Calcium Does Not Inhibit Iron Absorption or Alter Iron Status in Infant Piglets Adapted to a High Calcium Diet1,2,3

Ine P. M. Wauben and Stephanie A. Atkinson4

Department of Pediatrics, McMaster University, Hamilton ON L8N 3Z5, Canada.

ABSTRACT The purpose of this study was to investigate whether a dietary calcium:iron ratio similar to that often consumed by premature human infants inhibits iron absorption in infant piglets adapted to a high calcium diet. Male Yorkshire piglets were randomized at 3 to 4 d of age to a high calcium diet (4.67 g/L = HC) or a normal calcium diet (2.0 g/L = NC) and fed for 2 to 2.5 wk. An iron dextran injection was administered in amounts to achieve a marginal state of iron repletion to simulate iron status of premature infants. In vivo iron absorption from the diet was determined using the radiotracers 55Fe and 59Fe and whole body counting. Calcium:iron interactions at absorption sites in piglets fed HC and NC were investigated by measurements of time-dependent 59Fe uptake in response to different calcium:iron ratios in vitro in brush border membrane vesicles (BBMV). In vivo iron absorption from the diet did not differ between NC and HC diet groups [57 ± 8% versus 55 ± 17% (mean ± sd), respectively]. Iron status and iron concentrations in spleen, liver, intestine, kidney and heart did not differ between diet groups. Iron uptake in BBMV was significantly reduced by calcium in both HC and NC (P < 0.001); but there were no significant differences in iron uptake in response to different calcium:iron ratios between HC and NC. With feeding a HC diet for 2 wk there may be an adaptive response to counteract the inhibitory effects of calcium on iron absorption, thus resulting in similar in vivo iron absorption and iron status irrespective of the 1.3-fold difference in dietary calcium:iron ratio between piglet groups. However, future studies are needed to determine the specific sites of calcium:iron interactions and adaptation mechanisms. Since the calcium:iron ratios used in this study reflect the usual calcium:iron ratios in diets for premature infants, it is unlikely that interactive effects of calcium with iron will compromise iron status in this infant population when diets are supplemented with calcium. J. Nutr. 129: 707–711, 1999.

KEY WORDS: • infant piglets iron absorption • calcium: iron interactions • adaptation • high calcium diet.

Previous studies in animals and humans have demonstrated that calcium reduces iron absorption (Barton et al. 1983, Cook et al. 1991, Dawson-Hughes et al. 1986, Hallberg et al. 1991 and 1992b). Based on these findings, it was suggested that infants fed a diet with a high calcium content may be at risk for developing iron deficiency (Barton et al. 1983, Hallberg et al. 1992b).

The issue of calcium:iron interactions is important for premature infants. Supplementation of calcium and phosphorus is common practice for premature infants fed their mother’s milk or formulas designed for premature infants during the early neonatal period to prevent osteopenia (Schanler 1995). Furthermore, to optimize growth and bone mineralization calcium and phosphorus supplementation of formulas that are intended for feeding to premature infants after discharge from hospital is gaining interest (Chan 1993, Lucas et al. 1992). Of particular relevance is the fact that the premature infant has a marginal iron status at birth and is more vulnerable to becoming iron deficient compared to an infant born at term (Friel et al. 1990).

To extrapolate to premature infants the results of calcium:iron interaction studies of adult humans or animals requires caution for several reasons. The meals consumed in adult human or animal studies are more complex in composition than the liquid formulas or breast milk consumed by infants. Complex diets may contain specific components, such as fiber, which partially account for the reduction in iron absorption. The subjects in adult studies are in an iron replete status. Premature infants have high iron needs and thus might have a greater efficiency of absorption compared to adults. Further, most of the reported studies were performed with single test meals, ignoring the possibility that a subject may adapt its iron metabolism to counteract the inhibition of iron absorption by a diet high in calcium. Finally, the source of supplemental calcium may be important (Prather and Miller 1992). Studies in adults have used calcium chloride (Hallberg et al. 1992b), calcium carbonate, calcium citrate, hydroxyapatite or calcium phosphate (Cook et al. 1991, Dawson-Hughes et al. 1986,

1 Supported by the Dairy Farmers of Canada.
3 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.
4 To whom correspondence should be addressed.

0223-1669/99 $3.00 © 1999 American Society for Nutritional Sciences.
Hallberg et al. 1991). Premature infants, however, are usually supplemented with calcium tribasic or calcium gluconate and potassium phosphate. More recently, calcium glycerophosphate (CaGP) was used in mother's milk fortifiers (Schanler and Abrams 1995, Wauben et al. 1998).

