A, Paul AA, Watkinson M, Watkinson AA, Whitehead RG. Dietary supplementation of lactating Gambian women. I. Effect on breast-milk volume and quality. Hum Nutr Clin Nutr 1983;37C:53–64.
27. Prentice AM, Roberts SB, Watkinson M, Whitehead RG, Paul AA, Prentice A. Dietary supplementation of Gambian nursing mothers and lactational performance. Lancet 1980;2:886–8.
28. WHO. Quantité et qualité du lait maternel: rapport d'un comité d'experts. Geneva: World Health Organization, 1987.

Application of stable isotopic techniques in the prevention of degenerative diseases like obesity and NIDDM in developing societies

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Abstract

Economic development in developing societies characterized by idustrialization, urbanization, and globalization has seen the emergence of an epidemic of diet- and lifestyle-related chronic degenerative diseases. A research project was initiated under the aegis of the International Atomic Energy Agency (IAEA), Vienna, Austria under its Coordinated Research Programme (CRP) to promote the use of stable isotopic techniques to document the extent of the problem and to understand the determinants of this epidemic. The principal objectives of this CRP involving countries both in the North and the South are to define the magnitude of the problem of obesity and non-insulin dependant diabetes mellitus (NIDDM) in developing countries, to identify the vulnerable groups at increased risk, and to attempt to describe the metabolic and physiological mechanisms underlying this phenomenon. These comparative international studies of obesity and NIDDM are looking at the effects of childhood malnutrition (Brazil) and socioeconomic differentials (Mexico) on adult risk factors; the composition of the daily diet on obesity (Chile); levels of patterns of physical activity of older adults (China) as well as their influence on weight gain and obesity (Cuba, Nigeria); the impact of body composition and energy expenditure on the evolution frank diabetes from impaired glucose tolerance (Jamaica), and of body compositional changes and the role of inflammatory cytokines on impaired glucose tolerance (India). The last study conducted in New Zealand was aimed at comparing the energy expenditures of Maori (Pacific Island) with New Zealanders of European descent.

Key words: stable isotopes, chronic diseases, obesity, non-insulin dependent diabetes, cardiovascular disease, insulin resistance, body composition, inflammatory cytokines, glucose tolerance.

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Introduction

Non-communicable diseases (NCDs) account for nearly 60% of deaths globally mostly due to heart disease, stroke, cancer, diabetes, and lung diseases. The rapid rise of NCDs represents one of the major health challenges to global development in the 21st century and threatens economic and social development of nations as well as the lives and health of millions of their subjects. In 1998 alone, NCDs were estimated to have contributed to 31.7 million deaths globally and 43% of the global burden of disease [1]. Based on current trends, it is expected that by the year 2020, NCDs will account for 73% of deaths and 60% of the disease burden. A recent analysis of mortality trends from NCDs suggests that large increases in NCDs have occurred in developing countries [2], particularly those in rapid transition like China, Brazil, and India. The rapid increase in these diseases is seen disproportionately in poor and disadvantaged populations and is contributing to widening health gaps between and within countries. In 1998, of the total number of deaths attributable to NCDs, 77% occurred in develop-

ing countries, and of the disease burden they represent, 85% was borne by low and middle-income countries [1]. This increase in the incidence of chronic degenerative diseases is due to a complex range of factors that interact to determine the nature and course of this epidemic [3].

As developing societies industrialize and urbanize, and as standards of living continue to rise, weight gain and obesity will pose a growing threat to the health of the citizens. Obesity is now widely prevalent in several developing countries, particularly those in rapid transition, and affects both children and adults, and is a significant contributor to the ill health of people in developing countries. Obesity is a key determinant and important risk factor for other NCDs such as non-insulin dependant diabetes mellitus (NIDDM), cardiovascular disease (CVD) including hypertension, and certain cancers. The increasing prevalence of obesity in a population in developing societies is an early indicator of an emerging health burden due to the increasing mortality and morbidity from NCDs.

The principal objectives of this Coordinated Research Programme (CRP) of the International Atomic Agency (IAEA), Vienna Austria; involving countries both in the North and the South are to define the magnitude of the problem specifically of obesity and non-insulin dependant diabetes mellitus (NIDDM) in several developing countries, to identify the vulnerable groups at increased risk, and to attempt to describe the metabolic and physiological mechanisms underlying this phenomenon. This CRP was set up with the following objectives:

» to promote the use of stable isotopic techniques to document the extent of the problem and to understand the determinants of obesity and non-insulin dependant diabetes mellitus (NIDDM) in developing societies,
» to define the magnitude of the problem of Obesity and NIDDM in developing countries,
» to identify the vulnerable groups at increased risk and to attempt to describe the metabolic and physiological mechanisms underlying this phenomenon, and
» to enhance North-South collaboration and transfer of know-how and technology.

