Dose Effect of Bovine Lactoferrin Fortification on Iron Metabolism of Anemic Infants

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Summary
To evaluate the effect of iron-fortified formula with different concentrations of bovine lactoferrin (bLF) on improvement of anemic status in term infants who were previously breast-fed. A randomized, controlled, open, and post-market intervention study. A total of 108 infants aged 6–9 mo who were previously breast-fed and weaned were selected. The subjects were divided into three groups with the sequence of outpatient: fortified group 1 (FG1) with a bLF concentration of 38 mg/100 g, FG2 with 76 mg/100 g bLF, FG0 with no bLF. The intervention duration was 3 mo. Weight, height, head circumference and the concentration of hemoglobin (Hb), serum ferritin (SF), serum transferring receptor (sTfR) were measured and sTfR-SF index (TFR-F index) and total body iron content (TBIC) were computed before and after intervention. The primary outcome measures were obtained from 96 infants (35, 33 and 28 for FG0, FG1 and FG2, respectively). After 1 mo of intervention, the changes of Hb level showed no significant difference (p > 0.05) among the three groups, however, the Hb level of infants in FG2 were significantly higher than those of infants in the other two groups after 3 mo of intervention (p < 0.05). The present data indicated that the formula fortified with 76 mg/100 g bLF positively affected the Hb of anemic infants who were previously breastfed when compared with fortification with 38 mg/100 g bLF and no bLF fortification.

Key Words
weaned infant, anthropometric index, hemoglobin, ferritin, transferring receptor, TFR-F index, total body iron content

Anemia, which has been associated with increased morbidity and mortality and especially for child with mental and motor development problems, is a global public health issue that impacts health of 47.4% of preschool children, 41.8% of pregnant women, and 30.2% of non-pregnant women (1, 2). Despite considerable advancement in understanding of iron metabolism (3), the global prevalence of anemia has barely changed in the past decade. The World Health Organization (WHO) has suggested increasing iron intake, controlling infection, and improving nutritional status as preventive measures for iron deficiency (ID) and anemia (4), with the priority being enhancing iron absorption (4, 5), particularly for areas with a high prevalence of anemia, poor economy, and underdeveloped nutrient fortification technology.

Human milk has a low concentration of iron, which decreases during the course of lactation (6), but iron bioavailability in breast milk is high due to the presence of lactoferrin (LF) that can promote iron absorption from breast milk (7, 8). As the major iron-binding protein in human milk, LF serves as a delivery system of iron (9). Bovine LF (bLF) has similar structure, biochemical properties, and bioactivity in comparison to human LF and can be used as a supplement to promote iron absorption (10).

Studies have explored the effect of bLF on iron absorption of infant and suggested that formula fortified with bLF could significantly increase hematocrit, serum ferritin (SF), and reticulocyte levels (11–16). Our previous study (17) also indicated that formula fortified with 38 mg/100 g of bLF positively affected the hemoglobin (Hb) and iron status of infants who were previously exclusively breastfed and suggested that the effects of bLF fortification on iron metabolic homeostasis were mainly exerted through promoting total body iron...
content (TBIC) and iron absorption in intestine. However, most of the subjects in our previous study were in good health and their Hb concentrations were above the defined Hb levels of anemia. To our knowledge, no study has examined the effect of bLF fortification on health of anemic infant with imbalanced iron metabolic homeostasis.

In the present study, we explored the effect of iron-fortified infant formula containing different concentrations of bLF on iron metabolism of anemic infants who were previously breastfed and then formula-fed at 6 to 9 mo. The primary goal was to evaluate whether the iron-fortified formula with different concentrations of bLF would significantly improve the anemic status in term infants and to determine the appropriate dose of bLF.

METHODS

Participants and ethical approval. This randomized, controlled, and open intervention study was performed in the Baoxing County of Ya’an City and Qingyang District, Qingbaijing District, Dayi County, and Hehuachi Community of Chengdu City, Sichuan Province, western China from March 2017 to March 2018. One hundred and eight infants, who were previously breastfed, but weaned and formula-fed at 6 to 9 mo, were randomly recruited from five Women and Children’s Health Care Hospitals in the five described regions.