There is no information as to whether calcium supplementation inhibits iron absorption or alters iron status in infant populations. Because calcium supplementation of premature infants is maintained over a prolonged period of time, it is important to determine if continuous exposure to a high calcium diet inhibits iron absorption. In previous studies, the infant piglet was used as a model for premature infants to study mineral metabolism (Atkinson et al. 1993). The benefits of using the piglet as a model for infant nutrition research is that gut structure and functions are similar to that of humans (Moughan and Rowan 1989). Further, the digestive system in the newborn piglet is less developed at birth than the human newborn infant (Moughan et al. 1992) making the piglet a more appropriate model for the preterm infant than other animal models.

To address the issue of calcium:iron interactions in infant populations, we hypothesized that, when consumed over a prolonged period of time, a diet high in calcium will not inhibit iron absorption or compromise iron status. Our objectives were to study in infant piglets the influence of a diet high in calcium on 1) in vivo iron absorption, 2) in vitro iron uptake at intestinal absorption sites and 3) iron status and tissue iron stores.

MATERIALS AND METHODS

**Animals.** Eighteen 3–5-d-old male Yorkshire piglets were removed from the sows at the Swine Research Facility, Arkell Farms (Guelph, Ontario, Canada) and transported to the McMaster University Central Animal Facility. All procedures were in agreement with the Guide for the Care and Use of Laboratory Animals (Canadian Council on Animal Care 1993). Upon arrival, the piglets were randomly assigned to a regular piglet formula (NC) (n = 9) or to a high calcium piglet formula (HC) (n = 9). At that time an iron dextran injection providing 100 mg elemental iron was administered intramuscularly. Iron concentration in the experimental diets was adjusted in combination with the modified iron dextran dose to achieve a marginal state of iron repletion (defined as a hemoglobin concentration below the normal range) to simulate iron status of the premature infant.

**Experimental diets.** The composition of the liquid piglet formulas is shown in Table 1. The high calcium concentration in HC was achieved by adding CaGP to the diet. The increase in the molar calcium:iron ratio in HC from NC (relative increase calcium:iron ratio of 1.3; calcium:iron ratio NC = 230 and HC = 540) was similar to the increase in molar calcium:iron ratio in premature infants fed mother's milk with additional calcium (relative increase calcium:iron ratio of 1.3; calcium:iron ratio of preterm mother's milk ∼400; and preterm mother's milk with calcium supplements ∼935), but was somewhat greater than the increase in the calcium:iron ratio when term infant formulas are supplemented with calcium (relative increase calcium:iron ratio of 0.4; calcium:iron ratio of term formula ∼540 and term formula plus additional calcium ∼780).

**Study design and analysis.** After arrival at our Central Animal Facilities the piglets were weaned to the regular liquid piglet formula (NC). At 4–5 d of age when the piglets tolerated feeds of 400 mL/kg·d, the HC formula was introduced to piglets assigned to that group. Weaning to the experimental diets was achieved by 7 d of age. The experimental diets were then fed for 2–2.5 wk and piglets were killed at 21–24 d of age.

**TABLE 1**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>NC/HC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, g/L</td>
<td>42</td>
</tr>
<tr>
<td>Whey powder2</td>
<td>6</td>
</tr>
<tr>
<td>Skim-milk powder3</td>
<td>36</td>
</tr>
<tr>
<td>Fat, g/L</td>
<td>36</td>
</tr>
<tr>
<td>Carbohydrate, g/L5</td>
<td>76</td>
</tr>
<tr>
<td>Energy, kJ/L</td>
<td>3308</td>
</tr>
<tr>
<td>Calcium, mg/L6</td>
<td>1996/46661</td>
</tr>
<tr>
<td>Phosphorus, mg/L7</td>
<td>1422/34851</td>
</tr>
<tr>
<td>Iron, mg/L8</td>
<td>11.8</td>
</tr>
<tr>
<td>Zinc, mg/L9</td>
<td>6.24</td>
</tr>
<tr>
<td>Copper, µg/L10</td>
<td>780</td>
</tr>
<tr>
<td>Vitamin mixture11</td>
<td></td>
</tr>
<tr>
<td>Methyl mixture, g/L12</td>
<td>4</td>
</tr>
<tr>
<td>Molar calcium:iron ratio</td>
<td>230/5401</td>
</tr>
</tbody>
</table>