The following sections of this paper provide information on each of the studies under this IAEA-CRP Investigative Programme country by country while the principal objectives of these studies are summarized in the table 1.

Brazil

The studies in Brazil were conducted by A. Sawaya and her colleagues at the Department of Fisiologia, Disciplina de Neurofisiologia e Fisiologia Endocrina, Universidade Federal de Sao Paulo, Sao Paulo, Brazil [4] and in partnership with S. Roberts at the USDA HNRC at Tufts University, Boston. Previous studies by this group demonstrated higher energy conservation mechanisms in marginally stunted Brazilian children exemplified by lower resting metabolic rates, lower fat oxidation, higher susceptibility to gain weight, and thus leading to a higher prevalence of stunted but obese individuals. The hypothesis being tested was that living in shantytowns in the city of Sao Paulo, Brazil impairs growth and increases risk of stunting and that this stunting may be associated with alterations in fat patterning, circulating hormones such as insulin and IGF-1 as well as alterations in plasma lipid profiles. The study recruited 58 children of both sexes aged 8 to11 years out of a total of 300 who were screened from a shantytown in Sao Paulo, half of whom were stunted and the other half had normal height-for-age (HAZ). Stunted children (n = 28 with HAZ Z scores < –1.5) were compared to normal children (n = 30 with HAZ Z scores > –1.5). About 98% of the population had low IGF-1 values indicating impaired growth. The stunted boys and girls demonstrated delayed Tanner stages of pubertal development and had lower systolic blood pressures, but higher diastolic blood pressures. Alterations in insulin profile by homeostatic model assess-

TABLE 1. Comparative international studies of obesity and NIDDM under the IAEA-CRP in developing countries

Developing countries	Objective of IAEA-CRP Investigation
Brazil	The effects of childhood malnutrition on adult risk factors
Mexico	The effects of socioeconomic differentials on adult risk factors
Chile	The composition of the daily diet specifically fatty acids in the diet on obesity
China	The physical activity of adults as risk factors for overweight and obesity
Cuba	Total energy expenditure as risk factors for chronic disease in older adults in rural communities.
Nigeria	The influence of physical activity on weight gain and obesity
Jamaica	The influence of physical activity on the evolution of frank diabetes from impaired glucose tolerance
India	Body composition and the role of inflammatory cytokines on impaired glucose tolerance
Maori and Pacific Island	Comparison of energy expenditure of Maori and Pacific Island with New Zealand adults of European descent

ment (HOMA) analysis manifested by lower fasting insulin, higher insulin sensitivity, and lower IGF-1 levels characterized the stunted children. The plasma lipid profile was not different between the two groups. It was concluded that the environment in shantytowns impairs growth potential and that stunted children growing up in these environments have abnormal beta cell function and lower fasting insulin and higher insulin sensitivity.

Mexico

The Mexican studies on the risk factors for NIDDM and cardiovascular disease in adults from different socioeconomic levels was lead by Mauro Valencia Juillerat, at the Centro de Investigacion en Alimentacion y Desarrollo at Hermosillo, Sonora, Mexico [5]. The earlier National Survey of Chronic Disease in Mexico involving 18,924 adults in 8,120 households throughout Mexico [6] found a relationship between educational achievement and prevalence of NIDDM, i.e., 15.6% prevalence in those with no education compared with 2.8% among postgraduates. The present study was conducted on 350 male and female adults over 20 years old in Hermosillo, Mexico. They were recruited from low- and high-income socioeconomic groups. The subjects provided blood samples for an oral glucose tolerance test (OGTT) and for plasma lipids, insulin, and leptin determinations. The prevalence of obesity (BMI > 30) in this sample of the 350 adult men and women was in 23.4%, and it was observed that subjects with abnormal glucose levels had higher body weight, BMI, weight-for-height (WH), % fat, and blood pressure than normo-glycemic subjects. Subjects with high central adiposity (combination of % fat and WH ratio) had higher triglycerides, 2-hour glucose levels, and lower HDL-cholesterol. Waist circumference, WH, BMI, and bio-impedance analysis (BIA) for body composition showed the best correlations with glycemic status, blood lipids, and insulin sensitivity. Detailed body composition studies comparing deuterium dilution with data from other measurements and analysis of physical activity patterns between the two income groups and their relationship to adult risk factors is in progress.