The eligibility criteria for participation were as follows:
1. Good health without common obstetric risk factors;
2. Hb concentration >60 g/L and diagnosed with anemia;
3. Serum C-reaction protein (CRP)<10 mg/L;
4. Parent’s or guardian’s approval for participating in all aspects of the study and the provision of voluntarily written informed consent from parents of the child;
5. Parent’s or guardian’s agreement to avoid additional use of infant formulas and foods and nutrients fortified by iron during the investigation.
6. No supplementation of iron before recruitment.

Exclusion criteria included history of severe, persistent, or chronic diarrhea; severe malnutrition; serious infections requiring hospitalization in the prior month; serious chronic illness; personal or family history of allergy to cow’s milk or infant formula; eczema; allergic rhinitis; and asthma.

Ethical consideration. The enrollment and research plan were reviewed and approved by the institutional ethics committee of the Chengdu Women’s and Children’s Central Hospital, School of Medicine, University of Electronic Science and Technology of China in Sichuan province, China (ethical approval number: 2014(11)). The present study complied with the code of ethics of the World Medical Association (Declaration of Helsinki).

Participation in the study was voluntary, and the parents have the right to withdraw their child from the study without providing a reason and with no loss of benefits to which the child is entitled. If parents chose to withdraw their child, the study personnel must be informed immediately. The investigator has the right to terminate participation of any child at any time if they deem it in the child’s best interest. Examples of possible reasons for premature withdrawal of a study subject include: child’s parents withdraw for personal reasons, child’s general condition contraindicates continuation of the study as judged by the study personnel or the medical expert (e.g., continuous decrease of the Hb level or no significant increase of Hb level after 1 mo of intervention), non-compliance with study protocol or lack of cooperation, serious adverse event (SAE), and lost to follow-up. In present study, an adverse event (AE) was defined as any new unfavorable and unintended sign, including abnormal laboratory findings, symptoms and/or disease (such as diarrheal, vomiting, abdominal pain, nausea, feverish, et al.), or worsening of existing symptoms, which was temporarily associated with the use of formula. The SAE was defined as any untoward medical occurrence that at any dose, such as results in death, life-threatening at the time of the event, requires inpatient hospitalization, results in persistent or significant disability or incapacity and another important medical event.

Intervention. Three different fortification dosages were used in the present study and eligible infants were randomly assigned to one of the three groups. Infants in the fortified group 1 (FG1) took a commercially available bLF-fortified formula (Beinmate Research Institute of Food Science Co. Ltd., Hangzhou, China, with the lactoferrin provided by Hilmar Cheese Company, CA, USA), which had a bLF concentration of 38 mg/100 g (Production No.: 20160125B). Fortified group 2 (FG2) was supplemented with 76 mg/100 g (Production No.: 20160125C) of bLF. Infants in fortified group 0 (FG0) took a formula free of bLF (Production No.: 20160125A). All the three formulas contained the same composition of nutrients except for bLF and the iron content of the three formulas were 4 mg/100 g. Immediately after recruitment, infants were assigned a study number that had been previously randomly assigned to the different fortified groups with fixed, equal allocation to each group prepared by a third party. The RAND function of Excel (Microsoft, Redmond, WA, USA) was used to generate randomly permuted codes. The laboratory personnel, data entry, manager and statistician remained blinded to group assignments until the end of data analysis. The total duration of fortification was 3 mo.

Questionnaire interview. Parents and main caregivers (who were responsible for at least half of the infant’s care time) were interviewed by a trained field health worker for approximately 30 min. Information such as demographics (infant’s age, sex, etc.), family socioeconomic status, educational levels of main caregivers, family monthly income, use of vitamin/mineral supplement, and feeding method (exclusive breast-fed and mixed feeding, which means the combination feeding with formula and breast milk) before trial were collected.

Anthropometric measurements. The same trained nurses (n=10) from each hospital conducted the
anthropometric examinations at their specific hospitals at baseline and 3-mo follow-up using standardized techniques to eliminate inter-examiner error. Duplicate measurements were performed for all infants. The inter-examiner coefficient of variation of weight, length, and head circumference (HC) for each examiner in each group were <5%. Weight was recorded using a weighing scale (100 Med. Beijing, China) to the nearest 100 g with infants in minimal clothing and bare feet. Similarly, length was measured in the standard supine position by a supine scale (Haode, Guangzhou, China) to the nearest 0.1 cm.