1 Normal (NC, 2.0 g/L Ca)/High (HC, 4.67 g/L Ca).
2 Electrolyzed whey (53 g/L) (Wyeth Canada, Windsor, ON, Canada).
3 Skim milk powder (82 g/L) (Wyeth Canada).
4 30% Corn oil (Best Foods Canada, Etobicoke, ON) and 70% Canola oil.
5 Lactose from skim milk.
6 CaGP (Paul Lohmann, Emmerthal, Germany) and CaCO3 (Fischer Scientific, Toronto, ON, Canada).
7 Phosphorus derived from native elements in skim milk and whey powders plus calcium glycerophosphate (as detailed in footnote8).
8 Iron from skim milk and whey powders plus added as FeSO4.
9 Zinc from skim milk and whey powders plus added as ZnSO4.
10 Copper from skim milk and whey powders and added as CuSO4.
11 Additions provided the following per L of liquid formula: 0.8 mg retinol, 0.6 mg thiamin, 8.8 mg niacin, 4 mg pantothenic acid, 0.5 mg vitamin B-6, 0.2 mg folacin, 8 µg vitamin B-12, 40 µg D-Biotin, 120 µg ascorbate, 0.6 g choline chloride, 2 mg all-rac-a-tocopherol acetate and 1.8 µg cholecalciferol acetate.
12 Methyl cellulose (BDH Chemicals, Prole, UK).

**Growth and iron status.** Piglets were weighed prior to each morning feeding. Weight was determined to 1 g (Sartorius, Goettingen, Germany). Length was measured at three time points during the protocol by measuring snout to rump length when piglets were anesthetized for blood sampling. Length was measured with a non-stretchable tape measure. Blood samples from food-deprived piglets were collected in dry heparin by using an intraluminal jugular blind-stab technique while piglets were under light anesthesia (isoflurane gas). Anesthetized piglets were dosed with an iron-containing radiotracer (55Fe) to determine iron absorption capacity between piglets.

Iron absorption was determined, in the absence of calcium, from a 5 mL reference dose consisting of an aqueous solution containing 3 mg ferrous sulphate, 30 mg ascorbic acid and 111 MBq 55Fe. 55Fe absorption was calculated from incorporation of 55Fe into red blood cells 6 h post dosing adjusting for isotopic decay, assuming a blood volume of 90 mL/kg body weight (Talbot & Swenson 1970) and assuming that 80% of absorbed iron was incorporated into red blood cells (Davidson et al. 1990). 55Fe was quantified in whole blood samples after digestion and decolorization by the modified method of

---

**Abbreviations used:** BBMV, brush border membrane vesicles; CaGP, calcium glycerophosphate; Hb, hemoglobin; HC, high calcium diet; Hct, hematocrit; NC, normal calcium diet.
Eakens and Brown (1966). The samples were counted using a Phillips PW4700 scintillation counter (Phillips, The Netherlands) with an efficiency of approximately 20%. Iron absorption from the experimental diets was determined with the tracer $^{55}$Fe. NC or HC formula (25 mL) was labeled with 74–148 MBq $^{59}$Fe and equilibrated over a 3-h period. Both the $^{55}$Fe and $^{59}$Fe doses were administered by oral gavage after a period of 9 h where food was withheld and followed by a 3-h period where food was withheld. $^{59}$Fe absorption from the diet was determined 6 d post dosing by whole body counting using iodide crystals (Engineered by the Department of Nuclear Medicine, McMaster University, Hamilton, ON). Whole body counts of $^{59}$Fe crystals (Engineered by the Department of Nuclear Medicine, McMaster University, Hamilton, ON) was stopped by addition of 100 mL of the reference $^{59}$Fe standard was used to adjust for radioactivity decay. The activity was determined immediately prior to tracer administration, and analyzed in a nuclear data multichannel analyzer system. Baseline activity was measured immediately prior to tracer administration, and subsequent activity was determined in quadruplicate at 3 h on d 0 and d 1–6. The coefficient of variation for each set of counts was generally <3%. Each measurement was adjusted for background counts, and a reference $^{59}$Fe standard was used to adjust for radioactivity decay. The fraction of the oral $^{59}$Fe dose retained by the piglet at a specific time was determined as follows:

$$\text{Fe absorption} = \frac{\text{CPM} - \text{BG} \times \text{ST} - \text{BG}}{\text{CPM}0 - \text{BG}0 \times \text{ST} - \text{BG}},$$

where CPM is the average counts per minute of the piglet, BG is background count, ST is CPM of the reference $^{59}$Fe standard and time 0 is CPM on d 0.