Chile

The role of the composition of the daily diet and more specifically the fatty acids in the diet on the risk of obesity was investigated by Eric Diaz and colleagues [7] at the Energy Metabolism and Stable Isotopes Laboratory, Institute of Nutrition and Food Technology (INTA), University of Chile, Santiago, Chile. The hypothesis is that the type of fatty acids ingested in the diet can modify the disposal of lipids in the body, and that this may be an additional factor in worsening the insulin resistance. The effects of changes in dietary fatty acid composition on macronutrient oxidation were studied in eight normal weight and six obese adult women between 30 to 45 years old. The study followed a cross-over design of one baseline measurement and two week periods of supplementation with either canola oil or sunflower oil with a washout period in between followed by the other oil. The intervention trial comparing sunflower oil with canola showed significant modifications of plasma fatty acid profiles depending on the oil supplemented. No differences were observed between controls and obese. However the control subjects had higher fat oxidation with sunflower compared to canola treatment, while the obese women showed higher carbohydrate oxidation associated with a greater insulin response during the sunflower treatment. Significant changes in plasma n6/n3 fatty acids (n6/n3) ratio were observed. The higher fat oxidation in controls was associated positively with changes in plasma n6/n3 ratio while the higher insulin response in the obese was not associated with changes in plasma n6/n3 ratio.

China

The risk factors for obesity in Chinese adults with obesity, an important public health concern in China, was investigated by Guansheng Ma at the Institute of Nutrition and Food Hygiene, Chinese Academy of Preventive Medicine, Beijing, China [8]. The basic premise for this investigation was the recognition that dietary energy and fat intakes and physical activity levels play a critical role in overweight and obesity, which in turn is a risk factor for hypertension and NIDDM. The objectives of the study were to compare dietary intakes, physical activity patterns, anthropometry, and plasma insulin, leptin, and lipid profiles between normal weight, overweight, and obese adults based on BMI. The study included 152 adults 35 to 52 years old of both sexes. Overweight and obese groups in China have significantly greater BMI, waist, hip, waist/hip ratio, and percent of body fat than the normal weight group. Their total energy and fat intakes were greater than those of the normal weight group, while they expended much less energy. The obese had a significantly higher risk of hypertension, and had higher fasting insulin and leptin levels than the normal weight group.

Cuba

The IAEA CRP in Cuba investigated the energy requirements and physical activity levels of active elderly adults in rural areas. This investigation was lead by Manuel

Hernandez-Triana of the Institute of Nutrition and Food Hygiene at Havana, Cuba [9]. The collaboration was South-South with INTA in Chile. Elderly subjects aged 60 to 74 years ($n = 48$) in a rural mountain community of Western Cuba (Las Terrazas) were studied. Of those, 40% had impaired glucose tolerance and 23% had hypertension. Estimates of total energy expenditure (TEE), as determined by the doubly-labeled water (DLW) method and physical activity levels (PAL) were much higher than that reported for similar age groups in other studies, while PALs estimated from questionnaires underestimated TEE as compared to PALs measured by the DLW method. Dietary energy intakes were underestimated by 11% for women and by 55% for men compared to TEE by DLW.

Nigeria

This investigation on the relative contributions of energy expenditure on physical activity, body composition, and weight gain to the evolution of impaired glucose tolerance into frank diabetes was carried out at the Department of Paediatrics & the Institute of Child Health, University College Hospital, Ibadan, Nigeria [10]. The investigation was lead by Adeyemo in Nigeria and is another example of South-South collaboration and cooperation under the IAEA CRP program as this study in Nigeria is linked with Tropical Metabolism Research Institute (TMRU) in Jamaica. A one year follow-up of a lean cohort of adults (BMI ~21–22) in Nigeria showed an increase in body weight and BMI, an increased prevalence of overweight from 21.3% to 23.9%, an increased prevalence of obesity from 5.2% to 7.7%, and an increased body fat (fat mass and percent body fat mass). There was also a change in physical activity levels with increased fasting insulin and insulin-glucose ratios but no increase in homeostatic model assessment-insulin resistance (HOMA-IR). The body weight changes occurred without any worsening in the glycemia status. The conclusions are that the population is yet to reach the BMI threshold above which worsening of glycemia status accompanies the increases in weight gain. A further follow-up over several years would be required.