**Blood sample collection and biochemical assessment.** Blood samples were collected at the beginning, 1 mo, and the end of the intervention. Approximately 1 mL of blood was collected from an antecubital vein of each infant before breakfast. The blood samples were immediately stored at 4°C to prevent microhemolysis. One quarter of a milliliter of the blood was drawn into a container containing heparin for Hb measurement using the hemoglobincyanide method (Maker, Chengdu, China) (18). The remaining blood was centrifuged at 3,000 × g, 4°C for 5 min. Serum samples were aliquoted and stored at −20°C. SF, serum soluble transferrin receptor (sTfR), and CRP were determined using a commercial enzyme-linked immunosorbent assay (ELISA) (19), a microparticle-enhanced immunoassay (20), and a particle-enhanced immunoturbidimetry (Sunbiote, Shanghai, China), respectively.

**Definition of outcomes.** The prevalence of deficiency was expressed as percentage of infants below the cut-off value for each biochemical index. The WHO criteria were used to determine the prevalence of anemia (Hb < 100 g/L for 4 to 6 mo; < 110 g/L for 6 mo to 6 y) (21) and iron deficiency (SF < 12 μg/L) (22). Iron deficiency anemia was defined as co-existence of anemia and iron deficiency. CRP levels of > 10 mg/L were used as an indicator of infection or inflammation (Roche Diagnostics, Basel, Switzerland). Transferrin receptor-ferritin (TFR-F) index and total body iron content (TBIC, mg/kg) were calculated using the following formulas:

\[ \text{TFR-F} = \frac{-\log(\text{sTfR}/\text{SF}) - 2.8229}{0.1207} \]

\[ \text{TBIC} = \frac{\log(\text{sTfR}/\text{SF})}{0.1207} \]

The unit for sTfR and SF is mg/L.

**Sample size.** A sample size of about 26 infants per group was sufficient to allow the detection of a 10 g/L Hb of difference after fortification with 95% power and \( \alpha = 0.05 \) for a two-sided t test. To allow for 40% of dropout rate over the duration of the study, we recruited 36 anemic infants per group.

**Outcome measures.** The primary objective for the present study was to measure the change in Hb before and after intervention. The secondary objectives were anthropometric index, serum SF, sTfR, and other indices.

**Statistical analyses.** Using the Kolmogorov–Smirnov goodness-of-fit test, the distribution of each set of data was tested for normality before analysis. Data were presented as the mean and standard deviation for normally distributed variables or median (25th, 75th) for skewed distribution variables. Tests of significance were two-tailed and \( p < 0.05 \) was considered statistically significant. Analysis of variance (ANOVA) was used to analyze the differences among group means for normally distributed data. Meanwhile, the Wilcoxon sign-rank test was used for skewed distribution. The \( \chi^2 \) test was used for categorical variables, with a Bonferroni correction for multiple comparisons among multiple groups. Data were analyzed using the SAS for Windows statistical software package version 8.1 (SAS Institute Inc., Cary, NC, USA).

**RESULTS**

One hundred and eight infants met the inclusion criteria (36 for each group). About 11.1% (12/108) dropped out during the course of the study. Three of these infants were rejected before intervention for not accepting the written informed consent and nine for the loss of Hb data during intervention. Thus, primary outcome measures including analyses of biochemical indices of blood and anthropometric indices at the end of fortification were obtained from 96 infants (35, 33 and 28 for FG0, FG1 and FG2, respectively) (Fig. 1). The average daily intake of formula per infant for FG0, FG1 and FG2 were 119.5 ± 7.7 g, 124.2 ± 11.6 g, and 120.4 ± 9.2 g, respectively, and the average daily intake of iron per infant was 4.8 ± 0.6 mg, 5.0 ± 0.4 mg, and 4.8 ± 0.6 mg, respectively. No significant difference in intakes were found between the three groups (\( p > 0.05 \)). The average amount of daily intake of bLF for infants in FG0, FG1 and FG2 were 0 mg, 47.2 ± 8.7 mg, and
Dose Effect of Lactoferrin on Anemic Infants

91.5±12.5 mg, respectively. No important adverse events or side effects in each intervention group was observed.

Baseline characteristics

There were no significant differences in gender, pregnant and gestational ages, infant’s age, weight, length, HC, years of education of main caregivers, average monthly household income, number of passive smoking exposure, living environment, resident population, or other demographic and clinic characteristics among the three groups (p>0.05; Table 1).