In vitro iron uptake in brush border membrane vesicles. After necropsy a segment of ~60 cm of the jejunum was removed. Segments were rinsed and scraped while on ice to obtain mucosa for the preparation of brush border membranes vesicles (BBMV). Intestinal BBMV were obtained by employing a magnesium precipitation/differential centrifugation method (Davidson and Lönnerdal 1988, Kessler et al 1978). Briefly, mucosa were homogenized in a hypotonic buffer, basolateral membranes and intracellular components were precipitated with MgCl$_2$, and differential centrifugation produced a final pellet of purified brush border membrane fragments. This final pellet was resuspended in a BBMV buffer solution (112 mmol NaCl/L, 100 mmol D-mannitol/L, 10 mmol tris-HEPES/L at pH 6.8). The prepared BBMV of five piglets in each diet group were used in $^{59}$Fe uptake studies the same day. To assess purity of BBMV, the content of the purified BBMV was measured by the colorimetric procedure outlined by Muir et al. (1984) was used. Initial experiments were performed to determine uptake kinetics, optimal pH, protein concentration and iron concentration conditions for iron uptake by BBMV of piglets. Iron uptake in response to different calcium:iron ratios in BBMV derived from seven piglets fed NC and five piglets fed HC.

To perform iron uptake experiments, a modification of the procedure outlined by Muir et al. (1984) was used. Initial experiments were performed to determine uptake kinetics, optimal pH, protein concentration and iron concentration conditions for iron uptake by BBMV of piglets. Iron uptake in response to different calcium:iron ratios in the BBMV derived from piglets fed HC or NC was determined as follows: 500 μL of BBMV (containing between 450 and 750 μg protein) was added to a 500 μL incubation solution (0–117 mmol CaCl$_2$/L, 0.25 mmol FeSO$_4$/L, 37 MBq $^{59}$Fe and 5 mmol ascorbic acid/L at pH 6.8). A 20-fold molar excess of ascorbate was added, and the incubation solution was saturated with N$_2$ to ensure that iron was maintained in the iron(II) form. Isoosmolarity was maintained by adjustment of NaCl concentration in the BBMV buffer solution. The maximum concentration of CaCl$_2$ that could be used to maintain isoosmolarity was 117 mmol/L. Thus the final molar calcium:iron ratios to which BBMV were exposed were 0.56, 112 and 235. After 1, 5, 10, 15 and 20 min incubation, 50 μL of the BBMV-incubation solution was transferred in triplicate to a Millipore filter (Type HA, 0.45μm, diameter 25 mm, Millipore, Groton, CT), and $^{59}$Fe uptake was stopped by addition of 100 μL ice cold stop solution (0.1 mmol FeCl$_3$/L, 100 μmol citrate/L at pH 7.0). Then the BBMV were collected on the Millipore filters using a vacuum. The filters were rinsed with 5 mmol EDTA/L to remove extracellular bound $^{59}$Fe. To determine iron uptake, the filters were counted using a γ-counter (Minimax, auto-gamma, Packard-Canberra, Canada). Iron uptake in response to different calcium:iron ratios was measured in BBMV digested serum and in organs was determined by flame atomic absorptionmetry (Perkin Elmer, Norwalk, CT).

**Statistical analysis.** The Student’s t-test was used to determine differences between diet groups for in vivo iron absorption and tissue iron concentrations. A two-way ANOVA followed by the Student-Newman-Keuls multiple means test was applied to determine differences between diet groups for hemoglobin (Hb) concentrations and hematocrit (Hct) at different time points and for iron uptake at different calcium:iron ratios in BBMV. The statistical analysis were performed with SigmaStat Software (Jandel Scientific, San Rafael CA). Data are expressed as the mean ± SD, unless stated otherwise. The level of significance for all tests was $P < 0.05$.

**RESULTS**

**Growth and iron status.** Initial body size and growth during the study did not differ between diet groups. The dextran dose given intramuscularly combined with a modified iron content in the liquid formula resulted in a marginal iron repletion state in the piglets. As shown in Fig. 1, the Hb concentration of all piglets was below the normal range but above iron deficiency concentrations (Underwood 1977) after adaptation to the experimental diets. No differences between diet groups were observed in Hb concentrations or Hct, Hb and Hct increased significantly ($P < 0.001$) over time. No differences were observed in serum iron at necropsy (24.6 ± 14.7 and 28.5 ± 12.9 mmol/L for NC and HC, respectively), but mean values were in the lower range of normal values [17.7–53.2 mmol/L (Pond and Houpt 1978)] with 3 piglets fed NC and two fed HC being below the normal range.

Iron concentrations in organs (spleen: 23.3 ± 5.5 and 23.8 ± 6.06; liver: 11.4 ± 5.6 and 14.2 ± 8.2; intestine: 3.6 ± 1.6 and 3.0 ± 0.7; heart: 6.0 ± 2.4 and 6.0 ± 1.8; kidney:...
4.1 ± 1.4 and 4.4 ± 1.7 µmol/g dry tissue for NC and HC, respectively) did not differ between diet groups.