Jamaica

A similar follow-up of adults to examine the relative contributions of energy expenditure on physical activity, body composition, and weight gain to the evolution of impaired glucose tolerance into frank diabetes was being carried out at the Tropical Metabolism Research Institute (TMRU), University of West Indies, Mona, Kingston, Jamaica [11]. This investigation was lead by Terrence Forrester with a collaborator (Farook Jahoor) in the North at the USDA Children's Nutrition Research Center, Houston, Texas. In a sample of 614 adults (239 men and 375 women) anthropometry and body composition, energy expenditure budgets and OGTTs were carried out. A follow-up four years later of the cohort of urban Jamaican adults showed that the prevalence of impaired glucose tolerance (IGT) and frank diabetes increased over the same period. Lower physical activity was significantly associated with poorer glucose tolerance status. All adiposity variables, e.g., BMI, percent fat, and waist circumference predicted worsening of glucose tolerance for men while change in waist circumference predicted worsening status in both men and women. It was concluded that interventions to improve levels of physical activity are crucial to reduce the burden of chronic diseases including obesity and NIDDM.

India

The IAEA CRP contract in India was lead by Chittaranjan Yajnik at the King Edward Memorial (KEM) Hospital research center in Pune. The relationships between total body fat, plasma pro-inflammatory cytokines and their role in insulin resistance in Indians was investigated [12]. Like the study in TMRU, Jamaica this study also establishes a South-South Collaboration with St Johns Medical College, Bangalore with Anura Kurpad and All India Institute of Medical sciences (AIIMS) New Delhi with Anoop Misra. It also has North-South collaboration with John Yudkin at University College, London and Prakash Shetty at the London School of Hygiene & Tropical Medicine. The hypothesis for investigation was that the excess risk of insulin resistance in urban as compared to rural populations in India was the result of increased total body fat in the former, and that the increased total body fat resulted in insulin resistance which is mediated by changes in circulating pro-inflammatory cytokines. Studies in Pune, India in three different communities (rural/urban, slum/urban, middle-class) showed that the prevalence of obesity and central obesity (deposition of adipose tissue centrally distributed around the abdomen) progressively increased from rural to urban slums to urban middle-class men. This was reflected in an increasing prevalence of diabetes (0, 4% and 10%), impaired glucose tolerance (9%, 12%, and 20%), hypertension (2%, 4%, and 10%), and plasma cholesterol and triglyceride levels, respectively. The percentage of body fat was a significant predictor of increasing cardiovascular risk in these populations; central obesity increased the risk further, although to a smaller extent. The study concluded that measurement of body fat and its central distribution by appropriate isotopic and other methods should form an essential part of further studies of insulin resistance and cardiovascular disease risk in South Asians.

Maoris and Pacific Islanders

Elaine Rush of the Auckland University of Technology, New Zealand led the IAEA-CRP investigation on central obesity and risk for NIDDM in Maori, Pacific Island, and European young men in New Zealand [13]. This study compared the characteristics of normoglycemic young men 18 to 27 years old of Maori, Pacific Island, and New Zealand European descent ($n = 10$ in each group). An increased body fat content and central obesity were associated with measurements of glucose, insulin, lipids, and leptin indicating an increased risk of NIDDM. Central obesity was negatively associated with dietary fiber intake. TEE data using the DLW method are in the process of being analyzed. Body fat content and distribution of body fat predicted at a young age the increase in risk of NIDDM in all three ethnic groups. Further investigations are required to investigate differences between groups.

Conclusions

The following conclusions can be arrived at from the preliminary data from several investigations in countries in Latin American and the Caribbean, African, and Asian, and the Far Eastern regions of the developing world:

» This IAEA-CRP offered a unique opportunity to study physical activity, body composition in relation to insulin resistance (NIDDM risk), and obesity risk in seven different countries (table 1) using stable isotopic and other techniques to investigate physical activity patterns, total energy expenditure, body composition, and anthropometric characteristics.

» The IAEA-CRP provided an opportunity to use standardized protocols for body composition and physical activity measurements as risk factors for chronic diseases (obesity and NIDDM) in several developing countries despite variations in age, ethnicity, and geographic locations of the study populations.