Effect of fortification on anthropometric indices

The present data indicated that there were no significant differences in the baseline levels of weight, length, and HC among the three groups (p>0.05, Table 2). In each group, all the anthropometric indices increased significantly (p<0.05) after 3 mo of fortification when compared with the baseline. No significant difference

Table 1. Comparison of clinical and socioeconomic data of these infants completing the whole study (m±SD).

<table>
<thead>
<tr>
<th>Index</th>
<th>FG0</th>
<th>FG1</th>
<th>FG2</th>
<th>F/χ² values</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>35</td>
<td>33</td>
<td>28</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Female [n (%)]*</td>
<td>14 (40.0)</td>
<td>12 (36.4)</td>
<td>12 (42.9)</td>
<td>0.2711</td>
<td>0.8733</td>
</tr>
<tr>
<td>Age when recruited (mo)*</td>
<td>8.55±2.6</td>
<td>7.65±2.5</td>
<td>8.39±2.2</td>
<td>1.2674</td>
<td>0.2864</td>
</tr>
<tr>
<td>Weight when recruited (kg)*</td>
<td>8.83±1.4</td>
<td>8.37±1.5</td>
<td>8.49±1.4</td>
<td>0.9381</td>
<td>0.3951</td>
</tr>
<tr>
<td>Length when recruited (cm)*</td>
<td>71.85±5.4</td>
<td>70.03±6.3</td>
<td>70.75±6.2</td>
<td>0.8061</td>
<td>0.4497</td>
</tr>
<tr>
<td>HC when recruited (cm)*</td>
<td>43.42±3.6</td>
<td>43.21±3.1</td>
<td>43.30±3.1</td>
<td>0.0349</td>
<td>1</td>
</tr>
<tr>
<td>Years of education of main caregivers (y)*</td>
<td>11.00±2.7</td>
<td>11.79±3.3</td>
<td>11.04±2.8</td>
<td>0.7446</td>
<td>0.4777</td>
</tr>
<tr>
<td>Average monthly household income per person (yuan)*</td>
<td>882.35±530.3</td>
<td>955.00±768.6</td>
<td>1026.79±758.7</td>
<td>0.3446</td>
<td>0.7094</td>
</tr>
<tr>
<td>Living environment*</td>
<td>1.5998</td>
<td>0.4494</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rural</td>
<td>32</td>
<td>30</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Town</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of resident population (n)*</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>34</td>
<td>27</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥4</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feeding method when recruited</td>
<td>0.117</td>
<td>0.9431</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exclusive breast-fed</td>
<td>16</td>
<td>14</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed feeding</td>
<td>19</td>
<td>19</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complementary feeding when recruited [n (%)]</td>
<td>30 (85.7)</td>
<td>28 (84.8)</td>
<td>22 (78.6)</td>
<td>0.6545</td>
<td>0.7209</td>
</tr>
</tbody>
</table>
| FG0, fortified formula with no lactoferrin; FG1, fortified formula with lactoferrin by 38 mg/100 g; FG2, fortified formula with lactoferrin by 76 mg/100 g; HC, head circumference.

* No significant difference among the three groups (p>0.05).

Table 2. Effect of bovine lactoferrin on anthropometric indices of infants (m±SD).