**In vivo iron absorption.** There were no significant differences in measurements of iron absorption from the diet between NC and HC piglets (55 ± 7 and 57 ± 17%, respectively). To control for variation in iron absorption capacity among piglets, 55Fe absorption from the diet was expressed as a ratio of 55Fe absorption. There were no differences between diet groups in the 55Fe/59Fe ratio, which was 0.66 ± 0.15 and 0.66 ± 0.17 for NC and HC, respectively.

**In vitro iron uptake in brush border membrane vesicles.** Initial experiments, measuring Fe uptake at 4 and 37°C, determined that iron uptake in BBMV was mediated by a facilitated transport mechanism. Other conditions for optimal iron uptake in BBMV were pH: 6.75–7.00; protein concentration: 0.10–0.50 mmol/L incubation solution; and optimal iron concentration: 0.10–0.50 mmol/L incubation solution. Iron uptake in BBMV in response to incubation with calcium:iron ratios of 56, 112 and 235 was significantly (P < 0.001) suppressed after 20 min compared to incubation with a calcium:iron ratio of 0 in both diet groups (Fig. 2). No significant differences in iron uptake in BBMV were present between HC and NC at any calcium:iron ratios (Fig. 2).

**FIGURE 2** Time-dependent iron uptake in brush border membrane vesicles of piglets fed normal (NC, 2.0 g/L) (n = 7) (a) and high (HC, 4.67 g/L) calcium diets (n = 5) (b) in response to calcium:iron ratios of 0, 56, 112 and 235. Values are means ± SEM. Values with different letters represent a significant difference (P < 0.001) in iron uptake at 20 min.

Thus a high efficiency of iron absorption in the infant piglets, possibly also as a result of their marginal iron status, may explain in part why the inhibitory effect of calcium on iron absorption was not observed in vivo. It is also possible that after adaptation to a diet high in calcium, an adaptive response to the inhibition of iron by calcium occurs, possibly involving upregulation of iron transfer across the brush border membrane. In adult humans, the findings of Minihane and Fairweather-Tait (1998) suggested an adaptive response to the inhibition in absorbable iron in the presence of calcium. Most previous studies, which have investigated calcium:iron interactions using single test meals, have ignored the possibility of the presence of an adaptation in iron metabolism in response to continuous inhibition of iron uptake by calcium.

The location(s) within the enterocyte where calcium may interact with iron uptake remains speculative. The inhibitory mechanism of calcium on iron absorption may involve transfer
from the mucosal cell into the circulation (Barton et al. 1993; Hallberg et al. 1991a; Wient et al. 1996). Alternatively, calcium may compete for iron binding sites on the intestinal shuttle protein mobilferrin, which could interfere with intestinal iron uptake and intracellular transport (Conrad et al. 1993; Wolf and Wesling-Resnick 1994). From the measurements of iron uptake in BBMV, it appears that calcium and iron do compete for uptake by the enterocyte. No differences, however, were found in iron uptake between BBMV from HC and NC piglets, suggesting that an adaptation to counteract the inhibitory effect of calcium on iron did not occur by upregulating iron binding sites on the brush border membrane. However, other possible sites of calcium:iron interactions or adaptation were not investigated in our in vitro experiments. Further, it is possible that the number of piglets used for BBMV experiments was too small. Another explanation for this may be that in vitro the chelation of iron on the inside of the membrane of BBMV is a reversible process in the presence of chelating agents such as EDTA (Muir et al. 1984). EDTA was used for removal of extracellular bound $^{59}$Fe in the BBMV experiments. In vivo, however, the chelation of iron inside the enterocyte is likely irreversible, as iron is further metabolized by intracellular mechanisms and is not available for transport out of the enterocyte. Future studies will be needed to investigate the specific sites of calcium:iron interactions and possible adaptation mechanisms.

In summary, this is the first report addressing calcium:iron interactions in an appropriate infant-animal model for extrapolation to the issues related to nutrition of premature infants. A diet high in calcium did not inhibit iron absorption and it can be speculated that there may be an adaptive response to the inhibition of iron absorption by calcium to meet the increased iron needs in the presence of a high calcium diet. The specific mechanisms of such an adaptation remains to be determined. At amounts currently used in premature infant diets, calcium supplementation will likely not compromise iron status in early neonatal life.

ACKNOWLEDGMENTS

We thank Colin Webber for his assistance with the whole body counter.

LITERATURE CITED


CALCIUM-IRON INTERACTIONS IN INFANT PIGLETS