» The preliminary results of this IAEA-CRP activity confirmed the increasing risk of obesity and NIDDM in developing societies due to changes in diet and physical activity patterns and suggest that total body fat and its topography are perhaps the most important predictors of the evolution of insulin resistance syndrome.

The current IAEA-CRP program on the application of stable isotopic techniques in the prevention of degenerative diseases like obesity and NIDDM in developing societies demonstrates, in addition, that both North-South and South-South collaboration and cooperation can be very successful under international programs like these.

References

1. World Health Organization. Global strategy for the prevention and control of non-communicable diseases. Geneva: World Health Organization, 2000.
2. Murray CJL, Lopez AD. Global comparative assessments in the health sector. Geneva: World Health Organization, 1994.
3. Shetty PS. Diet and life-style and chronic non-communicable diseases: what determines the epidemic in developing societies? In: Krishnaswami K, ed. Nutrition research: current scenario and future trends. New Delhi: Oxford & IBH Publishing Co., 2000:153–67.
4. Sawaya AL, Fernandes MTB, Martins PA. Effects of childhood stunting on the increase of risk factors for obesity, NIDDM and coronary heart disease. Progress report submitted to IAEA. Sao Paulo, Brazil: Department of Fisiologia, Disciplina de Neurofisiologia e Fisiologia Endocrina, Universidade Federal de Sao Paulo, June 2001.
5. Valencia-Juillerat M, Gallegos AC, Ballesteros MN, Macias N, Ortega MI, Esparza J, Tobles-Sardin A, Morales GG, Pacheco BI, Bolanos A, Cabrera RM, Calderon de la Barca AM. Risk factors for NIDDM and cardiovascular disease in adults from different socioeconomic levels. Progress report submitted to IAEA. Sonora, Mexico: Centro de Investigacion en Alimentacion y Desarrollo at Hermosillo, June 2001.
6. Secretaria de Salud. National survey of chronic disease in Mexico. Mexico, DF: Encuesta Nacional de Enfermedades Cronicas (ENEC), 1994.
7. Diaz E, Galgani J, Morales I, Salazar G, Uauy R. Fat and carbohydrates in the diet: its metabolic contribution to obesity in Chilean women. Progress report submitted to IAEA. Santiago, Chile: Energy Metabolism and Stable Isotopes Laboratory, Institute of Nutrition and Food Technology (INTA), University of Chile, June 2001.
8. Guansheng M. The risk factors for obesity in Chinese adults. Progress report submitted to IAEA. Beijing, China: Institute of Nutrition and Food Hygiene, Chinese Academy of Preventive Medicine, June 2001.
9. Hernandez-Triana M, Aleman-Mateo H, Vallencia-Juillerat M, Salazar G, Sanchez V, Basabe B, Gonzalez S, Diaz ME, Toledo E, Cabrera A, Diaz M, Quintero ME, Moreno R, Moreno V, Sanchez MS, Martin I, Miranda A. Energy requirements and physical activity levels of active elderly adults in rural areas of Cuba. Progress report submitted to IAEA. Havana, Cuba: Institute of Nutrition and Food Hygiene, June 2001.
10. Adeyemo AA, Omotade OO, Forrester TE, Luke A, Rotimi C, Owoaje ET. Relative contributions of energy expenditure on physical activity, body composition and weight gain to the evolution of impaired glucose tolerance into frank diabetes. Progress report submitted to IAEA. Ibadan, Nigeria Department of Paediatrics & Institute of Child Health, University College Hospital, June 2001.

11. Forrester T, Wiilks R, Jahoor F, Adeyemo A, Gaskin P, Luke A. Relative contributions of energy expenditure on physical activity, body composition and weight gain to the evolution of impaired glucose tolerance into frank diabetes. Progress report submitted to IAEA. Kingston, Jamaica: Tropical Metabolism Research Institute (TMRU), University of West Indies, June 2001.
12. Yajnik C, Yudkin JS, Shetty PS, Kurpad AV, Misra A. Relationships between total body fat, plasma pro-inflammatory cytokines and their role in insulin resistance in Indians. Progress report submitted to IAEA. Pune, India: King Edward Memorial EM Hospital Research Centre, June 2001.
13. Rush E, Laulu MS, Mitchelson E, Plank L. Central obesity and risk for NIDDM in Maori, Pacific Island and European young men in New Zealand. Progress report submitted to IAEA. Auckland: Department of Applied Science, Auckland University of Technology, June 2001.