<table>
<thead>
<tr>
<th>Index</th>
<th>FG0 (n=35)</th>
<th>FG1 (n=33)</th>
<th>FG2 (n=28)</th>
<th>F values</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg) BF*</td>
<td>8.83±1.4</td>
<td>8.37±1.5</td>
<td>8.49±1.4</td>
<td>0.9381</td>
<td>0.3951</td>
</tr>
<tr>
<td>1 mo*</td>
<td>9.15±1.4</td>
<td>9.04±3.0</td>
<td>8.77±1.2</td>
<td>0.2743</td>
<td>0.7607</td>
</tr>
<tr>
<td>2 mo*</td>
<td>9.68±1.4</td>
<td>8.99±1.5</td>
<td>9.12±1.4</td>
<td>2.207</td>
<td>0.1158</td>
</tr>
<tr>
<td>AF*</td>
<td>9.71±1.3†</td>
<td>9.35±1.5†</td>
<td>9.55±1.5†</td>
<td>0.5392</td>
<td>0.5850</td>
</tr>
<tr>
<td>Length (cm) BF*</td>
<td>71.85±5.4</td>
<td>70.03±6.3</td>
<td>70.75±6.2</td>
<td>0.8061</td>
<td>0.4497</td>
</tr>
<tr>
<td>1 mo*</td>
<td>73.69±5.5</td>
<td>71.47±6.1</td>
<td>72.94±6.0</td>
<td>1.2507</td>
<td>0.2911</td>
</tr>
<tr>
<td>2 mo*</td>
<td>75.08±5.2</td>
<td>72.34±6.0</td>
<td>73.56±5.6</td>
<td>2.0435</td>
<td>0.1354</td>
</tr>
<tr>
<td>AF*</td>
<td>75.63±5.1†</td>
<td>73.95±6.5†</td>
<td>75.47±6.0†</td>
<td>0.8183</td>
<td>0.4444</td>
</tr>
<tr>
<td>HC (cm) BF*</td>
<td>43.42±3.6</td>
<td>43.21±3.1</td>
<td>43.30±3.1</td>
<td>0.0349</td>
<td>1</td>
</tr>
<tr>
<td>1 mo*</td>
<td>44.55±3.0</td>
<td>44.22±2.7</td>
<td>44.34±3.0</td>
<td>0.1129</td>
<td>1</td>
</tr>
<tr>
<td>2 mo*</td>
<td>44.60±1.9</td>
<td>44.46±2.2</td>
<td>44.46±2.4</td>
<td>0.0468</td>
<td>1</td>
</tr>
<tr>
<td>AF*</td>
<td>44.65±6.7†</td>
<td>45.58±2.4†</td>
<td>45.31±2.3†</td>
<td>0.3902</td>
<td>0.678</td>
</tr>
</tbody>
</table>

FG0, fortified formula with no lactoferrin; FG1, fortified formula with lactoferrin by 38 mg/100 g; FG2, fortified formula with lactoferrin by 76 mg/100 g; BF, before fortification; AF, after fortification; HC, head circumference.

* No significant difference before or after fortification in each group (p<0.05); † significant difference before and after fortification in each group (p<0.05).
was found among the three fortification methods after 1 and 3 mo of intervention (p>0.05).

Effect of fortification on serum biochemical indices

The present data indicated that there were no significant differences in the baseline levels of Hb, SF, sTfR, TFR-F index, and TBIC among the three groups (p>0.05, Table 3). In each group, all the biochemical indices mentioned above improved significantly after 3 mo of fortification when compared with the baseline (p<0.05), except for the sTfR level (p>0.05). After 1 mo of intervention, the changes of Hb level showed no significant difference, however, the Hb level of infants in FG2 were significantly higher than those of infants in the other two groups after 3 mo of intervention (p>0.05). No remarkable difference between FG0 and FG1 groups (p>0.05) was found after 1 and 3 mo of intervention. No significant difference in levels of SF, sTfR, TFR-F index, and TBIC of infants was found among the three groups after intervention (p>0.05).

**DISCUSSION**

Potential benefits of bLF fortification in infant formula was observed in conjunction with iron supplementation. There was a trend toward increased Hb with higher level of bLF fortification for 3 mo during the first 6–9 mo of age. Fortification with bLF is safe. No adverse effects was observed at the tested bLF fortification levels, 38 or 76 mg/100 g. For infants who consume formula derived from cow’s milk (15, 24, 25), bLF fortification does not put them at additional risks.

Dose effect of bLF fortification on growth and development

No significant difference in linear growth changes (including weight, length, and HC) was observed among anemic infants after 3 mo of bLF fortification, which was not in agreement with another study (26) and our previous results (17). Some studies have observed that bLF treatment significantly increased the weight, height, or HC gains of infants due to bLF-promoted cellular proliferation and differentiation (27), increased total protein content of the treatment formula (by about 5%) (14), or reduced prevalence of diarrhea, upper respiratory tract infection, and anemia (17).

We cannot explain why the bLF fortification had no significant effect on linear growth of anemic infants in this study. Whether the ineffectiveness was due to suboptimal dosage or intervention duration that could not compensate the deleterious effect of anemia, infant age, or other non-homogeneous factors was unclear. Therefore, the task remains to further investigate the effect of bLF fortification on growth and development of formula-fed infants.

Dose effect of bLF fortification on Hb level

Compared with the baseline, the levels of Hb increased markedly in all three groups, regardless of the duration of fortification and the concentration of bLF. Fortification with 76 mg/100 g of bLF for 3 mo was the most effective in improving Hb levels. To our knowledge, there is no study that investigated the dose effect of bLF fortification on the improvement of Hb, although many studies have reported the beneficial effects of bLF on iron metabolism (14, 28–31). In the present study, the average daily intake of bLF for infants in FG1 and FG2 were 47.2±8.7 and 91.5±12.5 mg, respectively. The anemic infants with an average daily intake of 47.2 mg bLF showed similar changes in Hb as compared with the anemic infants receiving no fortified bLF after the 1 and 3 mo of intervention. Our previous study (17) reported that daily intake of 35.8 mg bLF significantly increased the Hb level in healthy infants when compared to infants fed no additional bLF from the formula. Chierici et al. (13) showed that there were no statistically significant differences in Hb and hematocrit between different feeding groups (exclusively breastfeeding, formula feeding with 0, 10 mg/100 mL and 100 mg/100 mL bLF) at

<table>
<thead>
<tr>
<th>Index</th>
<th>FG0 (n=35)</th>
<th>FG1 (n=33)</th>
<th>FG2 (n=28)</th>
<th>F values</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/L)</td>
<td>BF</td>
<td>102.9±8.0</td>
<td>100.76±7.8</td>
<td>105.08±8.1</td>
<td>2.235</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>113.18±10.2*</td>
<td>109.78±8.3*</td>
<td>111.83±8.6*</td>
<td>1.9195</td>
</tr>
<tr>
<td></td>
<td>SF</td>
<td>116.49±8.0*</td>
<td>116.58±6.4*</td>
<td>121.50±5.1*</td>
<td>5.4316</td>
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<tr>
<td>SF (μg/L)</td>
<td>BF</td>
<td>8.4±1.2</td>
<td>8.9±1.3</td>
<td>8.2±1.2</td>
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<td>AF</td>
<td>25.6±3.7*</td>
<td>26.9±4.4*</td>
<td>26.2±3.1*</td>
<td>0.9935</td>
</tr>
<tr>
<td>sTfR (mg/L)</td>
<td>BF</td>
<td>1.45±0.13</td>
<td>1.40±0.21</td>
<td>1.51±0.22</td>
<td>2.5885</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>1.51±0.24†</td>
<td>1.54±0.22†</td>
<td>1.53±0.17†</td>
<td>0.1722</td>
</tr>
<tr>
<td>TFR-F index</td>
<td>BF</td>
<td>0.99±0.11</td>
<td>1.02±0.13</td>
<td>0.97±0.13</td>
<td>1.2867</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>1.65±0.41*</td>
<td>1.71±0.31*</td>
<td>1.69±0.23*</td>
<td>0.2889</td>
</tr>
<tr>
<td>TBIC (mg/kg)</td>
<td>BF</td>
<td>4.12±0.35</td>
<td>4.25±0.29</td>
<td>4.23±0.22</td>
<td>1.8838</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>6.62±0.51*</td>
<td>6.53±0.47*</td>
<td>6.78±0.63*</td>
<td>1.6770</td>
</tr>
</tbody>
</table>

FG0, fortified formula with no lactoferrin; FG1, fortified formula with lactoferrin by 38 mg/100 g; FG2, fortified formula with lactoferrin by 76 mg/100 g; BF, before fortification; AF, after fortification; SF, serum ferritin; sTfR, serum transferring receptor; TFR-F index, sTfR-SF index; TBIC, total body iron content.

* Significant difference before and after fortification in each group (p<0.05); † no significant difference before and after fortification in each group (p>0.05); ‡ significant difference before or after fortification among the groups (p>0.05); § no significant difference before or after fortification among the groups (p>0.05).
any sampling time, however, the dosage of bLF was not reported in this study. Another field study (26) also concluded that fortification with bLF did not benefit either iron status or erythrocyte fatty acids, however, the concentration of fortified bLF was not provide in the study.

Although administration of 76 mg/100 g of bLF effectively increased the concentration of Hb after 3 mo of intervention, there was no obvious change in Hb after 1 mo of intervention. It prompts the possibility that the efficacy of bLF on Hb is dependent on the intervention time, which should be more than 1 mo.

The theory of intake–response curve for a typical nutrient (32, 33) may partially explain why fortification with 38 mg/100 g of bLF was effective in improving Hb of healthy infants but not for anemic infants. Figure 2 demonstrated that an identical nutrient intake may result in different responses in people with different baseline values. For example, the anemic infants with the baseline iron level of “A” has a response of “a,” which means supplementation with 38 mg/100 g of bLF may not produce a measurable response in anemic infants with serious iron deficiency. On the contrary, healthy infants with a baseline iron level of “B” has only mild to moderate risk of iron deficiency and can produce a much higher response of “b” when supplemented with the same amount of bLF. Other non-homogeneous factors, such as age, sex, nutritional status, other micronutrients levels, disease during intervention may also contribute to the discrepancy between healthy and anemic infants. The present study suggested that when considering the bLF supplementation dosage for infants, attention should be paid to the baseline nutritional status, especially the iron status.

**Dose effect of bLF fortification on iron metabolism indicators**

SF is an iron storage marker and positive acute phase response protein whose concentration increases during inflammation regardless of true iron status (34). STfR is a transmembrane protein that mediates iron delivery from the extracellular pool into erythroblasts and increases with elevated cellular iron needs and cellular proliferation (35). Because of a close linear relationship between TFR-F index and the total body iron content expressed as milligram per kilogram body weight, TFR-F index is suggested to be a more reliable indicator of iron status. The supplementation of bLF in formula for anemic infants, regardless of the bLF concentration, showed no improvement on serum SF, sTfR, TFR-F index, and TBIC when compared with formula free of bLF.

Our previous study (17) found that supplementation of 38 mg/100 g of bLF in formula significantly increased the levels of Hb, SF, TFR-F index, and TBIC, but not the sTfR level in healthy infants who were previously exclusively breastfed when compared with infants without bLF supplementation. Hernell and Lönnerdal (26) found that infants fed formula containing 1.8 mg Fe/L (1.3 mg of which being bLF) for 4 and 6 mo had markedly higher SF levels than those fed formula containing 4 mg Fe/L, as ferrous sulfate. Chierici et al. (13) reported that formula supplemented with bLF induced significantly higher SF levels at day 90 and day 150 in comparison to formula without bLF supplementation. These observations suggested that LF may be involved in iron absorption. Similar to the present study, another fortification study (26) also reported no significant difference of sTfR levels in infants who were breast-fed or fed formula containing 1.6 mg Fe/L, 1.8 mg Fe/L (1.3 mg of which being bLF), 2.2 mg Fe/L with nucleotides as monophosphates, and 4 mg Fe/L in the form of ferrous sulfate.

With no change of sTfR and increased Hb level after bLF supplementation, we conclude that bLF had no apparent effect on erythropoiesis. The theory of intake–response curve for a typical nutrient explains the effect of bLF fortification on SF; that is, the basic status of iron nutrition determines the supplementation efficacy. For anemic infants, whose iron storage was exhausted (most serious iron deficiency), neither the low (38 mg/100 g) nor high intake (76 mg/100 g) of bLF for 3 mo produced a measurable response. An extended intervention duration or higher bLF dosage may be needed to exert a positive effect on SF, TFR-F index, and TBIC. On the contrary, for healthy infants, who were previously exclusively breast-fed with only mild to moderate iron deficiency, the low bLF intake (38 mg/100 g) for 3 mo is enough to produce a measurable response. The low iron intake in the present study (4.8–5.0 mg), can only improve the anemia status, but not the level of iron storage, which is measured by SF, TFR-F index, and TBIC.

**Strength and limitation analysis of the present study**

We believe our study has many strengths including 1) comparable anthropometric and biomedical characteristics of infants in the three fortification groups at baseline, 2) randomized and double-blinded group assignment, and 3) low attrition rate. Despite these advantages, we recognize a few limitations. Although most of the study factors were objective indicators, the questionnaire responses may be biased due to the fact that the formula was provided for free. Moreover, we did not analyze other micronutrients that could also influ-
ence Hb level and iron status such as vitamin B₁₂ (36), folate (37), zinc (38), and vitamin A (39). Finally, the limited duration of intervention (3 mo) may have prevented us from detecting long-term effects of bLF on infant health.

CONCLUSION

Our data indicated that formula fortified with 76 mg/100 g of bLF positively affected Hb of anemic infants who were previously breastfed when compared with bLF fortification at 38 mg/100 g or no bLF fortification. Our findings may be generalized to other infants living in similar socioeconomic districts.

Disclosure of state of COI

We confirm that all the listed authors have participated actively in the study, and have seen and approved the submitted manuscript. The authors do not have any possible conflicts of interest.

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This trial has been registered with ClinicalTrials.gov registry, registration number NCT 03534089.

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Authorship

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REFERENCES

Dose Effect of Lactoferrin on Anemic Infants


